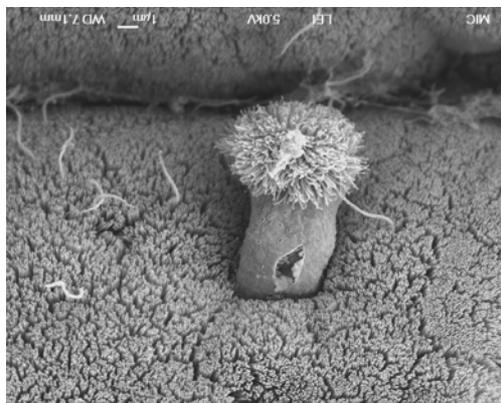


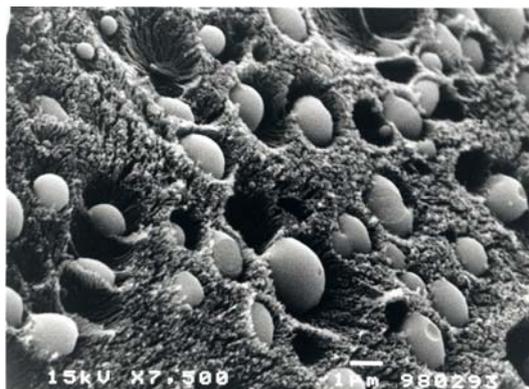


**Opinion of the Panel on Animal Feed of the  
Norwegian Scientific Committee for Food Safety**  
*05.02.2009*

**Criteria for safe use of plant ingredients in diets for aquacultured fish**



*Dead cell rejected by salmon intestine*



*Micelle absorption in salmon intestine*

*Photos: Rolf Erik Olsen*

## **Criteria for safe use of plant ingredients in diets for aquacultured fish**

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Chapter 5 is a revised version of a book chapter by Anne Marie Bakke-McKellep and Ståle Refstie: Alternative protein sources and digestive function alterations in teleost fishes. In *Feeding and Digestive Functions of Fishes* (ed. by J.E.P. Cyrino, R. Roubach, D. Bureau, B.G. Kapoor). Science Publishers Inc., Enfield NH, USA, in print.

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## 1. SUMMARY

A thorough review of different aspects on the health implications of using vegetable feed ingredients, both protein and lipid alternatives to fishmeal and fish oil, plus possible additions of immunostimulants, also including a short chapter on undesirable components, the use of genetically modified plants, and how processing may affect feed quality and availability, are given in the different chapters of the present risk assessment. Discussed in particular are Atlantic salmon (*Salmo salar*), rainbow trout (*Onchorhynchus mykiss*), Atlantic halibut (*Hippoglossus hippoglossus*) and Atlantic cod (*Gadus morhua*). Deemed necessary, since literature on these species is scarce, some theoretical background in the assessment chapter (Chapter 4) includes studies on other species when relevant for the present terms of reference, and to better be able to conclude on possible health implications due to changes in diet ingredients. The answers to the present Terms of Reference are given in Chapter 5 (Risk characterization and conclusions). Chapter 6 presents future challenges that need focus in research to be able to have healthy farming of fish even when volumes increase.

### *Plant proteins (Chapter 4.1 for details)*

The main challenges in using plant protein sources in diets for carnivorous fish lie in their often lower levels of protein and high levels of starch, unfavourable amino acid and mineral profiles, high levels of fibre and – perhaps most consequential – the presence of antinutritional factors (ANF) and/or antigens. Especially the long-term implications they may have on fish production, health, and product quality are largely unknown and deserve continued, substantial investments in research to preserve and enhance the sustainability of the aquaculture industry. Information regarding antinutritional factor levels in the plant ingredients and feeds containing them is rarely given in publications reporting data from feeding trials. The research on effects that various alternative protein-rich ingredients have on fish has largely been restricted to fish growth, feed conversion efficiency, and digestibility of the feed's nutrients. The current risk assessment attempts to examine more specific effects on gastrointestinal digestive function and health. Seemingly conflicting results from different studies as well as quantitative and qualitative differences in effects may be due to:

- Varying practices of fasting prior to sampling intestine for histological and physiological investigation;
- Tolerance level of various fish species;
- Developmental stage of the fish;
- Genetic and environmental influences that may govern the plants' nutrient and antinutrient composition;
- Type and degree of processing used for the alternative protein source;
- Protein level of the diets;
- Quality of the fishmeal or other protein source in the control diet.

Due to this large number of inconsistencies that can influence results within and between studies, little specific data can be used for a full risk assessment and the results of studies are discussed in more general terms.

Soybean meal (SBM) is one of the most commonly used protein sources in animal feeds due to its high protein content and favourable amino acid profile. However, even 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, lower feed intake, energy and fat digestibilities, and fecal dry matter in all salmonid species studied. Dehulled SBM as the sole protein source may lead to growth arrest and increased mortality in rainbow trout. These SBM products also cause an inflammatory response in the distal intestine (enteritis) of salmonids which may at least partially explain the effects on growth

parameters and feed utilization. Further processing of the SBM with alcohol extraction to produce soybean protein concentrate appears to remove the as yet unknown causatory agent(s) of the enteritis and supports very acceptable growth parameters and feed utilization. However, the added processing increases the market price. Atlantic cod and halibut appear to tolerate full-fat and standard SBM better than Atlantic salmon and rainbow trout. Levels up to 24% of total diet for cod and 36% of total diet for halibut did not markedly affect growth or feed utilization, nor cause inflammatory responses in their intestines.

As demonstrated in several fish species, the proteins in kernel meals of dehulled white (*Lupinus albus*), sweet (*L. angustifolius*) and yellow (*L. luteus*) lupins are highly digestible. It has been estimated that sweet lupin kernel meal may be included in diets for rainbow trout up to 30 or 40% of total diet without significantly influencing growth and nutrient utilization. No histological changes were observed in the pyloric caeca or distal intestine of rainbow trout fed up to 50% yellow lupin kernel meal (of total diet) although hepatocytes appeared to have a lower level of lipid droplets in the fish fed the 50% inclusion level. Nor were histological changes observed in the distal intestine of Atlantic salmon fed 24% of total diet of dehulled, low-alkaloid white lupin meal, or 30% of total diet of kernel meals or protein concentrates from yellow lupin or sweet lupin. In the stomachs of these fish, however, higher severity of ulcer-like lesions connected with the lupin-containing diets, were reported.

Pea meal has potential as an alternative protein source although low-processed pea meal in feeds for carnivorous fish is limited by its high starch content (ca. 50% of pea is starch). Pea protein concentrate or isolate is therefore more suitable. For Atlantic salmon, pea protein concentrate at inclusion levels of up to 28% of total diet led to apparent digestibility coefficients for dry matter, nitrogen and energy similar to those of a commercial diet and higher than a control diet containing fishmeal as the sole protein source. In another study, Atlantic salmon fed 20% pea protein concentrate (of total diet) led to lower digestibility of energy, but no significant differences in digestibilities of other macronutrients or amino acids, nor in growth performance, body composition, intestinal brush border maltase activities, fecal trypsin activities, or intestinal histology compared to the fishmeal control diet. However, Atlantic salmon in yet another feeding trial fed 30% pea protein concentrate (of total diet) exhibited lower growth rates, lipid and starch digestibilities, distal intestinal weight, as well as histological changes in the distal intestine characteristic of an inflammatory response. No other intestinal regions showed signs of inflammation. Rainbow trout also digest pea protein well at lower inclusion levels, although 30% pea meal in total diets led to lower dry matter, protein, energy and particularly phosphorus digestibilities compared to fishmeal-based diets. In Atlantic cod, apparent protein and energy digestibilities for pea protein concentrate (30% of total diet) has also been estimated to be moderate: 89.8 and 76.7% apparent digestibility coefficients (ADC), respectively.

Canola, developed from rapeseed to contain lower levels of glucosinolates, or heat-treated rapeseed meal appear promising as alternative protein sources for fish. In Atlantic salmon, 18.3% (of total diet) inclusion of low-glucosinolate, extracted and heat-treated rapeseed meal resulted in higher lipid digestibility but lower crude protein digestibility than a fishmeal control diet. Histological changes were not observed in the stomach or intestine. In rainbow trout, inclusion levels in total diet of canola protein concentrate of 19% to 38% led to reduced growth and feed intake compared to fishmeal controls. In Atlantic cod, apparent protein and energy digestibilities for canola meal has been estimated to be relatively low at 76.0 and 60.6% (ADCs), but higher for canola protein concentrate at 88.8 and 83.3% (ADCs), respectively.

Sunflower meal (partially dehulled) in diets for Atlantic salmon had ADC of 88% for protein and could be included in post-smolt diets up to 27% of total diet without adverse effects on growth performance, feed utilization or body composition. At a 22.9% inclusion

level (of total diet) of extracted and mildly heat-treated sunflower meal, higher lipid digestibility but lower crude protein digestibility than a fishmeal control diet was reported. Histological changes were not detected in the stomach or intestine. Sunflower meal at an inclusion level of 41% in a total diet for rainbow trout resulted in improved crude protein digestibility but reduced nitrogen-free extract and dry matter digestibilities compared to a fishmeal-based control diet.

Solvent-extracted cottonseed meal at inclusion levels of more than 50% in total diets for various fish species causes growth depression, but levels of up to 30% of total diet (replacing 50% of fishmeal protein) appeared to be well tolerated by rainbow trout juveniles and did not significantly infringe on growth parameters, feed conversion, nutrient digestibilities or mineral availability. A long-term feeding trial (35 months) showed that even inclusion levels as high as 59% of total diet, which was a complete replacement of fishmeal, did not impact fish growth negatively. However, female rainbow trout fertility was negatively affected by complete replacement of fishmeal with cottonseed meal.

Wheat gluten is a highly digestible protein source for rainbow trout, Atlantic salmon and Atlantic cod. It does not cause morphological changes in the intestinal tissues of salmon. Wheat gluten up to a level of 50% of dietary protein (29% of total diet) can be added to salmon diets without reducing protein, amino acid, fat, and energy digestibility, although  $\alpha$ -amylase inhibitors in wheat appear to reduce starch digestibility. Corn gluten protein as a protein source is also highly digestible for Atlantic salmon and Atlantic cod. In Atlantic salmon, morphological changes in the stomach, mid and distal intestinal tissues were not observed when using whole corn meal as a carbohydrate source in fishmeal-based diets for parr nor 20% corn gluten of total diet, added as a protein source in diets for post-smolts.

Potato protein concentrate in diets for rainbow trout resulted in severe appetite loss, even at dietary inclusion levels as low as 5% of total diet. However, if processed to remove inherent solanidine glycoalkaloids and protease inhibitors, up to 20% potato protein concentrate of total diet may be included in diets for salmonids without causing adverse effects on appetite, growth, nutrient digestibility, or nutrient retention.

Rice protein concentrate can be included at levels up to 20% in total diets for trout without negatively affecting growth performance. Faba beans have limited application in diets for carnivorous fish due to the high starch content. Peanut meal has not been tested in cold-water carnivorous species.

Mixing various plant protein feedstuffs in formulated feeds has been attempted in recent years with varying results. It appears that the proportion of feedstuffs from various sources and the degree of fishmeal substitution that is acceptable will vary depending on fish species and their dietary requirements and preferences. Possible additive/synergistic effects among ANFs, antigens, and/or toxins present in the various feedstuffs that comprise a mixture should also be taken into consideration (see below).

#### *Antinutritional factors (Chapter 4.2 for details)*

Antinutritional factors (ANFs) are defined as innate components of a food/feed ingredient that have a limiting effect on food/feed intake, digestion, and/or nutrient absorption. Possibly the most limiting factor for the use of plant feed ingredients as nutrient sources for fish are ANFs inherent to them. ANFs will most likely cause changes in nutrient availability and/or utilization, physiological responses, and ultimately metabolism, and they will therefore change recommended dietary levels of various nutrients in aquafeeds. The known ANFs and the plant feedstuff in which they are found are listed below. However, it is possible that hitherto unidentified compounds in plants may be important in fish nutrition and health. Little is available in the literature concerning effects of specific ANFs on fish or levels of various ANFs in feeds containing plant ingredients. Thus it is difficult to assess health

effects, especially long-term, on fish. No data has been found regarding effects of isolated ANFs on Atlantic cod and halibut.

<i>Plant feedstuff</i>	<i>Antinutrient present</i>
Soybean meal	Proteinase inhibitors, lectins, phytic acid, saponins, phytoestrogens, antivitamin, phytosterols, allergens
Kidney beans	Proteinase, amylase and lipase inhibitors, lectins, phytic acid, saponins, phytoestrogens, antivitamin, phytosterols, allergens
Rapeseed meal	Proteinase inhibitors, glucosinolates, phytic acid, tannins
Lupin seed meal	Proteinase inhibitors, saponins, phytoestrogens, alkaloids
Pea seed meal	Proteinase inhibitors, lectins, tannins, cyanogens, phytic acid, saponins, antivitamin
Sunflower oil cake	Proteinase inhibitors, saponins, arginase inhibitor
Cottonseed meal	Phytic acid, phytoestrogens, gossypol, antivitamin, cyclopropenoid acid
Alfalfa leaf meal	Proteinase inhibitors, saponins, phytoestrogens, antivitamin
Mustard oil cake	Glucosinolates, tannins
Sesame meal	Phytic acid, proteinase inhibitors

Various enzyme inhibitors, i.e. proteinase, amylase and lipase inhibitors, are proteins that inhibit the activity of the respective enzymes, often by forming stable complexes with the enzymes. By doing so they decrease protein, starch and lipid digestibilities, respectively, as well as often increase endogenous losses of the enzymes, and may cause feedback signals to the pancreas that result in increased secretion of the enzymes. This may have long-term implications on pancreas function so that even relatively low levels of enzyme inhibitors can have a significant impact on dietary nutrient levels needed to meet requirements caused by the endogenous losses over time. Effects of amylase inhibitors in diets for Atlantic salmon may be of limited practical significance however, since the amylase of this species seems to have a defective substrate anchor, reducing its catalytic ability and the ability of salmon to utilize starch. Proteinase and lipase inhibitor activities may account for the impaired protein and lipid digestibilities in Atlantic salmon fed on diets with an inclusion of solvent extracted SBM. However, no feeding trials for Atlantic salmon, rainbow trout, Atlantic cod or Atlantic halibut have been reported that investigated dietary effects of isolated plant lipase inhibitors, despite the economic implications of poor utilization of the expensive lipid by farmed fish.

Lectins (previously known as agglutinins or hemagglutinins) are a group of heterogeneous (glyco)proteins which bind reversibly to specific mono- or oligosaccharides. The mono- or oligosaccharides that lectins bind to may be an intrinsic part of many biologically important substances, so-called glycosubstances or glycoconjugates. These are present on cell surfaces, for example. By binding to cell surfaces, lectins may change cell functions and responses. Thus they can agglutinate cells, modulate the functioning of enzymes, transport proteins, receptors etc., act as growth promoters and immunostimulants, and mimic or block endogenous signalling substances. Thus they may have varied effects on digestive and absorptive processes. Furthermore, glycation of cells varies depending on many factors: animal species, its age, genetic make-up, blood group specificity, health status, diet, and bacterial flora in the intestine, as well as intestinal region and mucosal cell type. Therefore, there is a wide range of potential binding sites for lectins in the gut. This variability in glycation patterns may help explain the variability in biological effects of different lectins in different species of animals. The potency of the lectin's effect on cell metabolism appears to be correlated with the strength of its binding, which in turn is

dependent not only on its defined sugar/carbohydrate specificity, but also on other structural intricacies of the ligand. Binding of soybean lectin (agglutinin; SBA) to the intestinal brush border membrane of Atlantic salmon and rainbow trout has been demonstrated. Higher maximum binding and lower dissociation constants were observed in the distal intestine relative to the more proximal areas. This could indicate that the distal intestine would be more sensitive to a potentially toxic effect of soybean lectin or other antinutritional factors or antigens. However, soybean lectin has been largely ruled out as the sole cause of reduced digestive function and the inflammation caused by soybean meal in salmonids.

Saponins are amphipathic glycosides that disrupt cell membranes and can have antimicrobial, immunostimulatory, glucocorticoid and antioxidant activities. They inhibit protein and lipid digestion, vitamin absorption, and cholesterol metabolism. The involvement of saponins from soybeans in an inflammatory response in the distal intestine of salmon has recently been indicated, albeit not alone but in combination with unidentified components found in lupin meal.

The research focus on effects of phytoestrogens/-sterols in farmed fish has been on reproductive parameters. Little is known about other physiological responses following dietary intake of phytoestrogens in any fish.

Glucosinolates/goitrogens are a group of substances that disrupt thyroid hormone production, partially by interfering with iodine availability. They also cause reduced palatability and thus reduced growth, as well as affect liver and kidney functions.

Fibres are polymers of monosaccharides. They vary in their solubility in water, size and molecular structure. Dietary fibres alter flow, impair interactions, affect intestinal receptors, restrict nutrient diffusion, change microbial diversity and activities, and change absorptive surfaces. The variability of the compounds belonging to the fibre complex and the varying degrees that they affect these parameters make it difficult to conclude on a general basis regarding maximum inclusion level. Little is known on their specific effects in fish.

Tannins are phenols that may decrease feed digestibility by binding digestive enzymes or nutrients as well as reduce feed palatability due to their astringent flavour. Little is known on effects in fish. Therefore, tannin-rich seed hulls should be removed before adding to feeds.

Phytic acids and phytate are found in significant amounts in plant feedstuffs since they are the primary storage form of phosphate and inositol in seeds, they complex with mineral ions and possibly with protein, thus reducing their digestion /absorption. Dietary inclusion of phytic acid has been shown to reduce growth in salmonids as well as reduce digestibility and retention of protein and various minerals in Atlantic salmon. Effects in cod or halibut have apparently not been studied. Involvement of phytic acids in the development of skeletal deformities has not been fully resolved. However, diets for fish should probably not exceed 5 g per kg feed.

Oligosaccharides, i.e.  $\alpha$ -galactosyl homologues of sucrose, appear to interfere with nutrient digestion and may have osmotic activity in salmonids. They also appear to be fermentable by gut microbiota in Atlantic salmon. Effects in cod and halibut have apparently not been studied. An upper limit of 50 g per kg feed has been suggested.

Whether fish react to allergens, or feed antigens in general, is not known. It has not been conclusively demonstrated in fish that antigens/allergens can elicit hypersensitivity reactions, including allergic reactions, as they may in susceptible mammals. Nor has any conclusive data been presented in the literature that antigens are involved in feed-induced pathologies in fish, including soybean meal-induced inflammation in the distal intestine of salmonids.

The toxic, systemic effects of gossypol – reduced hematocrit, hemoglobin, reproductive capacity as well as lesions in liver, kidney, spleen and gonads – in various fish species have been documented but the mechanisms behind the reduced growth and nutrient

digestibilities are unknown. These responses were observed in rainbow trout fed cottonseed meal-containing diets with free gossypol levels of between 30 and 40 mg kg<sup>-1</sup> feed. Gossypol levels less than 20 mg kg<sup>-1</sup> feed, the maximum level allowed by the EU Commission, did not cause these reductions in growth and nutrient digestibility. Long-term effects and tolerance levels for salmon, cod and halibut are not known.

Among the various glycoalkaloids, those found in potatoes,  $\alpha$ -solanine and  $\alpha$ -chaconine are of the most practical importance. These potent toxins, which permeabilize cell membranes and inhibit cholinesterase, limit the use of potato protein concentrate, unless measures are taken during processing to remove them. The main antinutritional activity is in reduced feed intake due to their bitter taste. Tolerance levels are not known for any fish species but due to their toxicity they should not be present in feeds.

Arginase inhibitors, such as chlorogenic acid in sunflower seeds, may be associated with reduced protein digestibility of sunflower meal in salmon but the mechanisms are unknown. Tolerance levels and long-term effects are unknown.

Quinolizidine alkaloids, such as lupinine, gramine and sparteine in lupin meals, are toxins that inhibit motor coordination and muscular control in mammals. Data regarding this or other antinutritional activities are lacking in fish, but the bitter taste may be responsible for reduced feed intake in rainbow trout and Atlantic salmon. Tolerance levels for these fish may be between 100 and 500 mg kg<sup>-1</sup> dry diet. But tolerance levels for cod and halibut are unknown as are long-term effects for any fish.

No data on dietary intake effects of cyanide-releasing compounds were found. Nile tilapia consuming feed containing cassava meal, which contained cyanide-releasing compounds up to 71.1 mg kg<sup>-1</sup>, did not suppress growth. Long-term effects are not known.

Combined effects of ANFs have not been extensively studied in any animals. Tannins in combination with lectins, cyanogenic glycosides and saponins appear to reduce the deleterious effects of the individual ANFs. However, additive interactions with deleterious effects on intestinal function or structure have been reported for saponin and lectin on rabbit tissue *in vitro*, soybean lectin and protease inhibitor on Atlantic salmon intestinal tissue *in vitro*, and saponin and unidentified component(s) of lupin meal in Atlantic salmon distal intestine *in vivo*. Thus there is a need to test combinations of plant ingredients and ANFs on a case-by-case basis to assess any potential consequences to fish health.

#### *Vegetable lipids (Chapter 4.3 for details)*

All vegetable oils lack the long chain highly unsaturated fatty acids (HUFA) typical for marine oils (eicosapentaenoic acid, EPA, docosahexaenoic acid, DHA and arachidonic acid, ARA). The composition of vegetable oils does however differ from plant species to plant species. The predominant oils produced, such as soybean and canola oils, are rich in 18:2n-6 or saturated fatty acids as 16:0 in palm oil. Other oils are also available but are produced in smaller amounts. These include linseed oil (rich in 18:3n-3) and certain safflower species (rich in 18:1n-9) but may be even more important in future aquaculture feeds. The long chain plant fatty acids are often, and also in this report, categorized as PUFAs from plants (polyunsaturated fatty acids = PUFAs from plants).

One main use for plant oils is as energy source. In general these oils provide good energy sources. Medium chain triacylglycerols (MCT), such as those found in coconut oil, appear to be particularly good substrates increasing performance at relatively low inclusion levels. At higher levels poor performance and high mortality has been observed. The results differ with species and size of fish, and there is relatively little data available, particularly in cod and halibut. In general, adding 1-3% of total diet as MCT to fish larvae appears safe. Larger fish appear to tolerate more MCT. In salmonids up to 10% of total diet, and in cod 4% of total diet, seem relatively safe as judged from available literature. The maximum level of

inclusion may also depend on type of MCT. Shorter chain MCT ( $C_6$  or  $C_8$ ) appears to cause more problems than longer chain MCT like coconut oil ( $C_{10}$ ).

As dietary lipids also supply the fish with essential fatty acids (EFA), the level of these in practical diets must be controlled. As marine fish lack the ability to elongate and desaturate plant PUFAs to the longer chain HUFAs (EPA, DHA and ARA) needed for normal homeostasis, they have to be supplied by using marine raw materials in the diets. Thus, the highest level of vegetable oils inclusion is when essential fatty acid deficiency will develop. This depends on which marine lipids are used in the inclusion. For juvenile and adult marine fish the requirement of EPA and DHA is probably in the region 0.5-1.5% of total diet each, and somewhat higher for larvae. It is also possible that the requirement should be estimated at around 10-20% of the dietary lipid. The requirement of ARA may be in the region 0.3-0.5% of total diet. Existing data are however from other species than cod and halibut. Anadromous fish such as Atlantic salmon and rainbow trout do have some capacity to elongate and desaturate vegetable PUFA to EPA, DHA and ARA (from 18:3n-3 and 18:2n-6). Plant oils can thus to a large extent replace marine type oils in the diets for these fishes. For rainbow trout, the essential fatty acid requirement is around 1-2% of total diet if supplied as  $C_{18}$ , and half that if given as HUFAs. It has also been suggested that the level should be 10-20% of dietary lipid depending on the form of delivery used. In rainbow trout, it has been suggested that DHA should be regarded as an essential fatty acid due to too low a conversion rate from 18:3n-3. Similar mechanisms seem likely in Atlantic salmon.

If the essential fatty acid requirements are covered, vegetable oils do not seem to cause any major harm to marine fish. Some elongase activity of  $C_{18}$  PUFA to their  $C_{20}$  HUFA counterparts has been noted, although the activity appears very low in Atlantic cod and remains unknown in halibut. If produced, these compounds may compete with ARA and EPA for active sites for eicosanoids production. But the significance of this is unknown.

Fish probably have a high requirement of phospholipids (PL). If fishmeal is used as a protein source (fishmeal contains relatively large amounts of marine HUFAs), this is only a problem in larval fish where addition of soy lecithin is advised (in the range 2-6% of total diet). However, as other protein sources are now introduced, addition of some soybean lecithin (or marine PL) should be considered to larger fish as well (1-3% of total diet).

In freshwater fish, the effect of adding vegetable oils on fish immunity is inconclusive. Cases of increased disease resistance or immunocompetence in fish added high n-6 PUFA oils (mostly 18:2n-6) have been attributed to production of more eicosanoids from ARA with more potent activity in inflammatory processes than those from EPA. However, fish are different to mammals, and there are several studies suggesting that n-6 PUFA-rich oils will reduce fish immunocompetence. This may be related to altered eicosanoid cascade in addition to changes in membrane fluidity. The reasons for these discrepancies remains unknown and may be caused by several factors including type of study, environmental effects, strain, species and interference of other dietary components. At present however, exchanging up to 50% of the fish oil does not seem to be excessively harmful to the fish.

However, increased level of ARA (from 18:2n-6 rich oils) and its related eicosanoids may also enhance the stress response and may increase the level of subclinical stress that may affect the responsiveness to environmental stress or noxious substances affecting fish health in the long term. At present it would therefore be safe not to use very high levels of such oils.

Oxidative stress does not seem to be a major problem related to vegetable oils provided that they are of good quality. The reason is that most plant oil products have lower oxidative potential than most fish oils and therefore lower the potential oxidative burden. The only potential problem is found with high levels of linseed oil (high content of 18:3n-3), which may contribute to oxidative stress. Although no concrete figures are available for

salmonids, cod and halibut, around 25% addition of linseed oil (of dietary lipid) appears to be relatively safe.

The requirement for essential fatty acids in broodstock nutrition seems to be in the same range as that for fish essential fatty acid requirement in the grow-out stages. For sparids, 1-2% of total diet has been suggested, for salmonids 1% of total diet, and for turbot 20% n-3 HUFAs of dietary lipid. There are also reports suggesting that even higher levels of long chain polyunsaturated fatty acids should be used. But at very high levels of n-3 HUFAs negative effects are sometimes observed. It has also been argued in both cod and some salmonids that the level of ARA in broodstock diets should increase compared to normal standard diets. Cases of increased egg quality following this recommendation have been published. In rainbow trout no effect on fecundity or egg viability despite being fed corn oil as supplemental oil source was found.

As many of the potential disadvantages of using vegetable oils in salmonid diets are related to either very high levels of n-6 PUFA (most available oils) or very high levels of linseed oil, it would be recommended that mixtures of vegetable oils should be used as feed inclusions. By adjusting the ratio of n-6 and n-3 the level of eicosanoids can be controlled. By including palm oil, potential problems in lipid digestibility and transport can be controlled. A standard inclusion of soybean lecithin may also be advisory. These and other variants of mixtures of oil sources have been explored in recent years with some success in salmonid fish. Such mixtures do not seem to be necessary for marine fish.

With plant alternatives, care should be taken in selecting both types and qualities to prevent nutrition-related diseases such as skeletal deformities, cataracts, heart conditions, and other, unspecific symptoms.

#### *Undesirable substances (contaminants) (Chapter 4.4 for details)*

One premise for the use of feed ingredients of plant origin is that their use complies with current Norwegian and European feed legislation on the presence of undesirable substances. Available data on the occurrence and levels of undesirable substances in feed material of plant origin is, however, limited. It is important to continue the surveillance of undesirable substances in feed materials and feed, with special focus on pesticides, mycotoxins and PAHs (polyaromatic hydrocarbons), heavy metals, brominated flame retardants, dioxins and dioxin-like PCBs in feed materials of plant origin. With regard to pesticides, those in use in today's agriculture ought to be included in the surveillance.

The list of undesirable substances included in the feed legislation is, in general, sufficient, but it should be considered to include pesticides in use today and more of the mycotoxins. Currently only aflatoxin B<sub>1</sub> is included, while only recommendations (of maximum content) exist for other mycotoxins. Based on the recommendation, some European countries, including Norway, have national legislation for deoxynivalenol, fumonisins, ochratoxin A and zearalenone.

Studies of dietary exposure to pesticides and mycotoxins and their toxic effects and kinetics in fish are scarce. More studies, and especially long-term feeding trials, are needed for improved risk assessments.

#### *Genetically modified ingredients (Chapter 4.5 for details)*

Few studies are performed on the use of genetically modified (GM) plant proteins in fish diets, however, some publications exist on the use of Round-up Ready soybeans (RRS) and Bt-maize in salmon diets. The results in general show significant effects of including soya and / or maize in fish diets when compared to a reference diet based solely on fish meal and oil. In some studies GM-soybean resulted in larger spleen index compared to fish fed non-GM soybean. The increased spleen index was followed by increased number of red

blood cells of reduced size; these results were not consistent between studies. Bt-maize resulted in reduced feed intake followed by reduced growth in some, but not all studies. Some data show that parts of the immune system can be affected by GM. These results are however not consistent. The glucose transporter system in the pyloric region of the salmon intestine seemed to be altered by Bt-maize, and the stress resistance, measured as changes in mRNA expression of heat-shock proteins 27 and 70, was in some studies altered, but not in other studies. No clear conclusion is drawn on the effect on fish health and the use of neither RRS nor Bt-maize in salmon diets.

*Immunostimulants, prebiotics and nucleotides (Chapter 4.6 for details)*

Use of immunostimulants is a unique approach for fish culturists as they undertake methods of controlling disease losses in their facilities. The immune response can be modulated by  $\beta$ -glucans and high-M-alginate.  $\beta$ -glucans are glucose polymers that are major structural components of the cell wall of yeast, fungi, and bacteria, but also of cereals such as oat and barley. There is much structural variation in the  $\beta$ -glucans from these different sources, which may influence their physiological functions. Alginate is a polysaccharide and is composed of  $\beta$ -1,4-D-mannuronic acid (M) and  $\alpha$ -L-glucuronic acid (G). *In vitro* as well as *in vivo* studies in fish show that especially  $\beta$ -glucans derived from fungi and yeast and alginate have immune modulating properties. Most frequently evaluated are effects on macrophage activation, and on lysozyme, respiratory burst and leukocyte activity, which have been suggested to contribute to the increased resistance against infections, observed after immunostimulant exposure. Although more fish studies are needed, it is tempting to suggest that dietary  $\beta$ -glucans and alginate may be useful tools to prime the host immune system and increase resistance against invading pathogens.

Any reduction of both diversity and quantity of the gut microbiota is likely to reduce the effective barrier mechanism normally provided by the commensal microbiota; this leads to a reduction of competition against secondary potential pathogens from the surrounding environment. As information on this topic is nearly completely lacking with the respect to the effect of immunostimulants, this should be given high priority in future.

To date, the application of pre- and probiotics for the improvement of aquatic environmental quality and for disease control in aquaculture may be promising; however, the information is limited and sometimes contrasting. Owing to these uncertain and incomplete results, there are still no standardized protocols to test the effects of these products and their impact on farmed fish welfare, growth and health status.

Currently there are numerous gaps in existing knowledge about exogenous nucleotide application to fish including various aspects of digestion, absorption, metabolism, and influences on various physiological responses especially expression of immunogenes and modulation of immunoglobulin production. Additional information is also needed in regard to age/size-related responses and appropriate doses and timing of administration. Thus further research in these areas should be pursued.

As limited information is available about the effect of immunostimulants, prebiotics and nucleotides on gut morphology, this topic should be given high priority in future studies. Furthermore, most of the studies carried out on immunostimulants, prebiotics and nucleotides have only evaluated short-time effect. Therefore, we highly recommend that long-term studies are carried out in future.

*Processing (Chapter 4.7 for details)*

Plant ingredients will be subjected to processing before being used as feed. In the extrusion process, plant materials mixed with other ingredients will be heated to above 100 °C with water present. This process will alter the tri-dimensional structure of proteins and starch,

and will potentially induce new covalent bonds which will produce substances that may be harmful. Maillard products may be formed due to reactions between an amino acid and a reducing sugar, and these may be further modified to produce toxic substances. The extent to which these are formed in common feed processes has not been extensively studied, but it is likely that the negative effects are mainly associated with reduced protein digestibility.

Some plant ingredients are processed as raw materials, for example heated in conjunction with the extraction of the oil fraction or due to mechanical processes such as dehulling. These processes may improve nutritive value of the ingredient through elimination of antinutritive properties of proteins and through removal of harmful substances through removal of parts of the plant ingredient.

In conclusion, heat processing of raw materials and of the complete fish diets may potentially alter nutritional properties of plant materials. However, the negative effects appear to be modest under practical conditions.

#### *Pathologies linked to feed (for details see Chapter 4.8)*

Modern finfish aquaculture faces problems such as bone and skeletal deformities, cataracts, heart disorders, as well as unspecific ulceration, various digestive disorders including intestinal colic in Atlantic cod and gastric dilatation (bloat) in rainbow trout. Further, a focus has been laid on the occurrence of intestinal tumours, most of which have been related to malnutrition, feed, intensive growth and/or unfavourable environmental conditions. 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 increase the risk for such production related disorders in intensive fish farming.

Several nutrition-related cataracts have appeared as a consequence of introducing novel feed ingredients in fish feeds, such as “spleen and liver cataracts”, “white fishmeal cataract”, or “rancid low quality feed cataracts”, reflecting nutrient deficiencies, reduced nutrient availability and oxidative challenges.

Bone deformities in juvenile and adult fish are periodically observed with high prevalence in intensive aquaculture, and are also regarded as disorders of multidisciplinary origin. Several nutrients in deficiency and/or excess cause bone disorders. From a nutritional point of view, both development and maintenance of the bone tissue can be affected, and may include impairments in bone cell differentiation and function, matrix composition and bone tissue mineralization.

It is well documented that fish develop various heart disorders including lesions and arteriosclerosis both in wild and farmed. The dietary correlation to the progression of heart disorders is less well documented in fish, and there is particularly little information linking these conditions to feeding high levels of plant oils.

#### *Risk characterization/conclusions and challenges (gaps of knowledge) (Chapters 5 and 6)*

Chapter 5 (page 126); Risk characterization / conclusions, answers the present terms of reference, each question is quoted before the answer is given, while chapter 6 (page 130); Challenges (gaps of knowledge), summarizes weaknesses in the studies referred to, and suggests how and what to focus in future studies.

## **KEY WORDS**

*Plant ingredients, fish feed, undesirable substances, genetically modified plants, plant proteins, fibres, anti-nutritional factors, plant lipids, processing methods, fish growth, health, intestinal function, feed utilization, salmon, rainbow trout, cod, halibut.*

## 2. BACKGROUND

In autumn 2007, the Norwegian Food Safety Authorities (Mattilsynet) asked the Norwegian Committee for Food Safety, panel on Feed for Terrestrial and Aquatic Animals (Panel 6), to assess if the changes in fish diet ingredient composition seen in recent years were economically feasible, environmentally friendly and sustainable. Also asked was whether high levels of plant ingredients, plus additions of immunostimulants, would in any manner challenge fish health, and if any ingredient should be limited due to its negative effect. In focus should be Atlantic salmon, rainbow trout, Atlantic cod and Atlantic halibut.

On a worldwide scale, **fisheries** landings remain constant at about 90 million tons of fish whereas **aquaculture** supplies about 50 million tons and is increasing at a rate around 8% per annum. More than half of the fish products eaten by man come from aquaculture. This growth has resulted in an increased need for specialised compound feedstuffs, estimated at 20 to 25 million tons. Although this is only a small portion of the global animal feed production of around 620 million tons, the very specific nature of the aquafeeds, e.g. aquafeeds contain higher protein levels and lower carbohydrate levels than all other animal feeds, this results in special challenges if the traditional fishmeal and oil is replaced with plants, e.g. completely different nutrient profiles, and contents of molecules unknown to carnivorous fish in nature (e.g. antinutritional factors). The four species in focus in the present report, **Atlantic salmon** (*Salmo salar*), **rainbow trout** (*Onchorhynchus mykiss*), **Atlantic halibut** (*Hippoglossus hippoglossus*) and **Atlantic cod** (*Gadus morhua*), are all intensively farmed fish species in Norway, recognised to have high dietary protein requirements, and especially the two salmonids are reared with energy-dense, lipid-rich diets.

Worldwide annual production of **fishmeal** (about 6 million tons) and **fish oil** (less than 1 million tons) has remained fairly stable for the last 20 years. Fishmeal and fish oil are produced from designated pelagic fisheries, mainly from Chile, Peru and the Atlantic. Efforts are constantly underway to ensure that the marine fisheries on which fishmeal and fish oil depend remain sustainable and are not over-exploited. Fishmeal and oil are also produced from trimmings, offal and/or by-catch, although to a limited extent. But within the European Union, it is estimated that in 2002 about 33% of the fishmeal produced was manufactured from trimmings from food fish processing (Huntington et al., 2004). No comparable data is available at the global level (FAO 2005).

The traditional dependence of aquaculture of carnivorous and omnivorous species on fishmeal and fish oil raises questions as to the sustainability of this sector of the industry (FAO, 2002). Given the predictable increase in feed resources for the growing aquaculture industry, the risk of deficits in these ingredients is real. From a global perspective, it is recognised that the pressure on natural marine resources should be lowered. For the preservation and optimal use of wild fish stocks and for the healthy development of aquaculture, research on alternative protein and oil sources is therefore essential (FAO, 2003) and has gained momentum over the past decades. The main driving force is to meet the protein, amino acid and fatty acid requirements of farmed fish without relying too heavily on fishmeal and fish oil.

The change from fishmeal and oil to various **plant ingredients** or other marine alternatives might imply several metabolic and health challenges for the farmed fish (Figure 2.1). E.g. nature has equipped many plants with the capacity to synthesize a variety of chemical substances that are known to exert harmful effects when ingested.

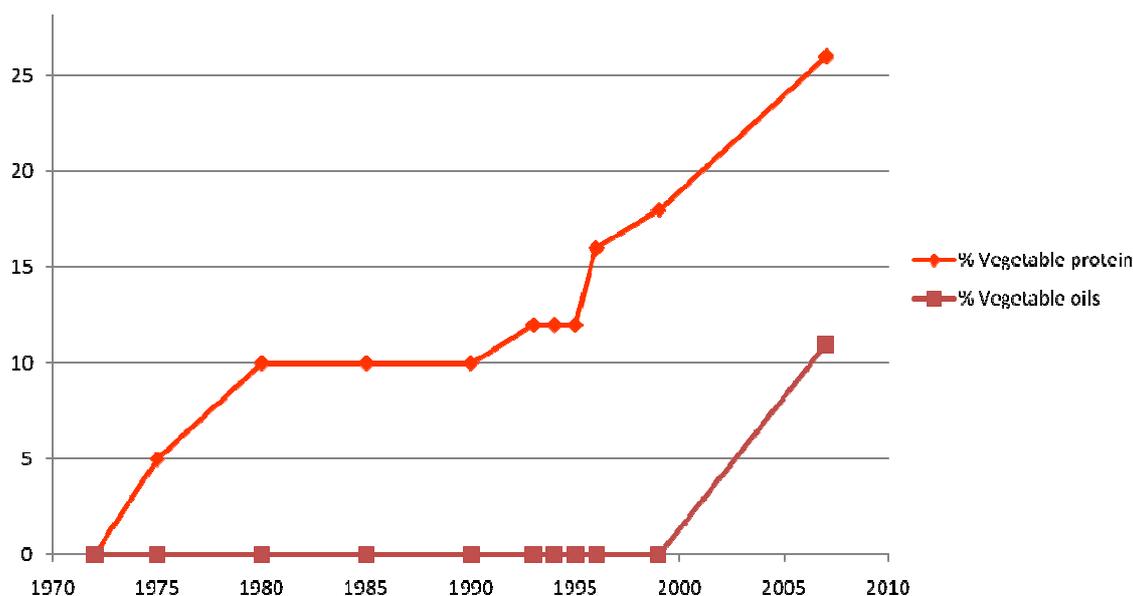


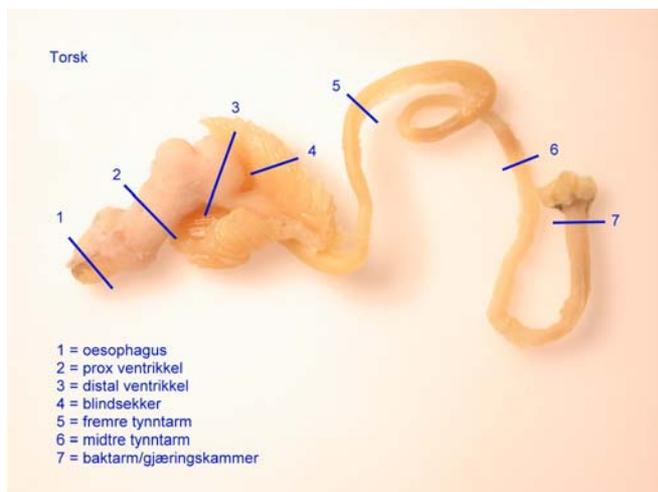
Figure 2.1. The use of plant protein mixtures and oil mixtures in fish diets in Norway from 1970 and until today. The data are based on information from Norwegian feed companies, and designed by Ole Torrissen, 2008. The Y-axis shows plant proteins and lipids as volume % of total diet.

Although there might not be an immediate violent reaction to a certain food there might still be a slow cumulative adverse effect resulting in overt disease or less than optimal health. This poses a great challenge, since knowledge of these effects is gained slowly and with difficulty, particularly if the causative principles remain unidentified (Liener, 1980). Further, fishmeal and oil are well balanced with regard to protein content, amino acid and fatty acid profiles, essential minerals and some B-vitamins, while plant fatty acid and amino acid profiles deviate strongly from the traditional marine resources. This places plant-derived feedstuffs at a disadvantage to fish-based ingredients in terms of their suitability for use in aquafeeds.

Specific **nutritional requirements** are scarcely described for Atlantic salmon, Atlantic halibut and Atlantic cod. The major body of literature on requirements is based on studies with small fish (mostly rainbow trout) fed either purified or semipurified diets. Changes in fish genetics, husbandry and management routines, huge improvements in **growth rates** and increased feed utilization have been obtained after establishment of most of the requirement data (NRC 1993). In 2008 fish diets in Norway were based on approximately 55% marine ingredients, the remaining being plant oils, plant proteins, binders, vitamins and antioxidants ([http://www.fhl.no/om\\_fiskefor/](http://www.fhl.no/om_fiskefor/)). The change to novel feed ingredients with different **nutrient profiles** has reinforced the need for updated requirement data, as we may assume that requirements have changed along with the other advances made in the aquaculture industry. Plant protein-based diets need to be adjusted for several limiting **amino acids, minerals** and **vitamins**, to mimic fishmeal and meet nutritional requirements for the various farmed fish species. Particularly the amino acids lysine and methionine, found to be low in several of the plant protein products on the market, have recently been in focus (Espe et al. 2006; Hansen et al. 2007). Even when levels of these amino acids were adjusted to meet requirements according to NRC (1993), the plant-based diets were found to result in enlarged lipid depots and liver sizes in Atlantic salmon, possibly signs of deficiencies in these amino acids (Espe et al. 2007, 2008). Most likely, the early requirement data (NRC, 1993) does not entirely cover the nutrient requirements for optimal fish health today because of

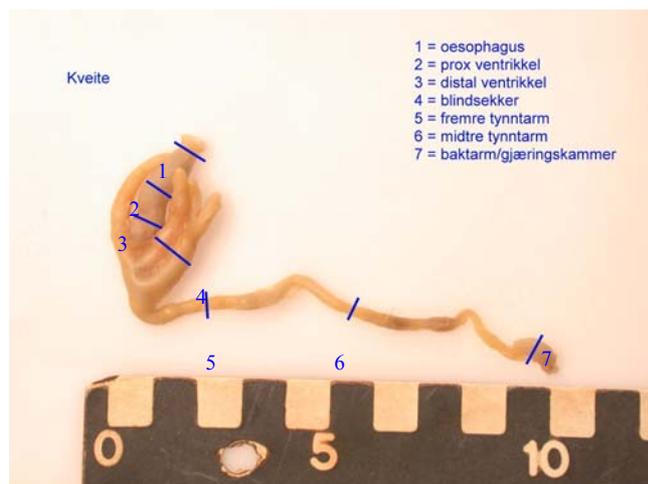
improvements over the years in e.g. fish growth rates due to selective breeding and other optimized production practices. Similar health implications were apparent when the marine oil fraction was substituted with plant oils in salmon diets (Ruyter et al. 2006; Jordal et al. 2007), despite the fact that the diets contained essential fatty acids from the fishmeal fraction.

In addition to the marked differences in growth rates within a species compared to when requirement data was established (1993), large species differences exist and we cannot use data from studies on one species and implement with success to another. For example, cod show a very different plant protein utilization capacity or tolerance to components present in protein rich plants, as compared to salmon (Krogdahl et al. 2005; Hansen et al. 2006). This illustrates the need for species specific data on health implications when introducing new plant-based diets. Some of the explanation of the huge species difference can be seen when studying the **gastrointestinal tract (GIT)** of the salmonids compared to halibut and cod. After metamorphosis all of them have a functional stomach, however, the length and size of the intestine (post-gastric alimentary tract) varies markedly, and may be indicative of a variable capacity to utilize plant ingredients. E.g. cod has a long intestinal tract with numerous pyloric caeca and a distal “fermentation chamber”, more consistent with an “omnivorous” type of GIT (Figure 2.2).



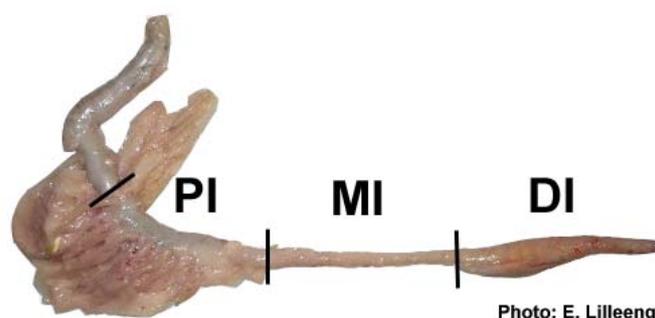
**Figure 2.2.** The gastrointestinal tract of Atlantic cod. The numbers designate: (1) oesophagus, (2) proximal stomach, (3) distal stomach, (4) pyloric caeca of the proximal intestine, (5) proximal mid intestine, (6) distal mid intestine, and (7) distal chamber/fermentation chamber. (photo by Thor Landsverk, NVH)

Ontogenic studies during early life stages describe the Atlantic halibut GIT (Gisbert et al. 2004). The findings, including the main observations of a short intestinal tract and 3-4 pyloric caeca, indicate that halibut have characteristics typical for a carnivorous species (Figure 2.3).



**Figure 2.3.** The gastrointestinal tract of Atlantic halibut. The numbers designate: (1) oesophagus, (2) proximal stomach, (3) distal stomach, (4) pyloric caeca of the proximal intestine, (5) proximal mid intestine, (6) distal mid intestine, and (7) distal chamber/fermentation chamber. (Photo by Thor Landsverk, NVH)

Atlantic salmon and rainbow trout also have a short intestine and a moderate number of pyloric caeca (Figure 2.4). The Atlantic salmon seems to be less tolerant to plant ingredients than cod, indicating that appearance of the GIT may give a first indication of the capacity to tolerate the fibre / anti-nutrient fraction of the plant ingredients.



**Figure 2.4.** The gastrointestinal tract of Atlantic salmon. The intestine is anatomically divided into the regions proximal intestine (PI) with the pyloric caeca, mid intestine (MI), and distal intestine (DI). The stomach is located to the left of the proximal intestine (PI).

Furthermore, with the increased inclusion of plant-based feedstuffs in diets, the intake of antinutritional factors (ANFs), including fibre, will increase. The various effects different ANFs have on digestive physiology and ultimately on metabolism will change utilization of specific nutrients (see review by Francis et al., 2001). This will change the dietary levels of specific nutrients needed to meet nutritional requirements. Such adjustments require extensive research in addition to the research needed to adjust recommended nutrient requirements for today's farmed fish.

From the official **statistics on production** in Norway, a total of 736,168 tons of salmon was sold in 2007. Rainbow trout sale was 77,578 tons and Atlantic cod sale was 9,611 tons. Cod production showed a small reduction compared to 2006. However, expectations for substantial production growth exist especially for the Atlantic cod. Only small volumes of other marine species were reported, among these Atlantic halibut, Arctic charr and plaice. The statistics in volume increases confirm an increased need for fish feed in the years to come (Statistikk for akvakultur, Fiskeridirektoratet 2008). With the volume increases come challenges for sufficient amounts of feed and the correct choice of feed ingredients that support nutritional requirements and promote optimal fish health.

Critical to the survival of any species is the optimal operation of their **immune defence systems**. Although fish have set the evolutionary paradigm for the ontogeny of all vertebrate immune systems, they possess and retain mechanisms unique to their own physiology, anatomy and environment. Facets of immune function span and integrate a variety of disciplines from endocrinology and neurophysiology to toxicology and microbiology. The last two decades have demonstrated the extraordinary nexus that immunology serves within these sub-disciplines of ichthyology.

The best way to get rid of disease problems in an animal system seems to be through effective management practices, i.e. management of stock, soil, water, nutrition and environment. A number of approaches have been applied in an attempt to address this problem, including sanitary prophylaxis, disinfection, and chemotherapy, with particular emphasis on the use of antibiotics. The application of antibiotics and other chemicals to aquaculture is quite expensive, undesirable due to contamination to the surrounding environment, and might lead to antibiotic resistance. The use of **antimicrobial drugs**, measured as active components, dropped from approximately 50 metric tonnes in 1987 to 746.5 kg in 1997 in Norway, and in 2007 is still at the same low level (less than 1,000 kg in salmonid production), in spite of substantial production increases (Verschuere et al. 2000; Fiskeridirektoratet 2008, Mattilsynet, 2008). The decreased use is partly due to widespread use of vaccination against specific diseases. However, there are practical difficulties and undesirable consequences associated with some of these approaches. An alternative approach has been the application of various compounds to boost or stimulate the innate immune system of cultured fish. These compounds, termed immunostimulants, are substances (drugs and nutrients) that stimulate the immune system by inducing activation or increasing activity of any of its components, and include bacteria and bacterial products, complex carbohydrates, nutritional factors such as vitamin E ( $\alpha$ -tocopherol), vitamin C, a combination of these two vitamins, vitamin A, lipids, lectins and plant extracts.

The use of feed ingredients, of both plant and animal origin, is set by the regulation "Forskrift 7. November 2002 nr 1290", and amendments. The objective of the regulation is to protect animals, consumers and the environment. For animals, the feed shall not pose a risk, or danger, to their health.

The different chapters of the present risk assessment will give a background and focus on possible consequences resulting from maximizing plant ingredients in fish diets. The focus is on knowledge from Atlantic salmon, rainbow trout, Atlantic halibut and Atlantic cod. However, since literature is scarce, the theoretical background includes studies on other fish species when relevant for the present terms of reference and to better be able to conclude on eventual health implications due to changes in diet ingredients.

### 3. TERMS OF REFERENCE

*The Norwegian Food Safety Authority asked the Scientific Committee for Food Safety, Panel 6 on Feed for Terrestrial and Aquatic Animals, to assess criteria to be applied when evaluating plant ingredients to be used in fish feed, so that these fulfil the Feed regulation §7 to "not induce health damages to the animal", and in this context aquacultured fish.*

*Further, in particular, to identify plant ingredients which might induce long-term negative effects affecting fish health, and should therefore be recommended limited. "Long-term effects" refers to substances that might affect the fish health beyond normal production time for consumption, e.g. when included in broodstock diets.*

*In particular the risk assessment should address the following issues:*

- *Assess if plant ingredients contain specific protein types or protein fractions that should be limited in fish diets, and identify these.*
- *Identify anti-nutrients in plants that are already in use or are planned to be used, and assess to what extent the various plant ingredients can be tolerated by the fish.*
- *Assess interactions between anti-nutrients, and how such interactions should be considered when plant ingredients are to be used in diets for various aquacultured fish species.*
- *Determine whether the use of plant ingredients with high fibre contents should be limited in fish diets.*
- *Assess if plant lipids should be limited in fish diets.*
- *Assess whether feed ingredients containing glucans, nucleotides or other potent molecules, added due to their immunostimulatory effects, should be limited in diets for aquacultured fish species.*
- *Assess if processing methods, including the use of processing aids, could influence the ingredient to such an extent that the processing aid might be a risk factor for the aquacultured fish species.*

*The risk assessment should identify if various aquacultured fish species have different tolerance to the various plant ingredients, and in which way. In particular Atlantic salmon, rainbow trout, Atlantic cod and Atlantic halibut should be included.*

## **4. ASSESSMENT**

### **4.1. UTILIZATION OF PLANT PROTEIN RESOURCES BY FISH**

As the use of fishmeal in the aquaculture industry decreases for various reasons, alternative, more cost-effective feedstuffs are being increasingly used as protein sources in formulated feeds for farmed fish. Various sources have been attempted from plant, microbial, and other animal sources. However, at least some of these alternative feed ingredients have been reported to have negative consequences on growth and feed utilization of farmed fish, depending on fish species and inclusion level in their diets. The causes, to name some possibilities that have been studied and documented, may lie in low levels of protein and  $\omega$ -3 fatty acids, unfavourable amino acid and mineral profiles, high levels of fibre 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. The causatory agent(s) in soybeans that lead to reduced nutrient digestibility and the inflammatory response in the distal intestine are still largely unknown, but effects on many specific digestive processes, from feed intake to enzyme activities to nutrient transport to gut histology, have been reported and are described in this chapter. However, such details are often lacking in studies focusing on other alternative protein sources that are already in use in formulated feeds for various fish species. Thus the long-term implications they may have on fish production, health, and product quality are questionable, and this topic deserves continued, substantial investments in further research to preserve and enhance the sustainability of the aquaculture industry.

#### 4.1.1. FISH HEALTH

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, ANFs and non-nutrients also comprising food antigens and microorganisms. The digestive apparatus adjusts to changing diet composition, and the mucosal defense system provided by the GIT must protect the body from injurious agents and at the same time develop oral tolerance to 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. Following are some:

- Nutrient requirements at various developmental stages
- Basic elements of digestive functions and suitable indicators of malfunction are major hindrances to 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 microflora 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.

#### 4.1.2. FISH HEALTH AND PLANT-BASED FEEDSTUFFS

The study of effects that various alternative protein-rich ingredients have on fish has largely been restricted to fish growth, feed conversion efficiency, and digestibility of the feed's nutrients. The current report is an attempt to examine more specific effects on gastrointestinal digestive function and health. Soybean products have been the focus of a large number of in-depth feeding trials and will therefore be the initial focus. If not otherwise stated, reported effects of the various protein sources on digestive function are in comparison to a fishmeal-containing diet, the protein source of choice for a control diet. Seemingly conflicting results from different studies as well as quantitative and qualitative differences in effects may be due to:

- Varying practices of fasting prior to sampling intestine for histological and physiological investigation (see Baeverfjord and Krogdahl, 1996; Krogdahl and Bakke-McKellep, 2005 for effects of fasting and re-feeding);
- Tolerance level of the fish species;
- Developmental stage of the fish;
- Genetic and environmental influences that may govern the plants' nutrient and antinutrient composition;
- Type and degree of processing used for the alternative protein source;
- Protein level of the diets;
- Quality of the fishmeal (see Hardy, 1996) or other protein source in the control diet.

Due to this number of unknowns that can influence results of a particular study, little specific data is presented and the results of studies are discussed in more general terms.

### 4.1.3. LEGUMES

#### 4.1.3.1. Soybeans (*Glycine max*)

Soybean meal (SBM) is one of the most commonly used protein sources 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 (Sandholm et al., 1976; Tacon et al., 1983; Davies and Morris, 1997). More specifically, SBM inclusion in the diet causes lower feed intake, weight gain, fecal dry matter, and energy and fat digestibilities in all salmonid species studied (Dabrowski et al., 1989; Rumsey et al., 1993 and 1994; Olli et al., 1994b; Olli and Krogdahl, 1994 and 1995; Kaushik et al., 1995; Refstie et al., 1997 and 1998; Bureau et al., 1998; Storebakken et al., 1998; Arndt et al., 1999; Burrells et al., 1999; Krogdahl et al., 2003). Dehulled SBM as the sole protein source may lead to growth arrest and increased mortality in rainbow trout, *Oncorhynchus mykiss* (Dabrowski et al., 1989; Rumsey et al., 1994). Full-fat SBM, however, appears to support better growth than solvent-extracted SBM in rainbow trout (Smith, 1977; Tacon et al., 1983; Oliva-Teles et al., 1994; Olli and Krogdahl, 1994) and Atlantic salmon, *Salmo salar* (Olli et al., 1994b). Dehulled, solvent-extracted SBM caused similar negative effects on growth and nutrient digestibility in Atlantic salmon as SBM produced from hulled soybeans (Olli et al., 1994b). White flakes, which are dehulled, moderately toasted solvent-extracted SBM, has been reported to cause similar reductions in growth, feed efficiency ratio, and nutrient digestibilities as standard SBM in Atlantic salmon (Refstie et al., 2005).

Apparent protein and energy digestibilities in Atlantic cod (*Gadus morhua*) for standard soybean meal has been estimated to be 92.3 and 88.1%, for soy protein concentrate 98.6 and 94.9%, and for soy protein isolate 97.4 and 92.1%, respectively (Tibbetts et al., 2006). One and two-year old Atlantic cod appear to tolerate up to 24% dietary inclusion of extracted soybean meal as well as a bioprocessed SBM (BPSBM), a process that reduces oligosaccharide and phytic acid content (Førde-Skjærvik et al., 2006; Refstie et al., 2006a and c). No effect was found on growth, nutrient utilization or body composition although digestibility of crude protein, amino acids and lipid, feed efficiency ratio, and protein retention were somewhat reduced in fish fed the soy containing diets following the 84-day trial (Førde-Skjærvik et al., 2006; Refstie et al., 2006a). Cod appear to compensate for lower digestibility of macronutrients in the diet by increasing feed intake (Hansen et al., 2006; Refstie et al., 2006a). Similar findings were observed in cod fed full-fat SBM at inclusion levels from 0-36%. In these fish the fatty acid composition of the muscle and liver were affected by the diet in a linear relationship (Karalazos et al., 2007).

Apparent protein and energy digestibility of dehulled soybean meal in juvenile haddock (*Melanogrammus aeglefinus*) was calculated to be 96.1 and 88.0%, respectively. There were no significant differences in nutrient digestibilities in the diets with SBM inclusion levels ranging from 10 to 40% (Kim et al., 2007).

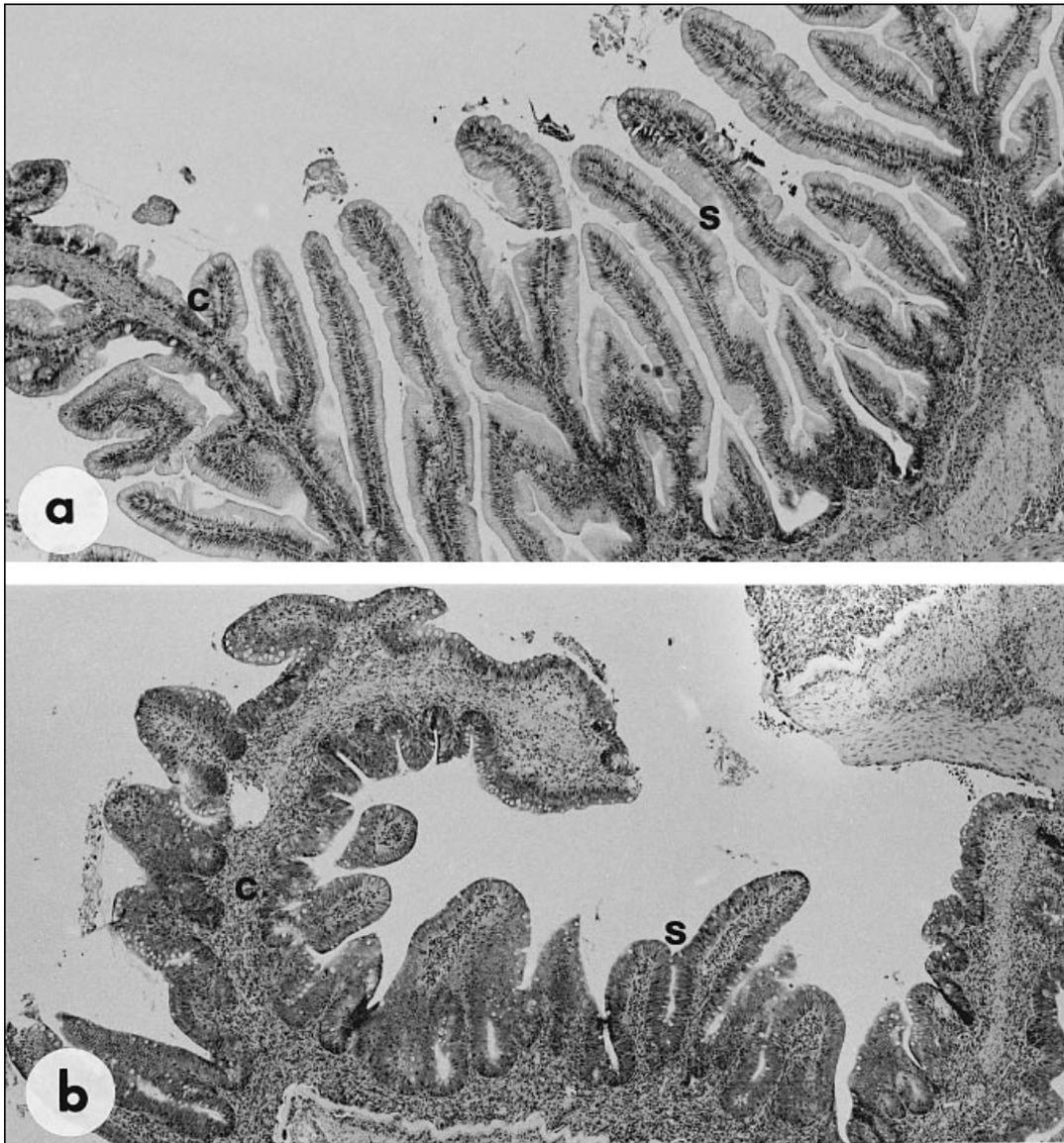
Lower growth performance and/or apparent digestibility coefficients for dry matter, energy, lipid, and/or protein in SBM-containing diets for other carnivorous species such as European sea bass, *Dicentrarchus labrax* (Tibaldi et al., 2006), largemouth bass, *Micropterus salmoides* (Portz and Cyrino, 2004), gilthead sea bream, *Sparus aurata* (Robaina et al., 1995; Venou et al., 2006), yellow perch, *Perca Flavescens* (Kasper et al., 2007), fingerling red drum, *Sciaenops ocellatus* (Reigh and Ellis, 1992), Australian snapper (red sea bream; squirefish), *Pagrus (Chrysophrys) auratus* (Quartararo et al., 1998) and Japanese flounder,

*Paralichthys olivaceus* (Deng et al., 2006) have also been observed. Lower weight gain, feed efficiency, and protein efficiency ratio at higher inclusion levels of SBM have been reported in juvenile coho, *Rachycentron canadum* (Chou et al., 2004; Zhou et al., 2005), although specific effects on digestive function are not known. In more omnivorous and herbivorous fish species, adequately heat-treated SBM does not always negatively affect digestive function, as indicated by weight gains, protein efficiency ratio (PER), net protein utilization (NPU), and/or protein and energy retention, in common carp, *Cyprinus carpio* (Viola et al., 1982), various tilapia (*Oreochromis*) species (Davis and Stickney, 1978; Shiao et al., 1987; El-Sayed, 1999), channel catfish, *Ictalurus punctatus* (Peres et al., 2003), and pacu, *Piaractus mesopotamicus* (Ostaszewska et al., 2005). However, growth performance of grass carp (*Ctenopharyngodon idellus*) fry (Dabrowski and Kozack, 1979) and tilapia, *Oreochromis mossambicus* (Jackson et al., 1982) declined with increasing commercial SBM inclusion. It has been suggested that heat-treated, solvent-extracted or full-fat SBM can completely replace fishmeal in common carp diets as long as limiting amino acids and minerals are supplemented (Viola et al., 1982; Abel et al., 1984). Studies in fingerling channel catfish in aquaria conducted in a similar time period by one group of scientists, however, suggest that dietary heat-treated, extracted SBM can result in considerably lower rates of weight gain (Gatlin and Wilson, 1984; Wilson and Poe, 1985), compared to fish fed comparable, purified casein diets (Gatlin et al., 1982; Wilson et al., 1983) in 10-week feeding trials. A more recent study confirms this: soy protein concentrate (SPC) as the sole protein source (41% of the diet) led to a 606% weight gain in an 8-week feeding trial whereas substituting the SPC incrementally with SBM from 14-55% of the diet led to dose-dependent reductions in weight gain (582-217%; Twibell and Wilson, 2004). Much of the differences in growth were explained by a negative impact of SBM on feed intake. Interestingly, the authors found that supplementing a diet with SBM as the sole protein source with 1% cholesterol significantly increased weight gain and feed intake. In another study, reducing the dietary content of solvent-extracted SBM from 45 to 22.5% led to improved growth and feed efficiency in channel catfish fingerlings (Barros et al., 2002).

The reasons for the lowered lipid and protein digestibility in Atlantic salmon fed SBM may at least be partly explained by reports of lowered bile acid concentrations (Kraugerud et al., 2007), which may affect lipid digestion and absorption, as well as trypsin activity (Olli et al., 1994a; Lilleeng et al., 2007), which may affect protein digestion, in the intestinal contents of the proximal (including the pyloric caeca) and mid intestine. Since these regions of the intestine together have been reported to be responsible for more than 90% of the total apparent absorptive capacity for lipid and amino acid nitrogen (Krogdahl et al., 1999), these decreases in bile acid concentration and trypsin activity can have substantial implications for the intestinal digestion and ultimate absorption of these nutrients. The mechanisms behind these decreases are not fully understood but the involvement of various antinutritional factors including trypsin inhibitors and saponins are likely (see Chapter 6 for further details concerning effects of antinutritional factors). Furthermore, histological changes in the pancreas and liver (Ostaszewska et al., 2005) and metabolic changes in the liver (Martin et al., 2003) of SBM-fed rainbow trout have been reported. The histological changes in the pancreas include adipose cell accumulation, and increased cytoplasmic density and reduced nucleus size in the exocrine cells of fish fed 32% soy protein concentrate and 44% SBM in their diets. These changes in the exocrine cells indicate a depletion of the enzyme-containing granules at high dietary levels of SBM (Ostaszewska et al., 2005) and may explain the reduced trypsin activity in intestinal contents mentioned above (Olli et al., 1994a; Lilleeng et al., 2007). In the liver, irregularly sized hepatocytes with pyknotic nuclei were observed in trout fed soy protein concentrate, and fatty accumulation in hepatocytes was seen in fish fed SBM (Ostaszewska et al., 2005). Protein profiling of liver proteins indicates shifts toward hepatic catabolic

pathways, increased or inefficient protein turnover, down-regulation of some structural protein expression, heightened immune response, altered levels of several stress proteins, and changes in cholesterol metabolizing enzymes in the SBM-fed fish (Martin et al., 2003). Changes in cholesterol metabolizing enzymes may alter bile acid production and can help explain the decreased bile acid concentrations in the intestinal content mentioned above (Kraugerud et al., 2007).

Structural and functional changes in the distal intestine may also explain SBM's deleterious effects on nutrient utilization. Morphological changes in this region of the intestine of salmonids (see Figure 5.3.1.1) are caused by dietary inclusion of full-fat as well as solvent-extracted SBM, with and without hulls, and also by the alcohol-extract (molasses) resulting from alcohol washing of SBM to produce soy protein concentrate (van den Ingh et al., 1991, 1996; Rumsey et al., 1994; Krogdahl et al., 1995; Baeverfjord and Krogdahl, 1996; Bureau et al., 1998; Ostaszewska et al., 2005; Aslaksen et al., 2007). They have been described in Atlantic salmon as followed: shortening of the simple and complex 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 putative T-cells (Bakke-McKellep et al., 2007a). Regarding the epithelial cells lining the mucosal folds, the number of cells in early stages of development are significantly increased, as are the number of cells undergoing cellular repair and programmed cell death (apoptosis) (Bakke-McKellep et al., 2007b).



**Figure 4.1.3.1.1.** Light microscopic image (taken with the x40 objective) of the distal intestinal mucosa of Atlantic salmon fed (a) a diet in which fishmeal was the sole protein source or (b) a diet containing 33% extracted soybean meal. In the latter, the lamina propria of the simple (s) and complex (c) intestinal folds are widened and infiltrated with a mixed population of leukocytes. The folds in (b) are also reduced in height compared to (a) (from Baeverfjord and Krogdahl, 1996).

In rainbow trout, the condition has been described similarly, with the 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. The distal intestine of rainbow trout may not be as detrimentally affected by soybean meal as Atlantic salmon. Rainbow trout have in fact been reported to adapt to soybean meal and resume acceptable growth and feed consumption rates following a period of acclimatization to the diet (Olli and Krogdahl, 1994; Refstie et al., 1997 and 2000). There are no reports of Atlantic salmon adapting to soybean meal. In recent studies, supplementation of bovine bile salts 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-supplemented 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 in Atlantic salmon has not been reported.

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 (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), although 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 increased general immunoglobulin levels (Rumsey et al., 1994). Serum and head kidney macrophage activities were depressed in trout fed diets containing high levels (60-80%) of dehulled 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.

Morphology has been studied but changes have not been observed in any regions of the intestine of Atlantic cod fed up to 24% dehulled, extracted SBM, bioprocessed SBM (BPSBM) or soy concentrate (Hansen et al., 2006; Førde-Skjærvi et al., 2006; Refstie et al., 2006c). The SBM diets did, however, increase the weight of intestinal regions proximal to the hindgut (Refstie et al., 2006c). Trypsin and leucine aminopeptidase activities were not affected by SBM, nor were rates of nutrient absorption. However BPSBM did slow absorption rates (Refstie et al., 2006c). Normal intestinal morphology was also observed in mangrove red snapper (*Lutjanus argentimaculatus* Forsskal 1775) fed up to 48% defatted SBM (Catacutan and Pagador, 2004), Atlantic halibut (*Hippoglossus hippoglossus*) fed up to 36% toasted full-fat SBM (Grisdale-Helland et al., 2002), Egyptian sole (*Solea aegyptiaca*) fed up to 30% SBM (48% crude protein; Bonaldo et al., 2006), pacu fed 50% extracted SBM (Ostaszewska et al., 2005), 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% dehulled, 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).

As mentioned above, SBM causes shortening of microvilli of the brush border membrane in Atlantic salmon (van den Ingh et al., 1991). Concomitantly, the activity of digestive enzymes in the epithelial cells' brush border membrane (e.g. alkaline phosphatase, maltase, leucine aminopeptidase, 5'-nucleotidase, and Mg<sup>2+</sup>-dependent ATPase) of the salmon distal intestine are significantly and dose-dependently reduced by including soybean meal in the diet (Krogdahl et al., 1995; Bakke-McKellep et al., 2000; Krogdahl et al., 2003). Cytosolic enzyme activities – alkaline and acid phosphatase, non-specific esterase, and alanine aminopeptidase – are also decreased (Bakke-McKellep et al., 2000), suggesting reduced supranuclear vacuole formation, number, size, and/or enzyme activity. These vacuoles have been suggested to function during endocytosis (McLean and Ash, 1987; Sire et al., 1992; Sire and Vernier, 1992). Macromolecular uptake of horseradish peroxidase (MW 44,000) from the distal intestine is reduced or slowed down in SBM-fed Atlantic salmon

(Bakke-McKellep, 1999), further suggesting that endocytosis is diminished during inflammation.

Reduced distal intestinal weight, along with the apparently decreased carrier-mediated transport, causing reduced activity and total capacity to absorb nutrients in both freshwater and seawater-adapted rainbow trout and Atlantic salmon (Nordrum et al., 2000), are most likely also contributing factors to the lower nutrient digestibilities as well. Dabrowski and co-authors (1989) also observed lower amino acid absorption in SBM-fed rainbow trout. The decreased digestive enzyme and nutrient absorptive capacity of the distal intestine, in conjunction with the increased trypsin activity in the feces in SBM-fed trout and salmon (Dabrowski et al., 1989; Olli et al., 1994a; Krogdahl et al., 2003; Romarheim et al., 2006) indicate increased losses of both dietary nutrients as well as a loss in the ability to reabsorb endogenous digestive secretions.

Numbers of proliferating cells lining the villous folds of the distal intestine of Atlantic salmon increased both in post-smolts fed a relatively high level (25%) of dehulled, toasted and extracted SBM (Bakke-McKellep et al., 2007b) as well as parr fed relatively low levels (12.5%) of full-fat soybean meal (Sanden et al., 2005). 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 enteritic changes. Immature cells have reduced function or number of integral proteins (enzymes, transporters, etc.) inserted in their apical membranes (Pusztai, 1989) and/or different surface receptor glycosylation profiles (Pusztai et al., 1995).

Krogdahl and co-workers (2000) carried out a challenge trial in an attempt to assess the consequences of the inflammatory response and other pathophysiological changes caused by SBM in salmon to their health, as measured by disease susceptibility. Following a three-week feeding trial, Atlantic salmon fed a fishmeal-based control diet, an extracted SBM-containing diet (24%) or a soy concentrate-containing diet (17.8%) were subsequently infected with *Aeromonas salmonicida* ssp. *salmonicida*, the furunculosis-causing bacterium, by infected cohabitants. Following 28 days, the cumulative mortality of the SBM-fed fish was significantly higher (65.6%) compared to the fishmeal-fed (62.9%) and soy concentrate-fed (60.5%) fish. Thus the SBM-induced enteritis may have detrimental consequences to the susceptibility of the fish to infectious agents.

#### 4.1.3.2. Lupin (*Lupinus* sp.)

As demonstrated in several fish species, the proteins in kernel meals of dehulled white (*Lupinus albus*), sweet (*L. angustifolius*) and yellow (*L. luteus*) lupins are highly digestible (Hughes, 1988; Robaina et al., 1995; Burel et al., 2000; Glencross and Hawkins, 2004; Glencross et al., 2003b and c, 2004a and b; Tibbetts et al., 2006; Aslaksen et al., 2007). There may be varietal differences in macronutrient digestibilities, however, as demonstrated for sweet lupin kernel meal fed to rainbow trout (Glencross et al., 2008b). Besides the relatively high oligosaccharide content (Glencross et al., 2003a) and quinolizidine alkaloids (Glencross et al., 2006), lupin species contain low levels of ANFs. Following dose-response studies, it has been estimated that sweet lupin kernel meal may be included in diets for rainbow trout up to 30 (Glencross et al., 2008a) or 40% (Farhangi and Carter, 2001) without significantly influencing growth and nutrient utilization. At 50% inclusion level, the maximum level tested (Farhangi and Carter, 2001), growth, feed conversion ratio, and energy efficiency ratio were negatively affected although trypsin and amylase activity in the pyloric caeca were not affected. In gilthead sea bream, however, 30% lupin seed meal in the diet retarded trypsin activity (Robaina et al., 1995). Villous height in the proximal intestine showed an insignificant tendency to become shorter with increasing lupin inclusion level in the diets

(Farhangi and Carter, 2001). Other intestinal regions were, however, not histologically assessed in this study. No histological changes were observed in the pyloric caeca or distal intestine of rainbow trout fed up to 50% yellow lupin kernel meal, although hepatocytes appeared to have a lower level of lipid droplets in the fish fed 50% inclusion level (Glencross et al., 2004b). Nor were histological changes observed in the distal intestine of Atlantic salmon fed 24% dehulled, low-alkaloid white lupin meal (Aslaksen et al., 2007), or 30% kernel meals or protein concentrates from yellow lupin or sweet lupin (Refstie et al., 2006b). In the stomachs of these fish, however, the latter study reported higher severity of ulcer-like lesions connected with the lupin-containing diets (Refstie et al., 2006b). In the same study, kernel meal and protein concentrate from yellow lupin also led to higher relative weights of the gastrointestinal tracts, although none of the lupin-containing diets led to changes in bile acid concentrations or trypsin activities in the intestinal contents, or leucine aminopeptidase or maltase activities in the intestinal walls of any intestinal region (Refstie et al., 2006b). In gilthead sea bream, up to 20% sweet lupin meal in the diets resulted in higher apparent digestibility coefficients than in the fishmeal control or 30% lupin meal containing diets (Robaina et al., 1995). Lupin seed meal up to 30% of the diet, irrespective of heat-treatment (de la Higuera et al., 1988), extruded lupin flour up to 50% (Burel et al., 1998), and yellow lupin kernel meal up to 37.5% (Glencross et al., 2004b) supports good growth in rainbow trout. Turbot (*Psetta maxima*) digested the dry matter, protein, energy and phosphorus of diets containing 30% extruded lupin equally well as did a fishmeal-based diet (Burel et al., 2000). Oligosaccharides and quinolizidine alkaloids may contribute to reduced utilization of lupin meal at high dietary inclusion levels in rainbow trout (Glencross et al., 2003a and 2006) but otherwise lupin meals may have considerable promise as an alternative protein source in fish feeds. Genetically modified narrow-leaf lupin (*L. angustifolius*) meal, modified to contain higher methionine levels, was as acceptable as non-modified lupin in diets for red sea bream, *Pagrus auratus* (Glencross et al., 2003c). Prototype lupin protein concentrates from sweet, *L. angustifolius*, and yellow lupin, *L. luteus*, also have substantial promise as prospective feed ingredients in rainbow trout. Levels up to 40% were fed trout without any adverse effects on palatability, feed intake, fish performance or feed utilization (Glencross et al., 2006). The occurrence of gastric ulcers was not reported in this study.

#### 4.1.3.3. Pea meal (*Pisum sativum*)

Pea meal has potential as an alternative protein source although low-processed pea meal in feeds for carnivorous fish is limited by its high starch content (ca. 50%). Pea protein concentrate or isolate is therefore more suitable. Milk fish (*Chanos chanos* Forsskal) fed 15% dietary protein (20% by weight) or more from oven-dried (60°C for 4 h), finely ground pea meal grew slower and feed conversion, protein utilization, and protein and dry matter digestibility were negatively influenced (Borlongan et al., 2003). For Atlantic salmon, pea protein concentrate, produced by air separation, at inclusion levels of 21 and 28% led to apparent digestibility coefficients for dry matter, nitrogen, and energy similar to those of a commercial diet and higher than a control diet containing fishmeal as the sole protein source (Carter and Hauler, 2000). In another study, Atlantic salmon fed 20% pea protein concentrate, also produced by air separation, with crude protein concentrations of either 35 or 50% led to lower digestibility of energy, but no significant differences in digestibilities of other macronutrients or amino acids, nor on growth performance, body composition, intestinal brush border maltase activities, fecal trypsin activities, or intestinal histology compared to the fishmeal control diet (Øverland et al., 2008). However, Atlantic salmon in yet another feeding trial fed a higher inclusion level of pea protein concentrate (30%) exhibited lower growth rates, lipid and starch digestibilities, distal intestinal weight, as well as histological changes in the distal intestine characterized by shortened mucosal folds with widened lamina propria

resulting from an increased leukocyte infiltration. The epithelial cells also exhibited reduced supranuclear vacuolization and most of the cells lining the mucosal folds were immature. No other intestinal regions showed signs of inflammation (Penn et al., 2008).

Rainbow trout also digest pea protein well at lower inclusion levels (25% dehulled pea meal and 20% air-classified pea protein concentrate, Thiessen et al., 2003; 12% pea meal, Drew et al., 2005), although 30% pea meal (24.6-26.0% crude protein) in diets led to lower dry matter, protein, energy (Gomes et al., 1995; Burel et al., 2000) and particularly phosphorus digestibilities (Burel et al., 2000) compared to fishmeal-based diets. Turbot, however, showed better apparent digestibility coefficients for macronutrients and phosphorus in the extruded pea containing diets (Burel et al., 2000). In Atlantic cod, apparent protein and energy digestibilities for pea protein concentrate have also been estimated to be relatively high: 89.8 and 76.7%, respectively (Tibbetts et al., 2006). In a dose-response study, juvenile tilapia fed 30% pea protein isolate exhibited similar growth performance and feed efficiency as groups fed a fishmeal-based control diet, while groups fed diets with 45 and 60% inclusion levels did not (Schulz et al., 2007). The intestines were not examined for morphological changes in these latter studies on rainbow trout, turbot, cod or tilapia.

#### **4.1.3.4. Faba bean meal (*Vicia faba*)**

Faba bean meal has potential as an alternative protein source although low-processed bean meal in feeds for carnivorous fish is limited by its high starch content (ca. 50%). Bean protein concentrate or isolate may therefore be more suitable. In Atlantic salmon, meal from whole as well as dehulled beans at inclusion levels of 22.0 and 18.7%, respectively, resulted in similar lipid and crude protein digestibilities as the fishmeal-based control diet. Histological changes were not detected in the stomach, mid or distal intestine (Aslaksen et al., 2007).

#### **4.1.3.5. Peanut (groundnut; *Arachis hypogaea*) meal**

Peanut meal and oilcake appear to be a highly digestible, palatable protein source in formulated feeds for striped bass (*Morone saxatilis*; Small et al., 1999), hybrid striped bass (*Morone chrysops* × *M. saxatilis*; Gaylord et al., 2004), silver perch (Allan et al., 2000), and mrigala carp (*Cirrhinus mrigala*; Singh et al., 2003). High levels (>25% inclusion) fed to tilapia led to depressed growth performance and feed conversion (Jackson et al., 1982). Specific effects of peanut meal on digestive physiology and morphology of digestive organs have not been evaluated.

### **4.1.4. OTHER OILSEEDS**

#### **4.1.4.1. Rapeseed/canola (*Brassica* sp.)**

Rapeseed contains glucosinolates, thioglucosides which in the *Cruciferae* family are always accompanied by thioglucosidases, although the two are kept in separate cellular compartments. During mastication/digestion with ensuing cellular damage to the plant material, thioglucosides are broken down by the enzymes and produce isothiocyanates and nitriles, which are toxic to the animals ingesting the plants. Tissue damage to liver and kidney and changes in thyroid structure and function have been reported (see Duncan, 1991). Feeding trials of rapeseed and glucosinolates in fish have concentrated on effects on thyroid effects and are summarised by Francis and co-authors (2001). Decreased growth performance and feed efficiency in fish has generally been explained as an effect of thyroid function and general metabolism rather than reduced digestive function. However, rapeseed also contains phytic acid, which may also contribute to the lowered protein and mineral digestibility (Singh et al., 2003). Solvent-extracted rapeseed up to 50% inclusion did not negatively impact

growth performance or feed conversion in tilapia (Jackson et al., 1982). In rainbow trout, 30% solvent-extracted rapeseed meal in diets resulted in higher dry matter, protein, and energy, but lower phosphorus digestibilities than diets containing equal amounts of heat-treated rapeseed meal. In turbot, however, the heat-treated rapeseed meal was more acceptable than the solvent-extracted meal (Burel et al., 2000). In Atlantic salmon, 18.3% inclusion of low-glucosinolate, extracted and heat-treated rapeseed meal resulted in higher lipid digestibility but lower crude protein digestibility than a fishmeal control diet. Histological changes were not detected in the stomach, mid or distal intestine (Aslaksen et al., 2007).

Canola, developed from rapeseed to contain lower levels of glucosinolates, or heat-treated rapeseed meal appear to have promise as alternative protein sources for fish (Higgs et al., 1982; Allan et al., 2000; Burel et al., 2000; Singh et al., 2003; Drew et al., 2007). In rainbow trout, inclusion levels of canola protein concentrate of 19% to 38% led to reduced growth and feed intake compared to fishmeal controls in rainbow trout (Drew et al., 2007). In Atlantic cod, apparent protein and energy digestibilities for canola meal has been estimated to be 76.0 and 60.6%, and for canola protein concentrate 88.8 and 83.3%, respectively (Tibbetts et al., 2006). Specific effects of rapeseed or canola meal on digestive physiology have not been evaluated.

#### **4.1.4.2. Sunflower (*Helianthus annuus*) seed meal**

There are very few recent studies reporting results of sunflower seed meal as an alternative protein source. One on Atlantic salmon (dose-response) reported that partially dehulled sunflower meal had 88% digestible protein and could be included in post-smolt diets up to 27% (22.7% of digestible protein) without adverse effects on growth performance, feed utilization or body composition (Gill et al., 2006). At a 22.9% inclusion level of extracted and mildly heat-treated sunflower meal, higher lipid digestibility but lower crude protein digestibility than a fishmeal control diet was reported. Histological changes were not detected in the stomach, mid or distal intestine (Aslaksen et al., 2007). Morales and co-workers (1999) demonstrated that sunflower meal at an inclusion level of 41% in a diet for rainbow trout resulted in improved crude protein digestibility but reduced nitrogen-free extract and dry matter digestibilities compared to a fishmeal-based control diet. Hybrid striped bass, however, fed 30% solvent-extracted, dehulled sunflower meal showed intermediate protein and amino acid digestibilities compared to groups fed other plant feedstuffs (Gaylord et al., 2004). Tilapia displayed no negative effects on growth performance or feed conversion when fed levels up to 75% of the diet (Jackson et al., 1982). Fingerlings of a carp species, *Cirrhinus mrigala*, fed sunflower oilcake at 65% inclusion level exhibited significantly poorer growth performance, protein digestibility, and feed, energy and protein utilization compared to a groundnut oilcake-containing diet (Singh et al., 2003). Lower digestive performance in fish fed sunflower seed products has been attributed to relatively high levels of protease inhibitors, arginase inhibitor, phytic acid, and the polyphenolic tannin chlorogenic acid (Tacon et al., 1984; Singh et al., 2003). Specific effects of sunflower seed meal on digestive physiology and morphology of digestive organs have not been evaluated.

#### **4.1.4.3. Cottonseed (*Gossypium* sp.) meal**

Cottonseed contains gossypol, a polyphenolic substance with toxic effects in fish that include reduced haematocrit, haemoglobin, and total plasma protein, and liver, spleen, and kidney damage, as well as growth depression (Herman, 1970; Dabrowski et al., 2000; Garcia-Abiado et al., 2004), the latter perhaps partly due to lower apparent digestibility of crude protein (Mbahinzireki et al., 2001). In dose-response studies conducted, more than 50% solvent-extracted cottonseed meal in diets for various fish species causes growth depression (tilapia, Jackson et al., 1982; rainbow trout, Lee et al., 2002 and 2006, and Luo et al., 2006),

but levels of up to 30% of the diet (replacing 50% of fishmeal protein) appear to be well tolerated by rainbow trout juveniles and did not significantly infringe on growth parameters, feed conversion, nutrient digestibilities or mineral availability (Lee et al., 2002; Luo et al., 2006). A long-term feeding trial (35 months) showed that even inclusion levels as high as 59%, which was a complete replacement of fishmeal, did not impact fish growth negatively (Lee et al., 2006). However, female rainbow trout fertility was negatively affected by complete replacement of fishmeal with cottonseed meal. Supplementation with lysine and methionine may positively influence protein utilization at higher inclusion levels (Luo et al., 2006).

Noteworthy is that acetone extraction apparently decreases gossypol and aflatoxin levels in the cottonseed meal more efficiently than hexane extraction and results in improved crude protein and amino acid digestibilities (Cheng and Hardy, 2002). Yet the authors suggested a maximum inclusion level of 10% of acetone extracted cottonseed meal in diets for rainbow trout. Specific effects of cottonseed meal on digestive physiology and morphology of digestive organs have not been evaluated.

#### **4.1.5. CEREALS AND OTHER PLANT SOURCES**

##### **4.1.5.1. Wheat (*Triticum aestivum*) gluten**

Wheat gluten is produced by washing out the starch fraction from wheat flour and recovering the insoluble part, the gluten. Wheat gluten contains 85% crude protein on dry matter basis, but is deficient in the amino acids lysine, arginine, and methionine. It is a highly digestible protein source for rainbow trout (Pfeffer et al., 1995; Sugiura et al., 1997), Coho salmon (Sugiura et al., 1997) and Atlantic salmon (Storebakken et al., 2000). It does not reduce digestibility of energy and macronutrients, or reduce availability of minerals in the diets. It does not cause morphological changes in the intestinal tissues (Storebakken et al., 2000). Wheat gluten up to a level of 50% of dietary protein (29% by weight) can be added to salmon diets without reducing protein, amino acid, fat, and energy digestibility, although  $\alpha$ -amylase inhibitors in wheat appear to reduce starch digestibility (Storebakken et al., 2000). In Atlantic cod, apparent protein and energy digestibilities for wheat gluten meal have been estimated to be 99.9 and 95.4%, respectively (Tibbetts et al., 2006).

##### **4.1.5.2. Corn (*Zea mays*) gluten**

Corn gluten is produced by fractioning shelled corn by wet milling. Following removal of germ, oil, and fibre, starch and gluten are separated by centrifuging the starch-gluten slurry. Corn gluten contains 67% crude protein on dry matter basis, but is deficient in the amino acids lysine and arginine, and contains high levels of leucine. The digestibility of corn gluten protein is relatively high in fish (Atlantic salmon, Anderson et al., 1992; four freshwater species, Watanabe et al., 1996; Atlantic cod, Tibbetts et al., 2006; Atlantic salmon, Aslaksen et al., 2007). In Atlantic salmon, morphological changes in the stomach, mid and distal intestinal tissues were not observed when using whole corn meal, genetically modified or not, as a carbohydrate source in fishmeal-based diets for parr (Sanden et al., 2005) nor 20.4% corn gluten as a protein source in diets for post-smolts (Aslaksen et al., 2007). Specific effects of corn gluten on digestive physiology have not been evaluated.

##### **4.1.5.3. Potato (*Solanum tuberosum* L.) protein concentrate**

Potato protein concentrate is produced by thermal coagulation and precipitation of soluble proteins in potato juice, as a by-product in the processing of potatoes for potato starch production. High quality potato protein concentrate contains 85% crude protein on a dry matter basis, and the amino acid composition of the protein is well balanced for fish (Moyano

et al., 1992; Xie and Jokumsen, 1997a). Potatoes contain solanidine glycoalkaloids (SGA), among which  $\alpha$ -solanine and  $\alpha$ -chaconine are the most well-known. SGA are glycoalkaloids with solanid-5-en-3 $\beta$ -ol as their steroidal aglycone (cyclic amino alcohols of steroidal structure). These are bitter-flavoured and toxic substances present near the peel of potato tubers to protect them against disease and insects. Potatoes also contain inhibitors of proteolytic enzymes. Both SGA and enzyme inhibitors dissolve in the potato juice, and are concentrated in and presumably absorbed by the protein during manufacture of standard potato protein concentrate (Bergers, 1980; Wojnowska et al., 1981). Thus, potato protein concentrate contains 1500 to 2500  $\mu\text{g g}^{-1}$  SGA, and often has a high trypsin inhibitor activity as well. In consequence, testing of potato protein concentrate in diets for rainbow trout resulted in severe appetite loss, even at dietary inclusion levels as low as 5% (Moyano et al., 1992; Xie and Jokumsen, 1997a and b, 1998). However, if processed to remove SGA and protease inhibitors, up to 20% potato protein concentrate may be included in diets for salmonids without causing adverse effects on appetite, growth, nutrient digestibility, or nutrient retention (Refstie and Tiekstra, 2003). Effects of potato protein concentrate on the intestinal morphology have not been evaluated.

#### **4.1.5.4. Rice protein concentrate**

Rice protein concentrate is of interest in carnivorous fish production due to its high protein and lipid content as well as favourable amino acid profile. In a dose-response study (up to 53% inclusion; Palmegiano et al., 2006), rice protein concentrate in diets for rainbow trout revealed a dose-dependent decrease in apparent digestibilities of dry matter, organic matter, crude protein and gross energy as inclusion level increased. Neither digestibility of ether-extract nor fatty acid composition of the muscle was significantly affected. The authors concluded that up to 20% rice protein concentrate can be included in diets for trout without negatively affecting growth performance. Specific effects of rice protein concentrate on digestive physiology and morphology of digestive organs have not been evaluated.

#### **4.1.6. MIXTURES OF VARIOUS PROTEIN-RICH FEEDSTUFFS**

The possibility of combining alternative high-protein feedstuffs from various sources, i.e. plant, microbial, and/or animal, to complement each other in terms of ANFs, toxins, limiting nutrients etc., appears to be gaining focus in recent years. These mixes have been shown to have some promise for tilapia (El-Saidy and Gaber, 2003), gilthead sea bream (Gómez-Requeni et al., 2004; De Francesco et al., 2007), Atlantic cod (Albrektsen et al., 2006; Hansen et al., 2006 and 2007) and rainbow trout (Lee et al., 2002; De Francesco et al., 2004; Vilhelmsson et al., 2004; Rahnema et al., 2007). Complete substitution of fishmeal for plant proteins in carnivorous species such as Atlantic cod, sea bream and rainbow trout have led to depressed feed intake, growth performance, feed efficiency, and/or protein efficiency ratio (De Francesco et al., 2004; Gómez-Requeni et al., 2004; Vilhelmsson et al., 2004; Barrows et al., 2007; Hansen et al., 2007). Supplementation of all- or high-plant diets with taurine in rainbow trout (Gaylord et al., 2007) and hydroxyproline in Atlantic salmon (Aksnes et al., 2008) has been shown to benefit growth. In another study, Atlantic salmon grew well, especially regarding protein gain, on high-plant protein diets (wheat gluten and corn gluten meal) which were carefully balanced regarding amino acids and micronutrients, and supplemented with attractants to improve palatability (Espe et al., 2006).

However, in Atlantic cod fed 100% plant proteins comprised of 50% wheat gluten, 36% soy protein concentrate and 14% bioprocessed soybean meal for 28 weeks (Olsen et al., 2007), a progression toward a diarrhea-like condition, goblet cell hypertrophy and hyperplasia, as well as inflammatory changes were observed, mostly in the distal intestine but some also in the mid intestine. In gilthead sea bream, 100% replacement of fishmeal for plant

protein from corn gluten, wheat gluten, extruded peas, rapeseed meal and sweet lupin resulted in depressed feed intake and growth, increased liver fat deposition, lipid and/or lipid droplet accumulation in enterocytes and cellular infiltration of the intestinal submucosa following a six-month feeding trial (Sitja-Bobadilla et al., 2005). Altered hepatic protein profiles for various metabolic pathways suggest increased primary energy generation, reducing potential, bile acid synthesis, and protein degradation in the trout fed only plant proteins (Vilhelmsson et al., 2004). Furthermore, organoleptic characteristics and colour of rainbow trout fillets were apparently negatively influenced by the all-plant protein diet (De Francesco et al., 2004).

Thus the proportion of feedstuffs from various sources and the degree of fishmeal substitution that is acceptable will vary depending on fish species and their dietary requirements and preferences. Possible additive/synergistic effects among ANFs, antigens, and/or toxins present in the various feedstuffs that comprise a mixture should also be taken into consideration (see below).

#### **4.1.7. CONCLUSIONS – PLANT INGREDIENT**

With the exception of full-fat and extracted soybean meal for salmonids, substituting at least part of the fishmeal fraction of aquafeeds with individual plant ingredients has promise, at least in the short to medium term. Indeed in some cases, diets containing up to 20% inclusion level of high-quality plant protein sources resulted in better nutrient digestibility and growth parameters than the fishmeal-based control diets. This reveals that not all fishmeals are of ideal nutritional quality. When substituting fishmeal with plant ingredients, however, the need to balance the diets regarding limiting amino acids and minerals may be necessary. Information regarding antinutrient levels in the plant ingredients and feeds containing them is rarely given in publications reporting data from feeding trials. The combination of various plant ingredients in feeds, thereby limiting the concentration of antinutritional factors/antigens inherent in single ingredients, has promise regarding complete substitution of fishmeal. However, each blend of plant ingredients needs to be thoroughly tested in order to rule out any detrimental effects caused by combinations of ANFs/antigens (see next chapter). Long-term investigations regarding metabolic and health aspects of individual plant ingredients as well as various mixes in aquafeeds are needed for all species. Also, little knowledge exists regarding consequences of adding various plant ingredients to diets for juveniles for the various fish species, nor effects on broodstock and subsequent reproductive parameters.

#### **4.2. ANTINUTRITIONAL FACTORS IN FEEDS**

The nondescript term “antinutrients” is commonly used to refer to those substances found in foods that produce negative effects on health and nutritional balance when ingested. Although some plants are known to produce obvious signs of poisoning, much more subtle effects produced only by prolonged ingestion of a given plant are more commonly observed. Such effects might include inhibition of growth, decreased food efficiency, goiterogenesis, pancreatic hypertrophy, hypoglycaemia, and liver damage. Other factors that should be taken into consideration 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 pure antinutrients, but rather using whole, or more or less refined, feed ingredients. Even though for some of the ingredients one or a few antinutrients dominate, several antinutrients are usually present at the same time. They may all have their distinct effects and may also interact to show significant effects not observed when they are present in

the diet alone. 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. (2001a) in a review regarding antinutritional factors in plant-derived alternate fish feed ingredients and their effects. As concluded by Francis et al. (2001a) seven years ago, we also have to state in 2008: There is an urgent need for studies concerning the effects of the individual antinutrients and effects of antinutrients in combination. It is most likely that antinutrients are involved in the aetiology of diseases related to the gut function and immune apparatus that emerge in the aquaculture industry today. Antinutrients may affect nutrient balances of the diets, alter gut microflora, and/or trigger immune modulation for better or worse. Efforts must be made to isolate the antinutrients in amounts needed for physiological studies. In vitro studies of effects of pure antinutrients on cells of the gut, liver and immune system would aid in the understanding of their mechanisms of action and effects. Moreover, during investigations on the effects in fish of a plant ingredient or combination of these in feeds, a greater effort to characterize, quantify and report the antinutrients present would provide valuable information. Similarly it would be helpful if the fish feed industry would provide such information. This would be a powerful tool in assessing longer-term effects of antinutritional factors on fish health.

#### 4.2.1. ANTINUTRIENTS OF RELEVANCE IN FISH NUTRITION

Plant feed ingredients currently used in aquaculture or with the potential for use as a nutrient source for fish are listed in Table 4.2.1.1 along with the known antinutrients found in each. Unknown factors are not listed for obvious reasons. However, it is likely that components of plants not harmful to traditional farm animals or model animals such as rats and mice may be harmful to various fish species in cultivation. Moreover, it is possible that hitherto unidentified compounds in plants may be important in fish nutrition and health.

Most of the compounds listed in Table 4.2.1.1 are addressed to varying degrees in the subchapters below. The information available for the different antinutrients varies greatly, a fact that is reflected in the volume of the subchapters.

Table 4.2.1.1 Important antinutrients present in some commonly used alternative fish feed ingredients (Francis et al., 2001a).

Plant feedstuff	Antinutrient present
Soybean meal	Proteinase inhibitors, lectins, phytic acid, saponins, phytoestrogens, antivitamin, phytosterols, allergens
Kidney beans	Proteinase, amylase and lipase inhibitors, lectins, phytic acid, saponins, phytoestrogens, antivitamin, phytosterols, allergens
Rapeseed meal	Proteinase inhibitors, glucosinolates, phytic acid, tannins
Lupin seed meal	Proteinase inhibitors, saponins, phytoestrogens, alkaloids
Pea seed meal	Proteinase inhibitors, lectins, tannins, cyanogens, phytic acid, saponins, antivitamin
Sunflower oil cake	Proteinase inhibitors, saponins, arginase inhibitor
Cottonseed meal	Phytic acid, phytoestrogens, gossypol, antivitamin, cyclopropenoid acid
Alfalfa leaf meal	Proteinase inhibitors, saponins, phytoestrogens, antivitamin
Mustard oil cake	Glucosinolates, tannins
Sesame meal	Phytic acid, proteinase inhibitors

#### 4.2.2. PROTEINASE INHIBITORS

##### General characteristics

Inhibitors of proteinases, i.e. of trypsin, chymotrypsin, elastases and carboxypeptidases, are proteins that form stoichiometric complexes with the enzymes and inhibit their activity in the gastrointestinal (GI) tract. Proteinase inhibitors are found in most plants (Liener, 1980). Also, enterokinase inhibitors have been described (Baht et al., 1981). Their molecular weight differs between 6,000 and 50,000 kD. The specificity of proteinase inhibitors varies greatly. Some inhibit only one type of enzyme, e.g. trypsin or carboxypeptidase, others two or three. Some inhibit one enzyme molecule per inhibitor molecule, others two or more.

Most proteinase inhibitors belong either to the Kunitz inhibitor family or the Bowman-Birk inhibitor, first observed in soybeans (Liener, 1980). The inhibitor has a molecular weight of about 8,000 kD and is characterized by the 7 S-S bridges which stabilize the molecule. This inhibitor may bind one trypsin and one chymotrypsin at the same time. Inhibitors of similar structure are found in several legumes. The larger (21,000 kD) Kunitz inhibitor with a specific site for trypsin also binds chymotrypsin but in a less stable complex.

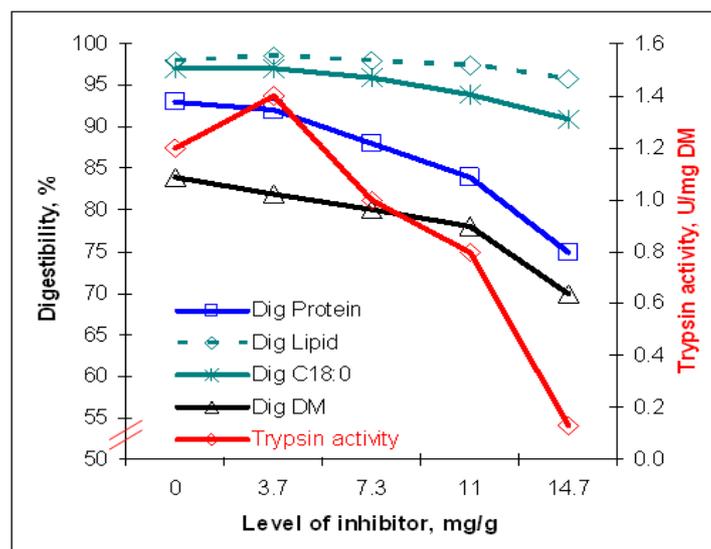
Proteinase inhibitors in unprocessed plant material are generally water soluble over a wide pH range. These inhibitors may be isolated by extraction of proteins, for example from bean meal, by solutions with pH at or above 7. The inhibitors can then, in many cases, be isolated in solution by adjusting pH, which will precipitate most other proteins (Duranti et al., 2003; Fan and Wu, 2005).

Isolation of inhibitors from heat-treated plant material represents challenges in analysis of inhibitor activity. Thorough grinding, extraction with pH adjustments both above and below 7 may be necessary. The required procedure depends on the heat processing method and conditions employed.

##### Biological effects in fish

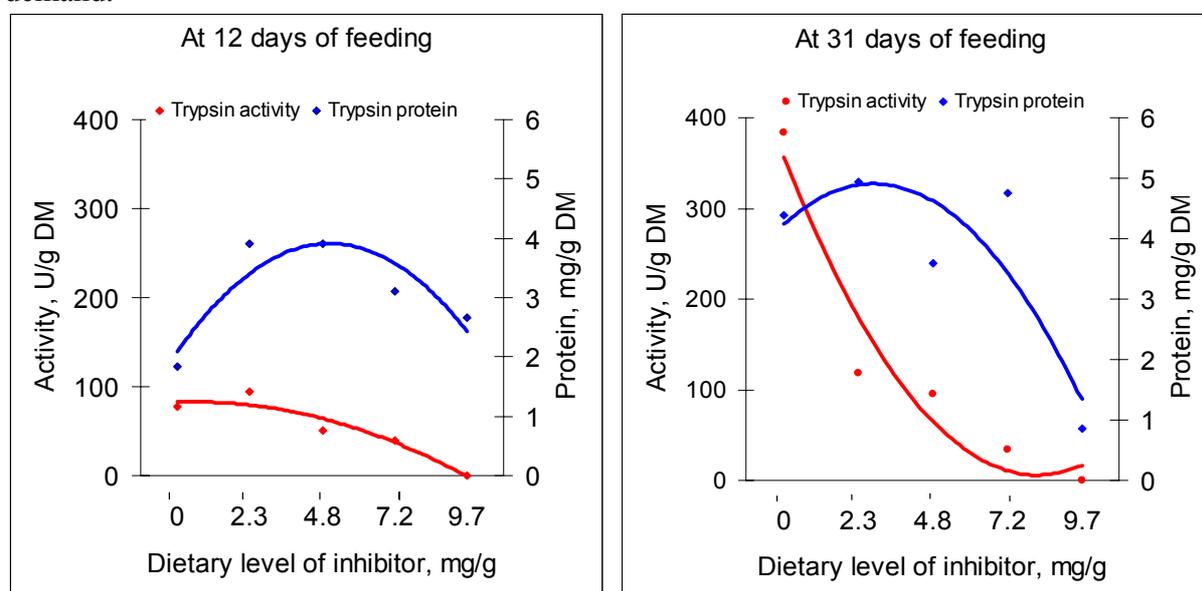
Discovered in 1947, proteinase inhibitors (Kunitz, 1947) and their effects in mammals and birds have been studied thoroughly. Based on these studies an understanding of their action has been developed (Liener, 1980). In the intestine, inhibitors first form a rather stable complex with trypsin, thereby reducing trypsin activity. This in turn stimulates secretion of PZ-CCK (pancreozymin-cholecystokinin) from the gut wall. This hormone stimulates secretion of trypsin from pancreatic tissue and stimulates the gall bladder to empty its content into the intestine. Trypsin synthesis in the pancreas is stimulated by the ingestion in particular of cysteine (and hence methionine) as trypsin is very rich in cysteine. In some animals, proteinase inhibitors cause pancreatic hypertrophy. Whether this also takes place in fish is not clear.

In studies with salmonids, proteinase inhibitors have been found to reduce apparent digestibility not only of protein but also of lipid (Berg-Lea et al., 1989; Krogdahl et al., 1994; Olli et al., 1994). The effects on digestibility correspond to a decrease in trypsin activity and presumably chymotrypsin, which is also inhibited by soybean proteinase inhibitors (Figure 6.2.1). Saturated fatty acids appear more severely affected than unsaturated.



**Figure 4.2.2.1. Effects of increasing levels of proteinase inhibitors on trypsin activity and trypsin protein in intestinal contents of rainbow trout. The samples were taken from the mid intestine between the distal most pyloric caecum and the distal intestine (Olli et al., 1994).**

The results indicate that the proteinase inhibitors stimulate pancreatic enzyme secretion causing the enzyme level of the intestinal content (trypsin protein) to increase. However, the activity in the intestinal content is not increased. The enzyme activity seems unaffected when fed diets with the lower inhibitor levels and short-term feeding (12 days; Figure 6.2.2), but higher levels decrease the activity. After longer-term feeding (Figure 6.2.2) it seems the pancreas may no longer manage to compensate for decreased enzyme activity by increasing secretion. Thus enzyme production does not appear to keep up with the increased demand.



**Figure 4.2.2.2. Effects of level of soybean proteinase inhibitors in diets for Atlantic salmon on nutrient digestibility and trypsin activity of proximal intestine (Berg-Lea et al 1989).**

A study from 1994 (Krogdahl et al., 1994) illustrates cumulative digestibilities of protein and cysteine in intestinal segments along the GI tract of rainbow trout as a function of dietary inhibitor level. The results support the findings of the previous study (Berg-Lea et al., 1989) indicating increased supply of cysteine-rich pancreatic enzymes into the GI tract. The

level of cysteine of the digesta increases sharply, giving a negative cumulative apparent digestibility in the pyloric region of the intestine.

No information has been found regarding effects of trypsin inhibitors in fish diets on gut microbiota, either qualitatively or quantitatively. It is, however, likely that by inhibiting proteolysis in the intestine and thereby increasing the substrate supply of the flora in the distal compartment, both increases in number of bacteria within species as well as shifts in the species profile of the microflora will follow.

#### 4.2.3. AMYLASE INHIBITORS

##### General characteristics

Several beans contain amylase inhibitors that may affect amylase activity in the GI tract of monogastric animals (Sawada et al., 2002). The presence of  $\alpha$ -amylase inhibitors in beans (*Phaseolus vulgaris*) was first reported in 1945 (reviewed by Gibbs and Alli, 1998; Whitaker, 1997). The  $\alpha$ -amylase inhibitor from the kidney bean has received considerable attention because of its high potency compared with inhibitors from other plants, and the possibility that it contains several distinct types of inhibitors. Some inhibitors from white kidney bean have been postulated to be glyco-proteins with an oligomere structure comprised of identical peptides or different polypeptides. The presence of  $\alpha$ -amylase inhibitors has also been reported in wheat, millet, sorghum, maize, barley, rye, mangoes, peanuts, bajra, *Colocasia esculenta* tubers and acorns.

##### Biological effects in fish

Studies of effects of amylase inhibitors in fish are limited. Studies by Fernandez et al. (2001) and Natarajan et al. (1992) have shown that an inhibitor from wheat is active against amylase from sea bream, carp and tilapia. Also, salmonid and cod amylase may be affected by an inhibitor from beans as seen with amylase from Atlantic salmon (Froystad et al., 2006). Effects of amylase inhibitors in diets for Atlantic salmon may be limited however, since the amylase of this species seems to have a defective substrate anchor, reducing its catalytic ability. Its secretion from the pancreas also seems limited.

In a recent feeding trial with Atlantic salmon in which an amylase inhibitor concentrate from common beans was included in a pelleted diet at 2g kg<sup>-1</sup>, no effect was observed on starch digestibility or digestibility of protein, total lipid and fatty acids (Chickwati, 2007). The level should be in the upper range of levels that may be present in extruded fish diets with significant inclusion of beans and other plant feedstuffs.

Regarding effects on gut microbiota of fish, no information was found. However, the general considerations made in Section 2.1.2 should be valid also for amylase inhibitors.

#### 4.2.4. LIPASE INHIBITORS

##### General characteristics

Oil seeds store energy reserves required for support of the growing embryonic axis during the post-germination period in the form of triacylglycerides (TAGs). Mobilization of these reserves is via gluconeogenesis starting with the hydrolysis of the TAGs to glycerol and fatty acids by lipase. Before germination, lipase in seeds has a subcellular location within protein storage vacuoles (PSVs) (Gupta et al., 2003) from which it relocates, in early seed germination, to the oil body, the site of TAG mobilization (Gupta and Bhatla, 2005). The presence of lipase inhibitors appears to be important in the prevention of auto-digestion of the lipid reserves by the seed lipases prior to seed germination (Gargouri et al., 1984; Wang and Huang, 1984; Bau et al., 1997; Gupta et al., 2003). The lipase inhibitors are protein in nature and have been described in seeds of soybean (Wang and Huang, 1984; Gargouri et al., 1984; Satouchi et al., 1998; Satouchi et al., 2002), sunflower (Wang and Huang, 1984; Chapman Jr.,

1987), peanut and cucumber (Wang and Huang, 1984). Lipase inhibitor proteins (LIPs) have also been demonstrated in a number of cereals including wheat (Borel et al., 1989; Cara et al., 1992; Tani et al., 1994; Tani et al., 1995; O'Connor et al., 2003), oat bran (O'Connor et al., 2003), millet, barley and sorghum (Cara et al., 1992). LIPs in soybeans and sunflowers have been reported to have a molecular weight of 70 kD (Gargouri et al., 1984) whereas those in wheat germ and bran have molecular weight 24 – 27 kD (Borel et al., 1989).

### **Biological effects in fish**

The lipase inhibitory effect in soybean is considered to be a result of prevention of normal lipase function through binding to the surface of the substrate micelles (Wang and Huang, 1984; Gargouri et al., 1984). In sunflower seed, however, a direct interaction between the enzyme and the inhibitor by formation of a tightly-bound inhibitor protein-lipase complex was postulated as the likely mode of interaction between the LIP and the lipase (Chapman Jr., 1987). The lipase inhibitors in wheat appear to behave in a comparable manner to the soybean lipase inhibitors (Borel et al., 1989).

In rats, both heated and raw soybean flour exhibited significant inhibitory effects on lipase activity in the pancreas and in its secretions (Khalifa et al., 1994). Presence of inhibitory activity in heat-treated soybean flour suggests a degree of thermostability of the lipase inhibitors and their possible presence in solvent-extracted soybean meal (SBM). Lipase inhibitor activity may account for the impaired lipid digestibility in Atlantic salmon fed on diets with an inclusion of solvent-extracted SBM (Storebakken et al., 1998a; Refstie et al., 2000; Refstie et al., 2001).

No feeding trials have been reported that investigated dietary effects of plant lipase inhibitors, despite the economic implications of poor utilization of the expensive lipid by farmed fish. Therefore *in vivo* biological activity in various fish species is not known and no maximum inclusion level can be estimated. Effects of LIPs from cereals on fish are possible confounders to the effects on fish macronutrient digestibilities that are attributed to cereal fibre. Presence of LIPs from dietary cereals may be more significant for fish species that tolerate high dietary carbohydrate and fibre and less in species such as Atlantic salmon that exhibit less tolerance. The effects of these lipase inhibitor proteins on lipid and other macronutrient digestibilities and on intestinal structure need to be investigated in different species of farmed fish. Regarding effects on gut microbiota of fish, no information was found. However, the general considerations made in Section 2.1.2 should be valid also for amylase inhibitors.

### **4.2.5. LECTINS**

#### **General characteristics**

Lectins (previously known as agglutinins or hemagglutinins) are a group of soluble, heterogeneous (glyco)proteins that “possess at least one non-catalytic domain which binds reversibly to a specific mono- or oligosaccharide” (Peumans and Van Damme, 1995). They were discovered first in plants (Stillmark, 1888), but later also in microorganisms and animals. On the basis of overall structure, lectins are divided into four major groups:

- Merolectins: small monovalent proteins with a single carbohydrate-binding site and thus unable to agglutinate cells or precipitate glycoconjugates (see below);
- Hololectins: proteins with two or more identical or homologous carbohydrate-binding domains, and therefore have agglutinating activity, distributed on one or more (usually 2 or 4) subunits or protomers;
- Chimerolectins: fusion proteins composed of a carbohydrate-binding domain (with one or more carbohydrate-binding sites) tandemly linked to an unrelated, often catalytic domain that acts independently of the carbohydrate-binding domain;

- Superlectins: a special type of chimerolectin built up of two tandemly linked carbohydrate-binding domains that are structurally different and recognize structurally unrelated sugars.

Sequence data obtained by molecular cloning and protein sequencing has led to a classification of plant lectins into four groups of evolutionary related proteins:

- Legume lectins;
- Chitin-binding lectins;
- Type 2 RIP (ribosome-inactivating proteins);
- Monocot mannose-binding lectins.

In addition, there are three small lectin families with distinct structures and sequences with prototypes amaranthin (from *Amaranthus caudatus*), jacalin (from jackfruit *Artocarpus integrifolia*) and the phloem lectin from *Cucurbita maxima*.

The legume (bean) lectins are a large family of homologous proteins (Sharon and Lis, 1990). Most are hololectins, although chimerolectins (e.g. Type 2 RIP) are also described in some species. The molecular sizes generally range from 40 to 160 kD. The different legume lectins all contain  $Mn^{2+}$  and  $Ca^{2+}$  ions, which are essential for the carbohydrate-binding activity. The amino acids involved in the carbohydrate-binding domain are less conserved however, and probably account for the different carbohydrate specificities among the different legume lectins (Van Damme et al. 1998).

Lectins are generally found in highest concentrations in seeds and other storage organs, but also in vegetative tissues – leaves, stems, bark, roots, root nodules, and flowers. Some seeds may contain as much as 20 g per kg on a DM basis, although most legume seeds contain 1-5 g per kg. On a protein basis, seed lectins account for 0.1-5% total protein, up to 50% in some *Phaseolus* species. The vegetative storage tissues – bark, bulbs, tubers, rhizomes, and corms – also contain 0.1-5% (up to 50%) lectin on a total protein basis. It appears that variations in lectin levels occur between different cultivars within a species, as well as with stress factors and ecological and climatological conditions that the plants are exposed to during growth.

There may be different forms of lectins, coded by different genes, located in different parts of the same plant. Lectins are found within the cells in protein bodies or vesicles, but some are also found in exsudate secreted following wounding or associated with cell walls.

## **Biological effects in fish**

### *General considerations*

The early names for lectin – agglutinin, hemagglutinin, and phytohemagglutinin – were coined when it was discovered that substances from different plants could agglutinate red blood cells *in vitro*. They were later given the name lectin from Latin “*legere*” (to select), when it was discovered that some selectively agglutinate blood cells of a specific animal or human blood group (Boyd and Shapleigh, 1954). An understanding of this ability to subtly differentiate between erythrocytes came with the discovery that the mono- or oligosaccharides that lectins bind to are often an intrinsic part of many biologically important substances, so-called glycosubstances or glycoconjugates. These are present on cell surfaces (receptors, antigens, structural proteins, transport molecules, enzymes, membrane lipids etc.), as well as free substances (hormones, growth factors and other signal substances).

This specific recognition of carbohydrates is one of lectins’ characteristics that have been used in their classification. Thus, plant lectins have been classified according to the mono- or oligosaccharide that prevents the lectin from agglutinating erythrocytes or precipitating glycoconjugates *in vitro* (from Van Damme et al., 1998):

- Mannose-binding lectins

- Mannose/maltose-binding lectins
- Mannose/glucose-binding lectins
- Glucosamine (GlcNAc)/(GlcNAc)<sub>n</sub>-binding lectins
- Galactose/Galactosamine (GalNAc)-binding lectins
- Fucose-binding lectins
- Sialic acid-binding lectins
- Lectins with complex, known specificity
- Lectins with complex, unknown specificity
- Lectins with dual specificity
- Lectins with undetermined specificity.

Plant lectins have been described as “one of the most important physiologically active ingredients and potent exogenous biological signals in the diet” (Pusztai and Bardocz, 1996). This is due to their widespread occurrence in plants and crops, their partial resistance to heat denaturation as well as to breakdown during passage through the digestive tract (Hara et al., 1984; Pusztai et al., 1990; Bardocz et al., 1995), and their interaction with endogenous surface receptors of the intestinal epithelial cells of all higher and lower animals that ingest them.

**Table 4.2.5.1.** Common legume lectins, the name of their lectins, and their sugar specificities (Van Damme et al., 1998)

LATIN NAME	COMMON NAMES(S)	LECTIN	MONO-/OLIGO-SACCHARIDE SPECIFICITY
<i>Abrus precatorius</i>	Rose-cocoa, crab-eye, jequirity, precatory bean	APA	Galactose/ N-acetylgalactosamine
<i>Arachis hypogaea</i>	Peanut, groundnut	Abrin PNA GNL MNL PRA-I PRA-II	Galactose Galactose Galactose Mannose/glucose Galactose Mannose/glucose
<i>Cajanus cajan</i>	Pigeon pea, yellow dahl, red gram	Unnamed	Mannose/glucose
<i>Canavalia ensiformis</i>	Jack bean	ConA	Mannose/glucose
<i>Cicer arietinum</i>	Chickpea, gram, Bengal gram, gram pea, garbanzo bean	CAA or CPA	Complex
<i>Glycine max</i>	Soybean, soya bean	SBA	N-acetylgalactosamine (>galactose)
<i>Lens culinaris</i>	Lentil	LcH or LCA	Mannose/glucose
<i>Lotus tetragonolobus</i>	Asparagus pea	LTA	Fucose
<i>Phaseolus acutifolius</i>	Tepary bean	Erythroagglutinin Lymphoagglutinin	Complex (unknown) Complex (unknown)
<i>Phaseolus coccineus</i>	Scarlet runner bean	PCA	Complex (unknown)
<i>Phaseolus lunatus</i> ( <i>Phaseolus limensis</i> )	Lima, butter or Sieva bean	Unnamed PLA LBA LBL	Complex (unknown) N-acetylgalactosamine
<i>Phaseolus vulgaris</i>	Kidney (dry) bean red, white, brown, black Common, dwarf, field,	PHA-E PHA-L	Complex Complex

	flageolet, French, French filet, garden, green, haricot, navy, Pinto, pop or popping, snap, string, wax bean	Pinto lectin	Complex
<i>Pisum sativum</i>	Garden pea	PSA or PsA	Mannose/glucose
<i>Vicia faba</i>	Broad, field or faba bean	Unnamed VFA or favin	Mannose/glucose Mannose/glucose
<i>Vigna angularis</i> ( <i>Phaseolus angularis</i> )	Adzuki/aduki bean		
<i>Vigna mungo</i>	Black gram, urdi bean		
<i>Vigna radiata</i>	Mung bean	MBA	Galactose
<i>Vigna unguiculata</i> ( <i>Vigna sinensis</i> )	Cowpea, blackeye bean, black-eyed pea		

Membrane proteins such as transport proteins, brush-border enzymes, and hormone and growth factor receptors, are glycosylated (syn. glycosylated) before being embedded in the brush border membrane (Roth, 1987; Pusztai et al., 1995). Membrane lipids and gangliosides, and secreted mucins are also glycosylated. During migration along the crypt-villus axis, the maturing intestinal epithelial cells continuously change in terms of protein composition, activity of enzymes and transport proteins, and the glycosylation profile of components in the cellular membrane. Glycosylation also varies depending on other factors: animal species, its age, genetic make-up, blood group specificity, health status, diet, and bacterial flora in the intestine, as well as intestinal region and mucosal cell type. Therefore, there is a wide range of potential binding sites for lectins in the gut. This variability in glycosylation patterns may help explain the variability in biological effects of different lectins in different species of animals.

Due to their ability to bind to glycoconjugates on animal cell membranes, lectins can have striking biological activities on intestinal structure and function, as well as systemic effects.

- Agglutinating lectins that have more than one carbohydrate binding domain can disturb the organization of the epithelium by cross-linking cell surface glycoconjugates.
- Lectins that bind to transport proteins or enzymes may physically block their activities and thus reduce digestive processes and nutrient assimilation.
- Normally, the binding of a physiological signal substance (e.g. hormones, growth factors, cytokines etc.) to a receptor causes transmission of a message across the cellular membrane and activation of second messenger system(s), thus specifically changing cellular activity. Plant lectins can bind to sugar structures on or near the receptor's functional binding site and thus mimic, block, or additively reinforce the physiological ligand's cellular and/or systemic effects.
- Lectins that bind to epithelial cells can be endocytosed and transcytosed and directly influence the metabolic activity of cells (Pusztai et al., 1990).

Therefore, the specific effect of an ingested lectin is dependent on its carbohydrate-binding specificity, and thus where it binds (see below). The potency of the lectin's effect on cell metabolism appears to be correlated with the strength of its binding to a ligand, which in turn is dependent not only on its defined sugar/carbohydrate specificity, but also on other structural intricacies of the ligand (Table 4.2.5.2; Grant et al., 1983).

As early as 1917 it was recognized that the nutritive value of raw soybeans was poor and would not support the growth of rats unless the soybeans were subjected to some form of heat treatment (Osborne and Mendel, 1917). The connection between the low nutritional value/toxicity of raw legumes and their agglutinating lectins was not made until the 1940s and 1950s when it was demonstrated that intraperitoneal injections of extracts and isolated lectins

from raw kidney beans (Jaffé, 1949) and soybeans (Liener and Pallansch, 1952) were lethal to rats. Liener (1953) then added isolated soybean lectin to the diets of rats and showed that it would indeed inhibit rat growth. Since then, innumerable feeding trials using various animal models have confirmed that legume lectins contribute substantially to the nutritional toxicity of many raw legumes.

Binding of soybean lectin (agglutinin; SBA) to carbohydrate moieties of glycoconjugates, specifically to N-acetyl-d-galactosamine, on the intestinal brush border membrane of Atlantic salmon and rainbow trout has been demonstrated (Hendricks et al., 1990; Buttle et al., 2001), in which higher maximum binding and lower dissociation constants were observed in the distal intestine relative to the more proximal areas (Hendricks et al., 1990). These authors suggested that this could indicate that the distal intestine would be more sensitive to a potentially toxic effect of soybean lectin or other antinutritional factors or antigens. This was supported by the follow-up study by the same group of scientists in which full-fat soybean meal, but not soy protein concentrate, was found to cause morphological changes in the distal intestine of Atlantic salmon (van den Ingh et al., 1991).

**Table 4.2.5.2.** Legumes classified by degree of nutritional toxicity (survival and net protein utilization) in rats and in vitro hemagglutinating activity (rabbit, pronase-treated rat, trypsin-treated cow, and human group 0+ and AB+ blood cells) according to Grant et al. (1983).

Latin name	Common name(s)	Lectin (Van Damme et al. 1998)
<b>Group a: Highly toxic*, high lectin activity</b>		
<i>Phaseolus coccineus</i>	(Scarlet) Runner bean	PCA
<i>Phaseolus vulgaris</i>	Red or brown kidney bean	PHA
<i>Phaseolus vulgaris</i>	White kidney bean	PHA
<i>Phaseolus vulgaris</i>	Black kidney bean	PHA
<i>Phaseolus acutifolius</i>	Tepary bean	F <sub>2</sub> (erythroagglutinin) T <sub>2</sub> (lymphagglutinin)
<i>Phaseolus lunatus</i> (= <i>P. limensis</i> ) (2 of 6 samples)	Lima bean	PLA, LBA, LBL
<b>Group b: Depress growth, moderate lectin activity</b>		
<i>Psophocarpus tetragonolobus</i>	Winged bean	WBA
<i>Phaseolus lunatus</i> (= <i>P. limensis</i> ) (4 of 6 samples)	Lima bean, baby lima bean, butter bean	PLA, LBA, LBL
<b>Group c: Non-toxic, low lectin activity</b>		
<i>Lens culinaris</i>	Lentil	LcH, LCA
<i>Pisum sativum</i>	Pea	PSA
<i>Cicer arietinum</i>	Chickpea	CAA, CPA
<i>Vigna sinensis</i> (= <i>V. unguiculata</i> )	Black-eyed pea	
<i>Cajanus cajan</i>	Pigeon pea, yellow dahl, red gram	
<i>Phaseolus aureus</i> (= <i>Vigna radiata</i> )	Mung bean	MBA

<i>Phaseolus aureus</i> (= <i>Vigna radiata</i> )	Black mung bean	
<i>Vicia faba</i>	Field or broad bean, fava bean	VFA, favin
<i>Phaseolus angularis</i> (= <i>Vigna angularis</i> )	Aduki bean	
<b>Group d: Low lectin activity, but growth inhibiting at higher inclusion levels**</b>		
<i>Glycine max</i>	Soya bean, soybean	SBA
<i>Phaseolus vulgaris</i>	Pinto bean	Pinto lectin

\*Diets containing 100 g seed protein kg<sup>-1</sup> caused death in all the rats within the 10 d experimental period.

\*\*Growth inhibition at 100 g seed protein kg<sup>-1</sup> dietary inclusion was suggested to be due to other antinutrients.

Lectins, like soybean trypsin inhibitors, were largely 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 extract, however, did cause the morphological changes when added to the salmonid diet. They suggested that alcohol-soluble oligosaccharides and saponins are the most likely causatory compounds. On the other hand, Buttle and co-workers (2001) observed morphological changes, especially damage to the villous tips and cellular infiltration in the submucosa/lamina propria, 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) indicate that phytohemagglutinin (PHA) from kidney beans (*Phaseolus vulgaris*) inhibit glucose transport into the intestinal epithelium of Atlantic salmon when tissue is exposed *in vitro*. SBA did not inhibit glucose absorption.

In other animals, soybean or other plant lectins have also been reported to cause hyperplastic growth of intestinal epithelial cells, especially of crypt cells (Oliveira et al., 1988; Tajiri et al., 1988; Pusztai et al., 1990; Bardocz et al., 1992 and 1995; Banwell et al., 1993); decreased cell turnover time (Pusztai et al., 1990); uptake of lectin via endocytosis by intestinal epithelial cells (Pusztai et al., 1981; Banwell et al., 1984; King et al., 1986; Bardocz et al., 1995); changes in pancreatic weight and enzyme secretion (Greer and Pusztai, 1985; Huisman et al., 1990; Banwell et al., 1993; Pusztai et al., 1993a; Grant et al., 1997); changes in liver weight (Oliveira et al., 1988; Aletor, 1989); reduced plasma insulin levels (Knott et al., 1992; Bardocz et al., 1996; Pusztai et al., 1998); increased cholecystokinin (CCK) release (Jordinson et al., 1996 and 1997); and reduced skeletal muscle mass (Oliveira et al., 1988; Bardocz et al., 1992).

Whether tissues and organs of lectin-fed fish respond similarly remains to be elucidated. Worth noting is a large-scale study carried out by Grant and co-workers (1983) on 15 species and varieties of edible legumes (see Table 4.2.5.2). The nutritional values for rats and *in vitro* hemagglutination properties were studied and revealed that these traits correlated well for highly toxic legumes that caused death at higher dietary inclusion (100 g seed protein kg<sup>-1</sup>) and severe growth depression at lower inclusion level (50 g seed protein kg<sup>-1</sup>), as well as for moderately toxic lectins, but not for legumes labelled non-toxic (summary in Table 4.2.5.2). The non-toxic legumes showed varying degrees of hemagglutination activity but generally acceptable net protein utilization by the rats at both levels of legume inclusion (50 and 100 g seed protein kg<sup>-1</sup>) in the experimental diets.

Maximum inclusion level of lectins from various plant protein sources have not been determined and will most likely vary considerably between specific lectins and fish species, as indicated by the discussion above and Table 4.2.5.2. Soybean lectin (SBA) inclusion of 35 g per kg feed, an inclusion level equivalent to a 600 g solvent-extracted soybean meal per kg

feed, reportedly caused histological changes in the distal intestine of Atlantic salmon (Buttle et al., 2001). Indeed, inclusion levels of solvent-extracted soybean meal as low as 100 g per kg feed have been demonstrated to cause histological changes and digestive disturbances in Atlantic salmon (Krogdahl et al., 2003). Thus SBA levels below 6 g per kg feed are advisable for salmon. The involvement of SBA alone in soybean meal-induced enteritis has not been unequivocally demonstrated, however. Francis et al. (2001a) suggest lectins' deleterious effects may be more potent when in the presence of other ANFs.

The contribution of the intestinal microbiota to the effects of lectins in the diet has been studied in mammals but not in fish. Raw red kidney beans as well as isolated PHA in diets for rats caused indigenous bacterial proliferation/overgrowth (e.g. aerobes, anaerobes and *E. coli*) in the jejunum and ileum (Wilson et al., 1980; Banwell et al., 1983 and 1988; Pusztai et al. 1993b). Bacterial proliferation was observed within 24 hours of PHA feeding, but counts fell progressively within 24-48 hours following reversion to a PHA-free diet (Banwell et al., 1988). Kidney beans of a less toxic variety ("Pinto III") did not cause the same degree of bacterial proliferation (Wilson et al., 1980). Some but not all of the diarrhea, increased small intestinal weight, crypt hyperplasia, increase in number of goblet cells, increased number of intraepithelial lymphocytes and lamina propria cells, decrease in disaccharidase activity, and nutrient malabsorption were due to the bacterial overgrowth as shown by treating the rats with antibiotics and/or feeding germ-free rats the kidney bean/PHA-containing diets (Banwell et al., 1993). Growth rate, however, was not affected by dietary PHA inclusion in the germ-free rats, whereas there was growth inhibition in the PHA-fed conventional rats.

Ten-day red kidney bean feeding (40% dietary inclusion) of conventional, growing rats resulted in the apparent translocation of indigenous intestinal bacteria (*Citrobacter* spp. and *E. coli*) to the mesenteric lymph nodes (Shoda et al., 1995). Thus septicemic complications may occur following prolonged feeding of kidney beans.

**Table 4.2.5.3.** Lectin concentrations in seeds of raw/dry common legume species

LATIN NAME	COMMON NAME	COMMENTS	LECTIN CONCENTRATION	REFERENCE
<i>Abrus precatorius</i>	Rose-cocoa, crab-eye, jequirity, precatory bean		Unknown but hemagglutinating activity present	Roy et al., 1976; Hegde et al., 1991
<i>Arachis hypogaea</i>	Peanut, groundnut		0.2 – 2 mg g <sup>-1</sup>	Reviewed by Peumans and Van Damme, 1996
<i>Cajanus cajan</i>	Yellow dahl, pigeon pea, red gram	Brown and cream	Unknown but hemagglutinating activity present	Oboh et al., 1998; Grant et al., 1983
<i>Cicer arietinum</i>	Chickpea, gram, Bengal gram, gram pea, garbanzo bean		Unknown but hemagglutinating activity present	Grant et al., 1983
<i>Glycine max</i>	Soybean, soya bean	Conventional "Williams 82"	8.0 – 8.3 mg g <sup>-1</sup> DM	Douglas et al., 1999

		Lectin-free variant (L90-8047), isogenic to Williams 82	<0.00015 mg g <sup>-1</sup> DM	Douglas et al., 1999
			0.2 – 2 mg g <sup>-1</sup>	Reviewed by Peumans and Van Damme, 1996
		Defatted raw meal	2.3 mg g <sup>-1</sup>	Maenz et al., 1999
			3.3 mg g <sup>-1</sup>	Marsman et al., 1995
		Raw seed meal from 102 different lines, 5 lines lacked detectable lectin levels	2.5 – 12.2 mg g <sup>-1</sup>	Pull et al., 1978
<i>Lens culinaris</i>	Lentil		0.1 – 1 mg g <sup>-1</sup>	Reviewed by Peumans and Van Damme, 1996
<i>Lotus tetragonolobus</i>	Asparagus pea		Unknown but hemagglutinating activity present	Pereira and Kabat, 1974
<i>Phaseolus acutifolius</i>	Tepary bean		1 – 10 mg g <sup>-1</sup>	Reviewed by Peumans and Van Damme, 1996
<i>Phaseolus aureus</i>	Mung bean		Unknown but hemagglutinating activity present	Wirawan et al., 1997; Grant et al., 1983
<i>Phaseolus coccineus</i>	Scarlet runner, runner bean		1 – 10 mg g <sup>-1</sup>	Reviewed by Peumans and Van Damme, 1996
<i>Phaseolus lunatus</i>	Lima, butter, Sieva bean		1 – 10 mg g <sup>-1</sup>	Reviewed by Peumans and Van Damme, 1996
<i>Phaseolus vulgaris</i>	Common, dwarf, field, flageolet, French, garden, green, haricot, kidney (red, white, brown, black kidney bean), navy, Pinto, pop or popping, snap, string, wax bean		1 – 10 mg g <sup>-1</sup>	Reviewed by Peumans and Van Damme, 1996

		Range reflects environmental and varietal differences	1.3 – 10.0 mg g <sup>-1</sup>	Muzquiz et al., 1999
		5 “wild types”	9.6 mg g <sup>-1</sup>	Sotelo et al., 1995
	White kidney bean (var. Processor)		18.5 mg g <sup>-1</sup>	Roozen and de Groot, 1991
	Black bean		1.7 mg g <sup>-1</sup>	Trugo et al., 1999
	Pinto bean		0 mg g <sup>-1</sup> PHA	Staswick and Chrispeels, 1984
	Kintoki beans		Unknown but high hemagglutinating activity present and toxic effect on mice	Miyoshi et al., 1982
	Wax bean		Unknown but hemagglutinating activity present	Sela et al., 1973
<i>Pisum sativum</i> var. <i>Solara</i>	Garden peas		0.2 – 2 mg g <sup>-1</sup>	Reviewed by Peumans and Van Damme, 1996
<i>Psophocarpus tetragonolobus</i>	Winged bean		2.8 mg g <sup>-1</sup> Unknown but hemagglutinating activity present	Tolman, 1995 Grant et al., 1983; Tan et al., 1983
<i>Vicia faba</i>	Faba, broad, field bean		0.1 – 1 mg g <sup>-1</sup>	Reviewed by Peumans and Van Damme, 1996
			1.9 – 2.3 mg g <sup>-1</sup>	Van der Poel et al., 1992
			0.34 mg g <sup>-1</sup>	Allen and Johnson, 1976
<i>Vigna (Phaseolus) angularis</i>	Adzuki/aduki bean		Unknown but hemagglutinating activity present	Grant et al., 1983
<i>Vigna mungo (Phaseolus mungo)</i>	Black gram, urdi bean		Unknown but hemagglutinating activity present	Sharma and Salahuddin, 1993; Suseelan et al., 1997
<i>Vigna radiata</i>	Mung bean		Unknown but hemagglutinating activity present	Hankins and Shannon, 1978
<i>Vigna unguiculata (sinensis)</i>	Cowpea, blackeye bean, black-eyed pea		Unknown but hemagglutinating activity present	Marconi et al., 1997; Grant et al., 1983 and 1995

#### 4.2.6. SAPONINS

##### General characteristics

Saponins are a highly diverse group of glycosides produced primarily by plants, particularly legumes, but also a few lower marine animals and some bacteria. Over 100 families of plants are known to contain saponins and considerable heterogeneity of chemical structures exists. Saponins are heat-stable, alcohol-soluble compounds. They are amphipathic molecules, containing a hydrophobic steroidal or triterpenoid aglycone to which one or more (hydrophilic) sugar chains are attached (Fig. 6.6.1). The sugar moiety (oligosaccharide) usually contains glucose, galactose, glucuronic acid, xylose, rhamnose or methyl pentose glycosidically linked to the hydrophobic aglycone (sapogenin).

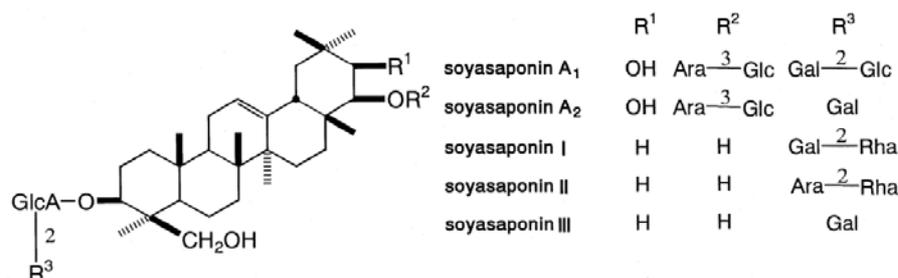


Figure 4.2.6.1. Chemical structures of soybean saponins showing different side chains attached to a triterpenoid backbone (Oda et al., 2000).

Monodesmosidic saponins have the oligosaccharide chain attached at the normal C<sub>3</sub> position while bisdesmosidic saponins have an additional sugar moiety at the C<sub>26</sub> or C<sub>28</sub> position. The complexity of saponin structure arises from the variability of the aglycone structure, the nature of the side chains and the position of attachment of these moieties on the aglycone (Francis et al., 2002b). Triterpenoid saponins are predominant in dicotyledonous angiosperms and the steroidal saponins are almost exclusively isolated from monocotyledonous angiosperms (reviewed by Sparg et al., 2004).

The mature soybean seed contains triterpenoid saponins divided into group A and group B soyasaponins based on the aglycone structure. The group A soyasaponins are bisdesmosidic (Shiraiwa et al., 1991a) and are found predominantly in the germ of the seed, which accounts for only 3% of the total bean, resulting in low levels of this group in extracts from whole beans (about one fifth the level of group B soyasaponins). The group B soyasaponins are nearly equally distributed between the germ and the cotyledons of the seed (Shiraiwa et al., 1991b; Berhow et al., 2006).

### Biological effects in fish

Saponins are reported to have diverse biological effects including antifungal and antiviral activity, immune stimulation, anticancer effects, antioxidant properties, inhibition of protein digestion and vitamin absorption, and glucocorticoid-like effects (reviewed by Francis et al., 2002a). The amphipathic nature of saponins is directly related to many of their biological activities. Saponins form micelles and can intercalate into cholesterol-containing membranes forming holes. Saponins also affect functions of mammalian intestinal epithelia by increasing the permeability of intestinal mucosal cells, inhibiting active mucosal transport and facilitating uptake of substances that are normally not absorbed, as shown by Johnson et al. (1986), such as allergens (Gee et al., 1996). Partially purified *Quillaja* saponins (Quil-A), when administered before or with human gamma globulin (HGG), was able to increase absorption and appearance of HGG in the bloodstream of Nile tilapia (Jenkins et al., 1991 and 1992). It is believed that saponins interact with enterocyte apical membranes but do not reach the circulation. Saponins when added to water are highly toxic to fish. They are the active ingredient in several traditional fish poisons, including mahua oil cake, and act by damaging the gill epithelium via their surface active and membranolytic activities.

Orally administered saponins that are incorporated into cell membranes will eventually be lost in the normal process of intestinal epithelial replacement (Sjolander and Cox, 1998). They can also be degraded by acid and alkaline hydrolysis (Cleland et al., 1996) and glucosidases of bacterial origin (Gestetner et al., 1968). Saponins are also lost due to binding with cholesterol, forming an insoluble complex that cannot be absorbed (Malinow et al., 1977). In the only report of the fate of orally administered saponins in fish, Knudsen et al. (2006) demonstrated that soyasaponins are not degraded during passage through the gastrointestinal tract in Atlantic salmon. The authors found a higher concentration

(approximately 2.6 times) of saponins in feces than in the diet, suggesting that bacterial degradation may be limited in fish.

Francis et al. (2005) summarise the results of several reports of the growth promoting effects of saponins in common carp and tilapia. The authors claim that maximum growth was obtained in carp fed diets containing 150 mg kg<sup>-1</sup> saponin from *Quillaja saponaria* while maximum growth in tilapia was obtained when diets contained saponins at 300 mg kg<sup>-1</sup> diet. However, the validity of the results is contentious. In the experiments with carp (Francis et al., 2001b and 2002c) very low numbers of individuals were used (five fish per diet) and fish were held individually in respiration chambers during the experiment. In the experiments with tilapia (Francis et al., 2001c and 2002b) a similar design was used. Additionally, a mixed sex population (unevenly distributed among diet groups) was used for the experiment, during which the authors noted that females exhibited mouth brooding and the control group experienced more spawning events during the experimental period than the other groups. For these reasons no reliable conclusions regarding saponin effects on growth can be made from these experiments.

Saponins have been implicated in the distal intestine enteritis that develops when Atlantic salmon are fed diets containing soybean meal at moderate to high levels. Their effects on protein digestion, cholesterol metabolism, and on functions of the immune and nervous systems (Francis et al., 2002a) also suggests possible involvement. 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, as had been previously found (van den Ingh et al., 1996), that soy molasses induced enteritis. Additionally, components in the butanol phase also elicited changes in the distal intestine consistent with those of soybean enteritis, suggesting that the yet to be identified responsible component resists fractionation with butanol and drying at 70 °C. However, saponins were 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. Knudsen et al. (2007) report that 68% of the fat and 13% of the protein contained in the molasses was recovered in the butanol phase. The findings of Knudsen et al. (2007) support the previously reported 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<sup>-1</sup> 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<sup>-1</sup>. Because of conflicting reports and the lack of direct evidence using purified reagents it cannot be concluded with certainty that saponins are responsible for the negative effects of soybeans in salmonids. Saponins are also present in lupins, sunflower and peas but no studies have specifically addressed the effects of saponins from these sources in fish diets.

Saponins have shown immunomodulating activities in fish. *Quillaja* saponin enhanced chemotactic activity of head kidney leukocytes after oral administration in yellowtail (Ninomiya et al., 1995). Grayson et al. (1987) report that adding Quil-A to a commercial *Yersinia ruckeri* immersion vaccine did not result in increased bacterial agglutination or serum bactericidal activity, but did increase *in vivo* bacterial clearance from the bloodstream. Ashida et al. (1999a and 1999b) report increased survival of Japanese flounder (*Paralichthys olivaceus*) to *Edwardsiella tarda* infection and yellowtail *Seriola quinqueradiata* to *Enterococcus seriolicida* infection in fish fed *Quillaja* saponins.

Saponins have reported antifungal, antiviral and antibacterial properties. However, many studies use crude extracts to test antimicrobial activity, making interpretation of results

difficult. The suggested mode of action for the antifungal and antibacterial properties of saponins is through interaction with membrane sterols of the target organism. At high concentration saponins will cause pore formation and membrane destabilization resulting in osmotic disturbances. Killeen et al. (1998) report that saponins inhibited microbial growth at low cell densities. However, because their impact was associated with adsorption to the microbes, they had no effect on dense microbial populations. Therefore, saponins may differentially affect gut microbes. Adherent bacteria (those associated with the epithelia) may accumulate saponins, whereas non-adherent bacteria (those flushed out synchronously with the digesta) should be protected by high population densities. Most studies investigating antimicrobial effects of saponins use *in vitro* testing systems. It is not known if, or how, these results apply to the *in vivo* situation.

At this time there is insufficient data to reliably predict safe levels of saponins in diets for fish. Caution should be exercised when deciding acceptable levels. Reduced feed intake has been observed in fish fed diets containing high levels of saponins due to effects on palatability. Specific responses or sensitivities of different fish species to the various saponins have not been adequately addressed (i.e. herbivorous or omnivorous fishes may exhibit different tolerances to saponins than carnivorous fishes). It should be noted that substantial chemical heterogeneity of saponins exists and different saponins exhibit varying levels of biological activity (Yoshiki et al., 1998; Oda et al., 2000).

#### 4.2.7. PHYTOESTROGENS

##### General characteristics

Phytoestrogens are reported to be present in several plant-based alternative fish feed ingredients including soy, lupin, cottonseed, and alfalfa (reviewed by Francis et al., 2001a). Oestrogenic activity has been reported in soybean, cottonseed, linseed and safflower among others. They include isoflavones coumestans, and lignans. Plant oestrogens are mostly isoflavones that occur as glycosides with a sugar moiety attached to one or more hydroxyl groups located at various positions (Figure 6.7.1). Oestrogenic compounds found in soybean meal include genistein, daidzein, coumestrol, formononetin, biochanin A, and equol (Pellisero et al., 1991a). The total isoflavones content of soybeans has been reported to vary and can reach levels as high as 4200 mg kg<sup>-1</sup> (Wang and Murphy, 1994a), but considerable variation exists and is influenced by variety, location and growing conditions (Wang and Murphy, 1994b). Isoflavones occur primarily as  $\beta$ -glucosides in the seed (genistin, daidzin and glycitein), but also as malonyl and acetyl glucosides, with only 2% occurring as aglycones (genistein, daidzein and glycitein).

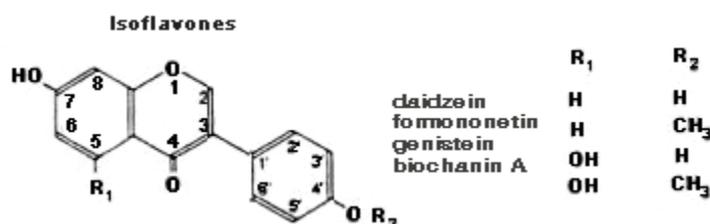


Figure 4.2.7.1. Chemical structure of isoflavones (Wikipedia, the free encyclopedia)

##### Biological effects in fish

One of the concerns regarding oestrogenic effects of phytoestrogens is their effect on reproductive performance, but only a few studies have addressed this issue. Oestrogen activity of plant feed ingredients was first reported in Siberian sturgeon (*Acipenser baeri*). Pelissero et al. (1991a) demonstrated increased vitellogenin in blood plasma of juvenile male and non-vitellogenic females fed diets containing soybean meal compared to individuals fed a casein-

based control diet. Pelissero et al. (1991b) subsequently reported direct evidence for oestrogenic activity of daidzein, biochanin A, genistein, equol and coumestrol after intraperitoneal injection. However, the activity was considerably less compared with estradiol. The affinity of genistein for blood plasma binding proteins in fish is much lower than endogenous oestrogens as shown by Tollefsen et al. (2004) and Milligan et al. (1998). Milligan et al. (1998) concluded that the phytoestrogens coumestrol, genistein and daidzein are unlikely to produce effects by displacing endogenous steroids from plasma binding proteins in rainbow trout. No differences in blood plasma estradiol concentrations were found in female rainbow trout fed diets containing 500 or 1,000 mg aglycone genistein kg<sup>-1</sup> for one year (Bennetau-Pelissero et al., 2001). Reproductive performance was affected, but only in females fed the diet containing genistein at 500 mg kg<sup>-1</sup>. However, male rainbow trout had decreased levels of 11-ketotestosterone and testosterone and reduced sperm motility but increased sperm volume. Spermatocrit was reduced in males at the higher dietary level of genistein. Inudo et al. (2004) found no effect on fecundity or fertility of Japanese medaka (*Oryzias latipes*) fed diets containing up to 58.5 mg kg<sup>-1</sup> genistein and 37.3 mg kg<sup>-1</sup> daidzein for over a 28-day period, although vitellogenesis was induced in males.

The information necessary to understand the significance of dietary isoflavonoids on other physiological processes in fish is incomplete. In soybeans, as previously mentioned, the majority of isoflavonoids exist as glycosides. D'Souza et al. (2005) fed rainbow trout genistein aglycone and found accumulation in muscle although no dose response or time relationship was found. Evidence in mammals suggests that enterohepatic recycling and reabsorption after glycoside hydrolysis and deglucuronidation by intestinal microbes is necessary to achieve a significant physiological role of dietary isoflavonoids (Gatlin et al., 2007). Additional research is needed to clarify the metabolism of native forms of oestrogenic compounds found in plant feedstuffs in fish. Additionally, the relationship between native forms and tissue deposition, and target tissue effects requires investigation.

The gut microflora may play an important role in the metabolism and activity of phytoestrogens. As previously mentioned, in mammals it appears that deglucuronidation of isoflavones by intestinal microbes is an important step in achieving physiological significance. Daidzein can be converted into equol (10 times more oestrogenic) by the intestinal microflora in mammals (Setchell and Cassidy, 1999), but Bennetau-Pelissero et al. (2001) suggest that this process is likely to be unimportant in fish because of the reduced influence of the microflora. However, recent evidence suggests that the intestinal microbiota in fish may be more diverse and important than previously thought (Bakke-McKellep et al., 2007b; Kim et al., 2007). No studies have addressed the effect of fish intestinal microflora on oestrogenic compounds derived from plants.

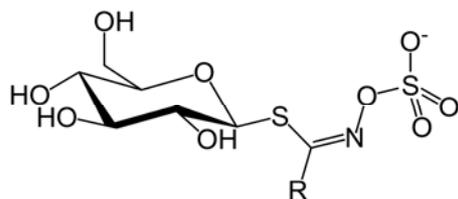
There is insufficient information regarding the effects of dietary phytoestrogens in fish to reliably predict safe inclusion levels. Isoflavonoids in plants occur mainly as glycosides, but the vast majority of research has focused on aglycone forms. It is not known whether, and to what extent, glycoside forms are converted in the fish gastrointestinal tract, and if they elicit the same changes when fish are fed aglycone isoflavonoids. The majority of studies reporting changes in vitellogenin synthesis and sex steroid alterations use intraperitoneal injection, rather than oral delivery, to administer the oestrogenic compounds. Bennetau-Pelissero et al. (2001) report changes in sex steroids in male fish fed diets containing high levels of dietary genistein (500 and 1,000 mg kg<sup>-1</sup> diet). The rationale for using these amounts was based on the work of Mambrini et al. (1999) who found high levels of isoflavones in soy protein concentrate (6,000 and 2,000 mg kg<sup>-1</sup> of genistein and daidzein, respectively), produced by aqueous alcohol extraction, far exceeding levels previously reported. This level of isoflavones content is unusual in soy protein concentrates, produced by aqueous alcohol extraction. While the authors suggest that the analytical method used to measure genistein and

daidzein was more efficient than those used in previous reports, it still does not entirely account for observed levels. Rainbow trout fed diets in which 75% or higher of the fishmeal was replaced with soy protein concentrate exhibited reduced growth. Genistein and daidzein contents in the diet with 100% replacement of fishmeal were 4042 and 1476 mg kg<sup>-1</sup>, respectively; levels in the 75% replacement diet were not reported. Growth of fish fed a diet with 50% replacement of fishmeal by soy protein concentrate, containing 1804 and 594 mg kg<sup>-1</sup> of genistein and daidzein, respectively, performed similar to controls. It appears rainbow trout can tolerate these levels. However, the authors did not examine oestrogenic effects. It is not known if different species vary in their sensitivity to dietary phytoestrogens.

#### 4.2.8. GLUCOSINOLATES/GOITROGENS

##### General characteristics

There are two general categories of foods and feeds that have been associated with disrupted thyroid hormone production, goiter, in animals and man: soybean-related and cruciferous such as rapeseed and cabbage. In addition, there are a few other foods not included in these categories such as peaches, strawberries and millet. The causative agents (goitrogens) of the thyroid disruption are glucosinolates. Content and composition of glucosinolates vary due to plant species, agronomic practices and climatic conditions (Figure 6.8.1). The glucosinolate content is generally higher in rapeseed meal varieties grown in a tropical environment than those in temperate regions. The rapeseed from the Indian sub-continent contains primarily 3-butenyl, 2-propenyl and 4-pentenyl glucosinolates. But 2-propenyl glucosinolate accounts for more than 95% of the total glucosinolates present in rapeseed meal of European and other temperate countries, and does not contain 4-pentenyl glucosinolates. Depending on the pH, cofactors and glucosinolate content and composition of rapeseed meal, major metabolites of glucosinolates are thiocyanates, isothiocyanates, nitriles, 5-vinyl-2-oxazolidinethione and 5-vinyl-1,3-oxazolodine-2-thione (VOT). Different glycosinolate profiles among the rapeseed meals induce varying levels of glucosinolate metabolites in animal tissues (Burel et al., 2001; Francis et al., 2001a; Tripathi and Mishra, 2007). Hydrolysis of the glucosinolates may be catalyzed by thioglucosidases present in the feedstuffs themselves, separated from the substrate by physical barriers. Damage to plant tissue will release the enzyme and initiate the hydrolysis which eventually will result in toxic products. Also, microbes of the gastrointestinal tract may possess thioglucosidases which will add to the hydrolysis (Fuller et al., 2007; Combourieu et al., 2001; Shapiro et al., 2001).



**Figure 4.2.8.1.** Glucosinolate structure. The side group R varies. (Wikipedia, the free encyclopedia)

##### Biological effects in fish

Tripathi and Mishra (2007) have recently presented a review of knowledge on glucosinolates in common feedstuffs and effects in animals, including fish. Major deleterious effects of glucosinolates ingestion in animals in general are reduced palatability, and decreased growth and production. The thiocyanates interfere with iodine availability, whereas VOT is responsible for the morphological and physiological changes of the thyroid. The nitriles are known to affect liver and kidney functions.

Regarding fish, Tripathi and Mishra (2007) report that the general response of fish to dietary inclusion of canola and rapeseed meals is favourable but all reports recommend a

limited inclusion level depending on the glucosinolate content of the meals. Diets containing 0.22–2.18 mmol TGIs kg<sup>-1</sup> did not affect intake, growth or thyroid hormone levels in red sea bream (*Pagrus auratus*) (Glencross et al., 2004c and 2004d), at 2.18 mmol kg<sup>-1</sup> TGIs reduced growth approximately by 0.15 level. The rainbow trout (*Oncorhynchus mykiss*), when fed diets containing 1.4–19.3 mmol glucosinolate kg<sup>-1</sup>, showed significant reduction in growth and changes in thyroid histology (Burel et al., 2000). A strong growth depression was observed at very low GIs intake of 30–47 mol kg<sup>-1</sup> fish body weight day<sup>-1</sup> and an intake of 422 mol decreased daily growth index and feed efficiency. Diets containing even 1.4 mol g<sup>-1</sup> diet decreased plasma T3 levels. Therefore, total glucosinolates content of 1.4 mol g<sup>-1</sup> in diet may be considered a safe upper limit, but this may induce low-level goitrogenicity.

No information has been found regarding effects of glucosinolates on microbial activity in the gastrointestinal tract. On the other hand, several investigations address the interaction between the intestinal microflora and glucosinolate metabolism in mammals (Fuller et al., 2007; Combourieu et al., 2001; Shapiro et al., 2001).

#### 4.2.9. FIBRE

##### General characteristics

Fibres, also called non-starch polysaccharides (NSP), are defined as the polysaccharides not digested by endogenous enzymes in monogastric animals. Dietary fibre is a mixture of substances derived from the structural materials of the plant cell wall and a range of non-structural polysaccharides either present naturally in foods or derived from food additives. Thoughts regarding fibre's impact on digestion and health are changing as knowledge on carbohydrate digestion develops (McCleary, 2003). Fibres can consist of one type of sugar units (homoglycans) or of several types of sugar units (heteroglycans). The monosaccharides may be joined in a linear pattern, as in cellulose, or in a branched fashion as in guaran. The frequency of branching sites and the length of side chains can vary greatly. The sequences may contain shorter or longer segments with periodically arranged residues separated by non-periodic segments or the sequence may be non-periodic all along the chain (Belitz and Grosch, 1999). Polysaccharides consist of monosaccharides bound to each other by glycoside linkages.

Some fibres are highly soluble in water; others are insoluble, whereas some are soluble under certain conditions. Solubility may be influenced by environmental conditions during the growing season and on the variety of the plant in question.

##### Biological effects in fish

The level of NSPs in diets for salmonids has increased due to the increased use of plant ingredients. In plants fibres have structural functions, bind water to variable degrees, act as ion exchangers, adsorb organic compounds and function as antioxidants. These characteristics are also expressed in the GI tract of animals ingesting the fibres. Effects of fibres on digestive function and physiology have been the focus of numerous studies in man and other monogastric, omnivorous homeotherm animals, and the effects are described in numerous papers. Less is known regarding effects in salmonids and other carnivorous fish species, and it is not known to what extent our knowledge regarding the effects in other animals is applicable to fish.

Effects of semi-purified and soluble NSPs, such as guar gum and alginates in fish diets, have been investigated and found to result in decreased dry matter of the faeces in both fish (Storebakken, 1985; Amirkolaie et al., 2005; Leenhouders et al., 2006) and mammals (Zentek, 1996). Many polysaccharides are widely used as thickeners, gelling agents and binders in nutritional products, since their hydroxyl groups are able to interact with nearby water molecules. Leenhouders et al. (2006) found increased intestinal viscosity in African

catfish, leading to decreased digestibility of main nutrients when including guar gum, at 40 g kg<sup>-1</sup> and 80 g kg<sup>-1</sup>, to the diets. Generally, no clear effect on intestinal viscosity by the presence of soluble NSPs in the diet has been observed (Amirkolaie et al., 2005; Leenhouders et al., 2006). Leenhouders et al. (2006) explained that the reduced digestibility of main nutrients was related to a reduced distribution of digestive enzymes in a viscous solution and decreased flow at the mucosal layer. Mwachireya et al. (1999) in general found a negative influence on protein digestibility when feeding NSP-rich diets to rainbow trout. Insoluble NSPs seem to have less influence on apparent digestibility of main nutrients than soluble NSPs. Studies with cellulose showed little to no effect on digestibility of main nutrients in fish fed diets with up to 200 g kg<sup>-1</sup> cellulose (Dias et al., 1998; Amirkolaie et al., 2005). To the contrary, Aslaksen et al. (2007) reported decreased fat digestibility in salmon when cellulose levels in the diets increased. A majority of NSPs in the relevant plant ingredients for use in fish feed are insoluble. Even though native NSPs are not degraded during extrusion, their properties may change (Wang et al., 1993; Rinaldi et al., 2000). Extrusion of NSP-rich diets may result in increased digestibility of main nutrients, although in most cases digestibility is not altered (Thiessen et al., 2003; Sklan et al., 2004; Barrows et al., 2007).

In a review by Storebakken et al. (2000), elevated faecal sodium excretion is pointed out as an effect of feeding soy products to Atlantic salmon. Increased sodium excretion was also observed when feeding protein-rich plant ingredients, SBM among others, at an inclusion level of 200 g kg<sup>-1</sup> to Atlantic salmon. However, no correlation was seen between inherent NSP content and sodium excretion (Aslaksen et al., 2007), while sodium excretion has been found to increase proportionally with dietary cellulose level in rainbow trout (Hansen and Storebakken, 2007). A negative influence on the faecal excretion of sodium and potassium by increasing the NSP levels in diets fed to monogastrics has been observed (Stanogias et al., 1994). Zentek (1996) registered an increase in sodium excretion in cellulose diets, compared to soluble NSP diets, given to beagles. Stanogias et al. (1994) found increased excretion of sodium and potassium when feeding increasing levels of complex NSPs to pigs.

In a recent study of effects of cellulose and a fibre fraction from soybean meal (soy-NSP), dietary cellulose inclusion increased faecal dry matter, while inclusion of native soy-NSP reduced it. This indicates that cellulose did not bind water in contrast to soy-NSP (Kraugerud et al., 2007). When diets with 75 or 100 g kg<sup>-1</sup> of native NSP and extruded soy-NSP were compared, fish fed native soy-NSP had reduced faecal dry matter, higher digestibility of starch, and increased faecal output of Cu, Fe, and K. Dry matter in faeces and faecal output of Cu was lower for the highest inclusion level, while digestibility of starch and faecal output of Mn and K were higher. In conclusion, soy-NSP was inert compared to the fishmeal reference, with respect to nutrient digestibilities and intestinal pathologies, but affected faecal mineral excretion in Atlantic salmon.

Dietary inclusion of soy-NSP, up to 100 g kg<sup>-1</sup>, did not result in reduced digestibility of main nutrients or cause enteritis in the distal intestine of Atlantic salmon. However, inclusion of soy-NSP in the diets did result in decreased faecal DM and increased faecal excretion of sodium, with a significant correlation between these two parameters, possibly indicating that increased drinking was causing the high levels of sodium in faeces. Extruding the soy-NSP did not significantly alter the properties of the native soy-NSP versus the extruded soy-NSP diets, even though some parameters were different in the processing and inclusion level model. The diets with cellulose lowered the digestibility of starch when compared to the native soy-NSP diet.

It can be concluded that dietary fibres alter flow, impair interactions, affect intestinal receptors, restrict nutrient diffusion, change microbial diversity and activities, and change absorptive surfaces. The variability of the compounds belonging to the fibre complex and the varying degrees that they affect these parameters makes it impossible to conclude on a general

basis regarding maximum inclusion level. For a fibre such as the highly water-soluble guar gum, which is used as a binder in fish diets at levels below  $10 \text{ g kg}^{-1}$ , negative effects are observed at levels of a few percent in diets for rainbow trout (Storebakken, 1985). Cellulose type fibres on the other hand, may apparently be present in salmonid diet at levels above 15% without effects on feed intake, nutrient digestibility and growth (Hansen and Storebakken, 2007). The only significant effect observed was on dry matter content of faeces, which increased with increasing cellulose level, an indication that cellulose adsorbs less water than the other dietary ingredients.

Material reaching the distal compartments of the GI tract, originating from the diet or from digestive juices and dead epithelial cells, feed the microflora of the intestinal tract. The lower the diet digestibility the more dietary nutrients and components from the GI tract are delivered to the flora. Depending on the nutrient profile of the matter reaching the distal compartments, microbial species will be stimulated differently. It is of utmost importance for the health of the animal that microbes that improve gut function and the immune apparatus dominate. The gut microflora constitutes a greater number of cells than the entire body of the host and the metabolites of the microflora are important for the gut wall itself as well as for the metabolism in the body. Great efforts have been made over the last 20 years to find dietary supplements that ensure that benign or beneficial microbes dominate the gut flora. Such compounds are called prebiotics. Ingredients that stimulate microbes producing butyrate are considered particularly beneficial (Noack et al., 1998; Gudiel-Urbano and Goni, 2002; Kimura et al., 2002; Swanson et al., 2002; Tsukahara et al., 2002).

Research showing effects of dietary NSP on the microflora in the fish intestine is limited. However, one study (Bakke-McKellep et al., 2007b) investigated the microflora of seawater-adapted Atlantic salmon fed diets containing fishmeal as the sole protein source (FM), 250  $\text{g kg}^{-1}$  dehulled, extracted and toasted soybean meal partially replacing the fishmeal (SBM), or a diet containing 75  $\text{g kg}^{-1}$  inulin (IN), a polymeric fructan in which the monomers are linked by  $\beta$  (2-1) bonds indigestible to digestive enzymes of animals but fermentable by beneficial bifidobacteria and other lactic acid-producing bacteria in the large intestine of mammals (Pool-Zobel et al. 2002). The SBM and IN diets contained comparable levels of nitrogen-free extract (NFE) – 9.4 and 8.9%, respectively – whereas the FM diet contained only 3% NFE. Interestingly, the IN fed fish had the lowest number of total viable bacterial counts in the digesta of both mid and distal intestine. The SBM fed salmon had the highest counts whereas the FM-fed fish had intermediate counts. There were diet-dependent differences in the diversity of bacterial strains identified. The IN fed fish also had the lowest diversity (16 genera and strains) compared to SBM-fed (26) and FM-fed (24) fish. The number of isolated lactic acid species, *Marinilactibacillus psychrotolerans* and *Carnobacterium piscicola* were highest in the FM fed salmon compared to the SBM and IN-fed fish. However, *Brevibacterium* and *Enterococcus* spp. were detected in the latter two groups but not in the FM groups. Similar findings have been reported in Arctic charr (*Salvelinus alpinus* L.) fed a diet containing inulin (Ringø et al., 2006a). Thus, as opposed to findings in mammals (Gibson and Roberfroid, 1995; Pool-Zobel et al. 2002; Xu et al., 2002), fibre may decrease the numbers and diversity of bacterial strains present in the intestines of fish. More studies are needed, however, to further investigate this and elucidate the impact on fish health.

#### 4.2.10. TANNINS

##### General characteristics

Tannins are water-soluble phenolic compounds with molecular weights between 500 and 3,000. They are grouped into two classes, the hydrolysable and the non-hydrolysable or condensed (Marquardt, 1989). Hydrolysable tannins are polyesters of phenolic acids such as

gallic acid, hexahydroxydiphenic acid and/or their derivatives and D-glucose or quinic acid. The condensed tannins are polymers of flavan-3-ols, flavan-3,4-diols, or related flavanol residues linked via carbon-carbon bonds, which do not have carbohydrate cores like the hydrolyzable tannins (Mueller-Harvey and McAllan, 1992). The seed coat of brown legumes and rapeseeds are rich in condensed tannins, which are brown pigments colouring the seeds (Rao and Prabhavathi, 1982; Francis et al., 2001a). Such condensed tannins may disturb digestion by binding digestive enzymes or complexing to feed components such as proteins or minerals, and also have an astringent, bitter flavour (Liener, 1989; Marquardt, 1989; Mueller-Harvey and McAllan, 1992; Sandoval and Carmona, 1998).

### Biological effects in fish

Effects of tannins in fish are little investigated. Common carp have been shown to tolerate 20 g condensed (quebracho) tannin powder  $\text{kg}^{-1}$  diet without any effect on feed intake and growth (Becker and Makkar, 1999). It is, however, an open question how far commercially available purified tannins simulate condensed tannins naturally occurring in plant seeds. In light of this, broad bean (*Vicia faba*) meal with a high content of condensed tannin had lower *in vitro* protein digestibility than soybean, and the differences were more pronounced when simulating carp digestion compared to rainbow trout (Grabner and Hofer, 1985). This indicates species differences in tannin interactions with other dietary components in the gut. It is also speculated that the content of condensed tannins (24 g  $\text{kg}^{-1}$ ) in copra was the cause for observed growth depression in tilapia and rohu (*Labeo rohita*) fingerlings when feeding diets with 250 g  $\text{kg}^{-1}$  or 200 g  $\text{kg}^{-1}$  copra (Jackson et al., 1982; Mukhopadhyay and Ray, 1999).

As the information about tannins in fish is so limited, caution should be exercised in incorporating feedstuffs containing high amounts of tannins in fish feeds. If tannin-rich seeds are to be used in aquafeeds, the dark seed coats should be removed, and only dehulled seeds (kernels) should be used in the feed. Most plant seeds used in salmonid feeds are, however, light coloured. When feeding such ingredients, tannins should not be problematic.

Animals living on tannin-rich diets, such as reindeer and koala, have a microflora that hydrolyses the tannins and reduces or eliminates the harmful effects. Under experimental conditions alterations in microflora towards species that can hydrolyse tannins have been observed also in horses and chickens (Shanmugavelu et al., 2006; Goel et al., 2005; Nemoto et al., 1995). No information regarding effects of tannin in feed for fish was found in the electronically available scientific literature.

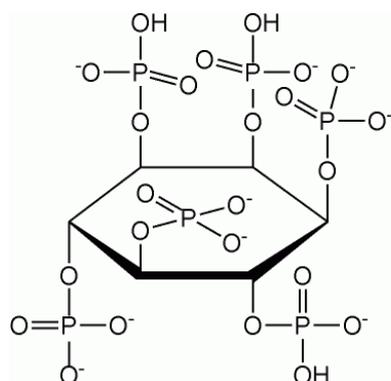
To the extent that tannins complex with proteins in the diet, reducing protein digestibility, a change in the microbial population of the gut is expected. Energy and nitrogen escaping absorption in the gut will serve as nutrients for the microbes of the gut and stimulating growth of some more than others and thereby shifting the status between the species of the gut content and among the species inhabiting the gut wall surface.

## 4.2.11. PHYTIC ACID

### General characteristics

Phytic acid (myoinositol, 1, 2, 3, 4, 5, 6 hexakis-dihydrogen phosphate; Figure 6.11.1) and phytate (a mixed cation salt of phytic acid) are found in significant amounts in plant material, and are generally regarded as 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  $\text{kg}^{-1}$ , respectively (Francis et al., 2001a). Phytic acid has 12 hydrogens (on the 6 phosphate groups) that are titratable in water. These negatively charged sites bind mainly  $\text{K}^+$  and  $\text{Mg}^{2+}$ , but may also form salts and precipitate with other cations including  $\text{Ca}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Ba}^{2+}$ , or  $\text{Fe}^{3+}$  at alkaline pH (Lott et al., 2000; 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 hydrolysed by pepsin (Spinelli et al., 1983), and phytic acid furthermore reduces the stability of trypsin (Caldwell, 1992).



**Figure 4.2.11.1** The structure of phytic acid (Wikipedia, the free encyclopedia)

### Biological effects in fish

In fish, a supplement of 5 g phytic acid  $\text{kg}^{-1}$  diet slowed growth by 10% in rainbow trout (Spinelli et al., 1983), while a dietary supplement of 26 g phytic acid  $\text{kg}^{-1}$  dramatically depressed growth in Chinook salmon (Richardson et al., 1985). Richardson et al. (1985) also found an increased incidence of cataracts when feeding diets high in phytic acid but low in Zn. Dietary supplementation of 5 - 10 g phytic acid  $\text{kg}^{-1}$  diet furthermore slowed growth in common carp (Hossain and Jauncey, 1993). In line with this, improved growth and higher digestibility and retention of protein and mineral elements was shown in rainbow trout when comparing dephytinised with standard soy protein meal (Vielma et al., 2004), soy protein concentrate (Vielma et al., 2002 and 2004), or rapeseed protein concentrate (Teskeredzic et al., 1995).

However, when comparing standard (18 g phytic acid  $\text{kg}^{-1}$ ) to dephytinised soy protein concentrate at 50% dietary inclusion in Atlantic salmon, Storebakken et al. (1998b) found no effects on growth. Still, in line with the expected effects of dietary phytic acid, the apparent digestibility and retention of both protein and phosphorus as well as whole body concentrations of ash, Ca, Mg, and Zn were lowered. Supporting this, Denstadli et al. (2006) found a tolerance for sodium phytate with regard to feed intake and growth in Atlantic salmon between 4.7 and 10 g  $\text{kg}^{-1}$  diet. The apparent digestibilities of Mg and Zn were reduced by the dietary phytic acid in a dose-dependent manner, and this resulted in lower whole body concentrations of Ca and Mg, and a lower concentration of Zn in the vertebral column (Helland et al., 2006). The apparent digestibility and retention of protein was little affected. However, when feeding as much as 21 g phytic acid  $\text{kg}^{-1}$  diet the intestinal trypsin activity was significantly depressed in the Atlantic salmon (Denstadli et al., 2006).

Richardson et al. (1985) found abnormalities in thyroid, kidney and alimentary tract morphology of rainbow trout fed 26 g phytic acid  $\text{kg}^{-1}$  diet. Furthermore, the pyloric caeca were found to be abnormally hypertrophied and showing cytoplasmic vacuolation. Supporting this, Hossain and Jauncey (1993) found hypertrophy and vacuolisation of the cytoplasm of the intestinal epithelium in common carp fed 10 g phytic acid  $\text{kg}^{-1}$  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  $\text{kg}^{-1}$  diet did not, however, induce any histomorphological 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 this that the dietary levels of phytic acid in fish feeds should be monitored, and should probably not exceed 5 g kg<sup>-1</sup> diet. When using feed ingredients originating from crops with moderate phytic acid content such as cereals and legumes this should be unproblematic, but may be an issue when using high levels of ingredients originating from non-leguminous oilseeds such as rapeseed and sunflower.

No information has been found regarding effects of phytic acid on the microbial activity of the gastrointestinal tract of fish. Studies of microbial effects on metabolism of phytic acid and utilization of phytic acid phosphorus are numerous, however, for example Miyazawa et al. (1996), Steer and Gibson (2002), and Refstie et al. (2005).

Thus if dietary phytic acid may affect tissue mass, function of digestive organ, and may be toxic at high dietary levels, an important question arises. Can exogenous phytase in aquafeeds be used to reduce the potentially deleterious effects of phytic acid? As investigations on this topic have not been given priority in fish studies, this topic should be focused on in future studies. Furthermore, more effort should be focussed on phytase activity of intestinal gut bacteria.

#### **4.2.12. OLIGOSACCHARIDES**

##### **General characteristics**

With regard to plant feedstuffs, the term “oligosaccharides” refers to a variable mix of  $\alpha$ -galactosyl homologues of sucrose, which are important constituents of a wide variety of grain legumes and cereals (Saini, 1989). The main compounds of these  $\alpha$ -galactoside oligosaccharides are trisaccharide raffinose, the tetrasaccharide stachyose, and the pentasaccharide verbascose (Bach-Knudsen, 1997; Petterson, 2000). Legumes and oilseeds commonly used in fish feeds typically contain 4 – 14 g raffinose, 12 – 50 g, stachyose, and 0 – 34 g verbascose kg<sup>-1</sup> dry matter, adding up to typical total contents of 14 (rapeseed meal) to 80 (white lupin) g kg<sup>-1</sup> dry matter (Bach-Knudsen, 1997). Feedstuffs high in  $\alpha$ -galactoside oligosaccharides include legumes such as soybeans, peas, and broad beans, while sunflower and cereal grains contain less.

##### **Biological effects in fish**

High dietary levels of  $\alpha$ -galactoside oligosaccharides have negative nutritional effects, some of which may be applicable to fish. These include interference with the digestion of other nutrients, osmotic effects of oligosaccharides in the intestine and anaerobic fermentation of the sugars resulting in increased gas production (Wiggins, 1984; Cummings et al., 1986; van Barneveld, 1999). Although utilisation of  $\alpha$ -galactoside oligosaccharides has not been well defined in fish, studies with swine and poultry have shown that oligosaccharides are indigestible in the stomach or small intestine, primarily due to a lack of the enzyme  $\alpha$ -galactosidase (EC 3.2.1.23; Gdala et al., 1997).

It has been suggested that dietary  $\alpha$ -galactoside oligosaccharides is at least partly responsible for causing the diarrhoea resulting from feeding soybean meal to fish (Refstie et al., 2000, 2005, 2006) due to elevated intestinal osmotic pressure. Other reported effects of  $\alpha$ -galactoside oligosaccharides in fish are, however, 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. Krogdahl et al. (1995) on the other hand found no effects of dietary raffinose included at a level corresponding to 30 – 40% dietary soybean meal on apparent nutrient digestibility and growth in Atlantic salmon. Still, Glencross et al. (2003) indicated that dietary  $\alpha$ -galactoside oligosaccharides reduce the digestibility of macronutrients by rainbow trout, reporting that adding  $\alpha$ -galactosidase as a feed enzyme to a diet with 30% lupin meal or removing the oligosaccharides from the lupin meal by ethanol extraction before

adding it to the diet considerably improved the apparent digestibility of the lupin meal by the trout. This may indicate that the trisaccharide raffinose is not particularly problematic, while the tetra- and/or pentasaccharide stachyose and verbascose may be more potent antinutritional factors in fish.

Limited information is available in the scientific literature that may found a basis for suggesting maximum levels of oligosaccharides in fish diets. However, as they are indigestible their presence in the diet dilutes energy and nutrient concentration. Studies (Arnesen et al., 1990; Krogdahl et al., 1995) indicate that 5% may be an upper limit. Oligosaccharides affect the intestinal microflora and are in focus as possible prebiotics for use in regulation and improvement of human microflora. Several papers have been published recently in this area, for example Macfarlane et al., 2008; van Meer et al., 2008; Biggs et al., 2007. There are also indications that dietary  $\alpha$ -galactoside oligosaccharides play a role in changing the intestinal microflora in fish. Dietary soybean meal has been shown to increase the number and diversity of intestinal bacteria in both Atlantic salmon (Bakke-McKellep et al., 2007b) and Atlantic cod (Refstie et al., 2006; Ringø et al., 2006b). In line with this, 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<sup>-1</sup> to 55% when the diet contained 22 g raffinose kg<sup>-1</sup>. At the same time, the concentration of polysaccharides potentially originating from bacterial cell walls increased in the intestine of fish fed high levels of  $\alpha$ -galactoside oligosaccharides and plant non-starch polysaccharides. This strongly indicates that  $\alpha$ -galactoside oligosaccharides are digested and utilised by intestinal bacteria in the salmon gut.

#### **4.2.13. ALLERGENS**

##### **General characteristics**

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).

##### **Biological effects in fish**

Whether fish are able to 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, teleosts do not appear to have an analogous structure to monomeric IgE, only tetrameric IgM and possibly monomeric IgD (Wilson et al., 1997; Choi et al., 2007). On the other hand, the distal intestine of at least some fish appears to be sensitive to antigen stimulation and strong immune responses are achieved with antigens delivered to this region (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), which act as allergens to several animals and man. Rumsey et al. (1993 and 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 but glycinin and beta-conglycinin were detected in defatted, toasted soy flour. 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, protein or growth significantly different for the different soy products. Thus rainbow trout do not appear to react allergically to soybean meal. Van den Ingh et al. (1991 and 1996), Baeverfjord and Krogdahl (1996) and Krogdahl et al. (2000) observed enteritis-like changes in the distal intestine of Atlantic salmon (*Salmo salar* L.) fed diets containing solvent-extracted soybean meal or an alcohol extract of soybean meal. The antigenic compounds present in feed may trigger a variety of 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. The presence of allergens or antigens that fish may react to in common plant-derived feed ingredients, however, remains a matter of controversy.

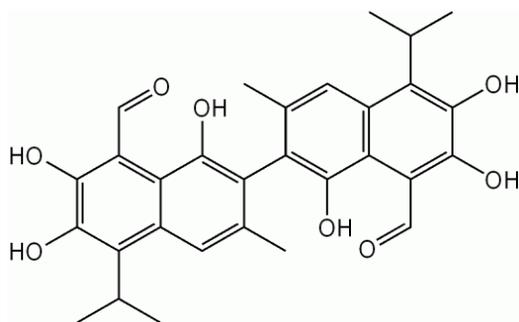
Recently, however, the involvement of cells positive for an antibody against human CD3-epsilon was observed in the distal intestine of Atlantic salmon exhibiting the inflammatory response against soybean meal (Bakke-McKellep et al. 2007a), suggesting that putative T cells may be involved in the enteropathy. 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 cause of the soybean meal-induced enteropathy in salmonids remains to be discovered.

No information regarding maximum inclusion levels and interactions with microflora exists.

#### **4.2.14. GOSSYPOL**

##### **General characteristics**

Gossypol (C<sub>30</sub>H<sub>30</sub>O<sub>8</sub>) is a yellow, lipid soluble polyphenolic aldehyde (Fig. 6.14.1) derived from the cotton plant (genus *Gossypium*, family Malvaceae). It is the major toxic constituent in cottonseeds and is found in discrete pigment glands located in various parts of the plant. Gossypol content of cottonseeds varies from a trace to >6% and is affected by plant species and variety as well as by environmental factors such as climate, soil type, and fertilization. Gossypol is a natural component of all except the “glandless” variety of cotton. Gossypol occurs in bound (combined with free amino groups) and free forms. The bound form is believed to be of little significance because it passes through the gastrointestinal tract without being absorbed. Free gossypol, including gossypol derivatives and transformation or breakdown products, can be toxic to single-stomached animals. The free form is found as two enantiomers, (+) and (-) (Huang et al., 1987) and the ratio may vary between varieties and processing methods (Gamboa et al., 2001).



**Figure 6.14.1.** The structure of gossypol (Wikipedia, the free encyclopedia)

### Biological effects in fish

Lipid-soluble gossypol is readily absorbed from the GI tract. It is highly protein-bound to amino acids, especially lysine, and to dietary iron. Bound gossypol is not readily absorbed, which may result in lysine deficiency. Conjugation, metabolism, and urinary excretion of gossypol are limited; most is eliminated in the feces.

The toxic effects of gossypol have been documented in several species of fish (reviewed by Li and Robinson, 2006). Different levels of toxicity have been reported for different species, and even within a species various toxicity levels have been reported. The reasons for these differences may be the various conditions, fish ages and sizes, diet formulations and analytical methods used. The toxic effects that have been reported include decreased feed consumption, growth, hematocrit, haemoglobin and reproductive capacity. Histological lesions have been found in liver, kidney, spleen and gonads. Despite several reports of toxic effects, death from gossypol toxicosis has rarely been reported in fish.

Recently Luo et al. (2006) reported that juvenile rainbow trout (*Oncorhynchus mykiss*) fed diets with cottonseed meal (465 g kg<sup>-1</sup> diet; 30.7 mg kg<sup>-1</sup> free gossypol) partially replacing fishmeal showed decreased feed conversion efficiency. At complete replacement (610 g cottonseed meal kg<sup>-1</sup> diet; 40.3 mg kg<sup>-1</sup> free gossypol) fish also had reduced apparent digestibility coefficients of crude protein and energy. No differences were observed when the level of free gossypol was 20 mg kg<sup>-1</sup> or less (305 g cottonseed meal kg<sup>-1</sup> diet). In juvenile Japanese flounder (*Paralichthys olivaceus*) Pham et al. (2007) report no differences in growth or feed utilization fed diets containing a mix of cottonseed meal and soybean meal (1:1 ratio) up to 400 g kg<sup>-1</sup> diet (total gossypol 320 mg kg<sup>-1</sup>). However, haemoglobin was reduced with increasing levels and at the highest level hematocrit was also reduced.

Yildirim et al. (2003) reported immunomodulation (improved macrophage chemotaxis ratio, serum lysozyme activity) and increased resistance of juvenile catfish (*Ictalurus punctatus*) to *Edwardsiella ictaluri* challenge at levels of 900 mg gossypol kg<sup>-1</sup> or higher of a casein-gelatin based diet. However, in a later study no differences to *E. ictaluri* challenge were observed in juvenile catfish fed up to 800 mg gossypol kg<sup>-1</sup> of a soy-based diet (Yildirim-Aksoy et al., 2004).

Gossypol is not expected to cause toxicity if cottonseed meal from “glandless” varieties of cotton plants is utilized in feeds. It has also been suggested that cottonseed meals containing gossypol should not be problematic given the expected low inclusion levels of cottonseed meal in fish feeds (Li and Robinson, 2006). The Commission of the European Communities limits the amount of free gossypol in feed materials (with the exception of cottonseed, cottonseed meal and cottonseed cakes) at 20 mg kg<sup>-1</sup>. The limits for cottonseed and cottonseed meal are 5,000 mg kg<sup>-1</sup> and 1,200 mg kg<sup>-1</sup>, respectively. However, the maximum allowable level in complete feeds (with the exception of feeds for cattle, sheep, goats, poultry, rabbits and pigs) is 20 mg kg<sup>-1</sup> (Commission of the European Communities, 2003).

No studies have directly addressed the effect of gossypol on the intestinal microflora. Gossypol functions as part of the plant's defense system and has anti-insecticidal and antifungal properties (reviewed by Gershenzon and Dudareva, 2007) as well as anti-viral activity (Baram et al., 2004).

#### 4.2.15. GLYCOALKALOIDS

##### General characteristics

Potatoes and other tubers contain secondary metabolites such as the steroid glycoalkaloids (SGA)  $\alpha$ -solanine and  $\alpha$ -chaconine. The two compounds differ only in glycosylation (Figure 6.15.1). The latter contains L-mannose instead of D-glucose. Excessive SGA contents, i.e. 20 mg 100 g<sup>-1</sup> fresh weight or more causes an unacceptable bitter taste and tubers become unfit for human consumption. SGA content varies quantitatively and qualitatively in response to photoperiod, wavelength and intensity of light, soil moisture, stage of growth, storage conditions, growing season, air and soil temperatures and wounding. Potatoes grown in a hot dry climate were reported to contain more glycoalkaloids than those grown in a high altitude, cooler climate. Thus the exposure of potato plants to physiological stress and especially to extreme climatic conditions may cause accumulation of SGA in the tubers. The role of SGA in the defence mechanism of potatoes is still questionable. A direct relationship has not always been found between SGA level and disease resistance. Moreover, SGA content dramatically decreased concomitantly with an increase in phytoalexins following inoculation of tuber slices with fungi or treatment with elicitors of fungal origin. However, this was shown in the pith of the tubers and not in the peel, which is the first tissue to be invaded by pathogens (reviewed by Dimenstein et al., 1997). Potatoes also contain other proteins and metabolites that may protect the tuber against attack from insects and microorganisms (reviewed by Friedman, 2006).

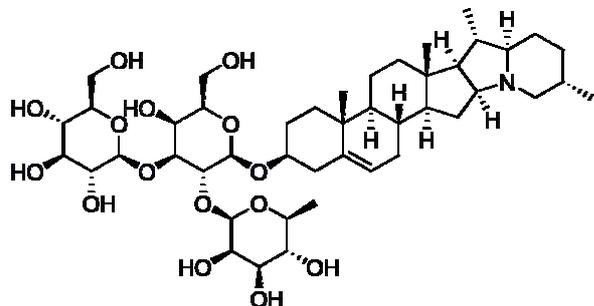


Figure 4.2.15.1. The structure of  $\alpha$ -solanine (Wikipedia, the free encyclopedia)

##### Biological effects in fish

The main effects of GAs on mammals are cell membrane permeabilization and cholinesterase activity inhibition. Due to the latter, GAs can cause prolongation of myorelaxant, anesthetic and analgesic actions, and may affect ester prodrug activation in blood serum. The main potato GAs,  $\alpha$ -solanine and  $\alpha$ -chaconine, in certain concentrations can provoke serious food poisoning, while the principal tomato alkaloid, tomatine, seems to be much safer for humans.

Cholinesterase is one of the crucial enzymes responsible for proper nervous system function. In fact, vertebrates possess two cholinesterases: acetyl cholinesterase (EC 3.1.1.7) and butyryl cholinesterase (EC 3.1.1.8) with different kinetic properties and specificity towards various substrates and inhibitors. In humans, acetyl cholinesterase (AcChE) is localized in neurons and erythrocytes; butyryl cholinesterase (BuChE) is localized in neurons, glia and blood serum. Some compounds, e.g. natural and synthetic drugs, and pesticides are known to be reversible or irreversible inhibitors of both cholinesterases. The mechanisms of inhibition of

cholinesterases by GAs are still poorly known. Kinetics of soluble cholinesterase inhibition by possible pharmacophores has been intensively studied during last years, but there are only few investigations on the kinetics of butyryl cholinesterase inhibition by solanaceous glycoalkaloids  $\alpha$ -solanine,  $\alpha$ -chaconine and tomatine (Benilova et al., 2006).

Solanine poisoning is primarily displayed by gastrointestinal and neurological disorders. Symptoms include nausea, diarrhea, vomiting, stomach cramps, burning of the throat, heart arrhythmia, headache and dizziness. Hallucinations, loss of sensation, paralysis, fever, jaundice, dilated pupils and hypothermia have been reported in more severe cases. In large quantities, solanine poisoning can cause death. Doses as low as 2 - 5 mg kg<sup>-1</sup> body weight can cause toxic symptoms, and doses of 3 - 6 mg kg<sup>-1</sup> body weight can be fatal. Symptoms usually occur 8 to 12 hours after ingestion, but may occur as rapidly as 30 minutes after eating high-solanine foods (Wikipedia).

Potato protein concentrate (PPC) is a candidate plant protein concentrate for fish. Standard PPC is produced as a co-product in the processing of potatoes for potato starch production. Potatoes contain proteins that are dissolved in the potato juice. To retrieve this protein, the potato juice is heated, resulting in precipitation of the protein. This process is known as thermal coagulation. After centrifuging and drying, the precipitate results in PPC. High-quality PPC contains  $\geq 85\%$  crude protein (CP; N $\times 6.25$ ) on a dry matter (DM) basis, and the amino acid composition of the protein is well balanced for fish. However, experimental testing of standard PPC in diets for rainbow trout resulted in severe appetite loss, even at dietary inclusion levels as low as 5%. This was attributed to the solanidine glycoalkaloids in the PPC, among which  $\alpha$ -solanine and  $\alpha$ -chaconine are the best known (Refstie and Tiekstra, 2003). Previous work has shown that rainbow trout embryos exhibit a toxic response to chaconine, solasidine, repin and solanine while Japanese medaka (*Oryzias latipes*) embryos were only affected by the compounds chaconine and solanine (Crawford and Kocan, 1993).

No estimate can be suggested for the maximum level of alkaloids in fish diets due to lack of relevant scientific information. It can however, be stated that the potato alkaloids are very toxic and should not be present in feed.

There is no available information on effects of potato alkaloids on the intestinal microflora.

#### 4.2.16. ARGINASE INHIBITORS

##### General characteristics

An inhibitor of the enzyme arginase occurs in sunflower seeds first reported by Reifer and Morawska (1963). The inhibitor is an unstable nitrogen-derivative of the phenolic compound, chlorogenic acid (Figure 6.17.1; Morawska-Muszynska and Reifer, 1965; Reifer and Augustyniak, 1968). The bond between the nitrogen component and the rest of the molecule is very labile as the compound rapidly decomposed in both acidic and alkaline conditions. 5-CQA is soluble in organic solvents such as ethanol, DMSO and dimethyl formamide.

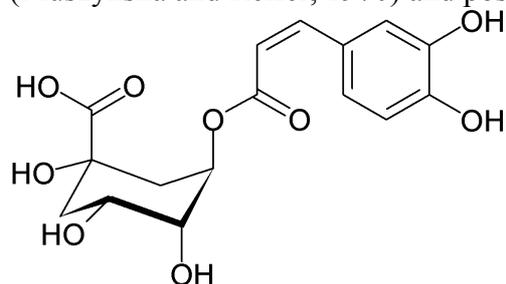
Chlorogenic acid (CGA) refers to a family of esters formed between L-quinic acid and certain *trans* hydroxycinnamic acids such as caffeic, ferulic, sinapic and *p*-coumaric acids (Clifford, 1999). The most common individual CGA is the caffeic/quinic acids' monoester, 5-O-caffeoylquinic acid (5-CQA), commonly referred to as chlorogenic acid.

Chlorogenic acids are present in many plants but only accumulate in a few plants such as sunflower and coffee to quantities sufficient to induce significant physiological effects. Sunflower and, to a lesser extent, soybean are the two plant protein sources with significant levels of CGAs. Chlorogenic acid levels in sunflower seed kernels are in the range 11-45 g kg<sup>-1</sup> (mean, 28 g kg<sup>-1</sup>) (Dorrell, 1976; Dreher and Holm, 1983). The CGA content in sunflower seeds varies with crop variety and is highly correlated to seed oil content. Levels do

not appear to be significantly affected by differences in climate (Dorrell, 1976). Levels of  $9.9 \text{ g kg}^{-1}$  (Pratt and Birac, 1979) are present in soybeans where the CGAs reportedly contribute significantly to the antioxidant activity of the soybeans.

5-CQA is a known inhibitor of arginase as well as other enzymes such as glycerol-3-phosphate dehydrogenase and the translocase, T1, of the hepatic and renal glucose-6-phosphatase systems (Arion et al., 1997 and 1998). With regard to arginase, 5-CQA was shown to possess inhibitor activity in a linear, dose-dependent manner while the N-5CQA exhibited inhibitory activity following a logarithmic function of the inhibitor concentration (Reifer and Augustyniak, 1968). The nature of the inhibitor-enzyme interaction is not fully established. The arginase inhibition by N-5-CQA can be reversed and blocked, by organic reducing agents such as L-cysteine, reduced glutathione, 2-mercaptoethanol and ascorbate (Morawska-Muszynska and Reifer, 1965; Reifer and Augustyniak, 1968).

The arginase inhibitor, N-5-CQA, is unstable and inactivated by oxidation. Presence of arginase stabilises the inhibitor and protects it from oxidation by chlorogenic acid oxidase (Muszynska and Reifer, 1970) and possibly by air as well.



**Figure 4.2.16.1.** The structure of chlorogenic acid (Wikipedia, the free encyclopedia)

### Biological effects in fish

Arginase (L-arginase, L-arginine amidinohydrolase, EC 3.5.3.1) is a widely distributed and relatively conserved enzyme in the biosphere, present in bacteria, yeasts, plants, invertebrates and vertebrates (Jenkinson et al., 1996). Arginases occur in isoforms of varying sequential identity and immunological distinction in different organisms (Jenkinson et al., 1996; Morris et al., 1997; Ash, 2004). Arginase catalyses hydrolysis of L-arginine to form L-ornithine and urea. In ureotelic species, arginase activity is responsible for the cyclic nature of the urea cycle (Jenkinson et al., 1996), the essential (usually hepatic) pathway for ammonium ion excretion. Arginase is also involved in a number of biological functions unrelated to nitrogen excretion in many tissues that include the gastrointestinal tract (GIT), where it is thought to provide a source of ornithine, the biosynthetic precursor of proline and the polyamines (Ash, 2004). Proline is an essential requirement in the synthesis of collagen and wound healing while the polyamines are strong growth factors essential for cell proliferation and differentiation including in the GIT (McCormack and Johnson, 1991). Arginase is an important player in the biochemistry of the immune system, especially with regard to function of the macrophage (Joerink et al., 2006). Extracellular arginase activity can deplete L-arginine as a macrophage strategy to deny a necessary substrate for proliferation and survival to malignant cells, virus-infected cells, fungi and parasites (Currie et al., 1979; Vincendeau et al., 2003). Intracellular arginase activity affords the enzyme an even bigger influence on functions of macrophages and other cell types such as endothelial cells and myocytes through its association with the pathway of the nitric oxide synthase (NOS) enzymes that produce nitric oxide (NO).

The two pathways reciprocally regulate each other by modulating the flux of L-arginine through either pathway (Chang et al., 1998; Mori and Gotoh, 2000). Nitric oxide is a messenger molecule in neurons; a non-adrenergic, non-cholinergic (NANC) neurotransmitter in the autonomic nervous system in a pathway mediating smooth muscle relaxation in

cerebral circulation, gastrointestinal and respiratory tracts; involved in regulation of contractility of, and exercise-induced glucose uptake by skeletal muscle (Bredt, 1999) and its sustained production endows macrophages with cytostatic or cytotoxic activity against viruses, bacteria, fungi, protozoa, helminths, and tumor cells (MacMicking et al., 1997). NO is implicated in disorders of the various processes it mediates in, such as pathophysiological conditions of the autonomic nervous system, muscular dystrophy (Bredt, 1999), endothelial cell dysfunction including contributing to the angiogenic properties of endothelial growth factor in tumours (Papapetropoulos et al., 1997). Overproduction of NO suppresses lymphocyte proliferation (MacMicking et al., 1997), mediates cellular toxicity by mediating in DNA damage, affecting cellular transcription machinery (Kröncke et al., 1997) and damaging critical enzymes leading to apoptosis or necrosis and by reacting with superoxide to form an even more potent oxidant, peroxynitrite (Bredt, 1999). Most of these physiological and pathological implications of arginase and nitric oxide activities are reported in mammalian species and are yet to be fully explored in teleosts.

Arginases in fish are reported to participate in L-arginine metabolism and biosynthesis (Wright, 1995; Jenkinson et al., 1996; Berge et al., 1997), alternative activation of macrophages (Joerink et al., 2006), and in biochemical survival strategies in harsh environments (Randall et al., 1989; Wright and Land, 1998; Steele et al., 2001). It can be speculated that arginase inhibition potentially impacts negatively on fish cellular proliferation and differentiation by disrupting polyamine biosynthesis in many tissues including in the intestine where proliferation, differentiation and migration form the basis of homeostasis and adaptive responses of the enterocytes. Furthermore, inhibition of arginase activity may result in overproduction of NO with consequent NO-mediated cytotoxicity, and related complications.

Chlorogenic acid is absorbed intact in the rat (Gonthier et al., 2002; Lafay et al., 2007), human (Olthof et al., 2001) and in mice absorption occurs in the stomach (Lafay et al., 2006) and in all cases it is detectable in plasma and urine. Chlorogenic acid in the distal aspects of the GIT from the small intestine downwards is mainly hydrolysed by the gut microflora into the constituent components which possess highly variable bioavailabilities (Gonthier et al., 2002; Lafay et al., 2007).

There is a deficiency of data on the bioavailability of the arginase inhibitor in any animal species, on the possible *in vivo* modification of chlorogenic acid into the N-derivative with arginase inhibitory activity and on the effects of chlorogenic acid and related phenolic compounds as antinutritional factors in fish or other animal species. A number of studies have been conducted on the nutritional value of sunflower meal (SFM) in diets for aquaculture fish species. Dietary SFM inclusion levels of up to 40% for rainbow trout (*Oncorhynchus mykiss*) (Sanz et al., 1994; Gill et al., 2006), 64% for European eel (*Anguilla anguilla*), (Garcia-Gallego et al., 1998), 20% for tilapia (*Tilapia rendalii*), at least 22.7% for Atlantic salmon (*Salmo salar* L.) (Gill et al., 2006) and 12% for gilthead sea bream (*Sparus aurata*) (Gill et al., 2006; Lozano et al., 2007) have been reported to cause no adverse effects on growth and feed utilization performance. However, dietary inclusion of more than 20% SFM has been associated with a reduction in crude protein digestibility in Atlantic salmon (Gill et al., 2006; Aslaksen et al., 2007), reflecting reduced digestibilities of most amino acids. This finding is consistent with reported effects of chlorogenic, caffeic and quinic acids that are known to interact and reduce the bioavailability of amino acids including lysine, cysteine, methionine (Pierpoint, 1970), alanine, phenylalanine and glutamic acid (Sripad et al., 1982).

No information on the effect of arginase inhibitors on gut microflora is available in the scientific literature.

#### 4.2.17. QUINOLIZIDINE ALKALOIDS

##### General characteristics

Alkaloids are secondary metabolites produced by plants often serving a role in defence. They are derived from amino acids, containing nitrogen in a heterocyclic ring. Quinolizidine alkaloids are found in a wide variety of plants and are the predominant antinutrient in lupins (genus *Lupinus*). The levels and specific alkaloid profiles vary among the over 300 species of lupins (Wink et al., 1995). While lupins also contain other alkaloids the quinolizidine alkaloids are of most concern for animal health.

Different varieties of lupins contain different alkaloid profiles which may be influenced by variety, location and growing conditions. The predominant alkaloids in *L. albus* are lupanine (70%), albine (15%), 13 $\alpha$ -hydroxylupanine (8%) and multiflorine (3%), whereas in *L. angustifolius* lupanine (70%) 13 $\alpha$ -hydroxylupanine (12%), and angustifoline (10%) predominate, and in *L. luteus* the predominant alkaloids are lupanine (60%), sparteine (30%) and p-coumaryl-lupanine (5%) (Wink et al., 1995). Total alkaloid content has been shown to vary between species and cultivars of a single species (Sujak et al., 2006). Selective breeding has produced varieties of lupins with low alkaloid content (commonly called “sweet lupins”). Currently low alkaloid varieties contain less than 600 mg kg<sup>-1</sup> DM of alkaloids with some cultivars consistently containing less than 100 mg kg<sup>-1</sup> (Glencross, 2001). Wild type, high alkaloid varieties can contain up to 40,000 mg alkaloids kg<sup>-1</sup> seed meal.

##### Biological effects in fish

There are few reports regarding direct biological effects of quinolizidine alkaloids in fish. In mammals quinolizidine alkaloids appear to cause toxicity through neurological effects leading to loss of motor coordination and muscular control. All of the major quinolizidine alkaloids found in lupin exhibit anti-muscarinic acetylcholine receptor activity while several also exhibit anti-nicotinic activity (Wink et al., 1998). Lupanine and sparteine also inhibit Alpha<sub>2</sub> adrenergic receptors, while sparteine also inhibits butylcholine esterase (Wink et al., 1998). Direct evidence for similar effects in fish is lacking.

The organoleptic properties (i.e. bitter taste) of the lupin alkaloids are believed to be responsible for decreased feed intake in fish fed diets containing high levels of alkaloids. Low-alkaloid varieties of lupins do not appear to cause palatability problems, but may reduce growth performance at higher inclusion levels (Glencross et al., 2004a and 2004b). Glencross et al. (2006) added gramine, an indole alkaloid found in lupin, to rainbow trout in diets at levels up to 10,000 mg kg<sup>-1</sup> and reported decreased feed intake above 500 mg alkaloid kg<sup>-1</sup> diet and above. Fish fed the highest levels consumed too little food to supply maintenance protein and energy requirements. The authors noted increasing densities of melano-macrophage centres in the head kidney at increasing levels of dietary gramine but attributed the differences to starvation due to a lack of evidence for a direct toxic effect of alkaloids. No significant lesions were observed in the liver, kidney, spleen, pyloric caeca or intestine.

Chien and Chiu (2003) reported that alkaloid removal from *L. angustifolius* seed meal in diets (670 g meal kg<sup>-1</sup> diet) for juvenile tilapia (*Oreochromis niloticus* × *O. aureus*) increased feed performance but did not affect growth. The authors offer no explanation for this effect. However, the alkaloid content of seed before (40 mg kg<sup>-1</sup>) and after (20 mg kg<sup>-1</sup>) extraction were both low. Additionally the authors note that all of the experimental diets were deficient in methionine, lysine and threonine, but no supplementation was made. Therefore, whether the observed effects are attributable to the alkaloid content is questionable.

At this time there is insufficient data to reliably predict safe levels of lupin alkaloids in diets for fish. Caution should be exercised when determining inclusion levels. Reduced feed intake has been observed in fish fed diets containing high levels of alkaloids due to effects on palatability. Based on the results of Glencross et al. (2006) the highest demonstrated level of

dietary gramine that did not reduce feed intake in rainbow trout was 100 mg kg<sup>-1</sup> (reduced intake was observed at the next higher inclusion, 500 mg kg<sup>-1</sup> diet). Also, it is not known whether different fish species exhibit varying sensitivities to lupin alkaloids or whether safety levels of dietary lupin of one species or cultivar may differ.

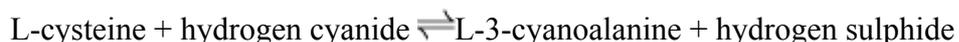
While lupin alkaloids (particularly sparteine) display toxicity and deterrence to tubifex worms and insects, Wink et al. (1998) did not observe antibacterial activity against *Escherichia coli*, *Serratia marcescens*, or *Bacillus subtilis*. In contrast, Erdemoglu et al. (2007) recently reported significant *in vitro* antibacterial activity of alkaloid extracts from *L. angustifolius* against *Staphylococcus aureus*, *B. subtilis*, and *Pseudomonas aeruginosa*, and moderate antifungal activity against *Candida albicans* and *Candida krusei*. Therefore, there is the potential for lupin alkaloids to affect the intestinal microbiota but there are currently no studies in the scientific literature specifically addressing antimicrobial effects *in vivo*.

#### 4.2.18. CYANIDE RELEASING COMPOUNDS

##### General characteristics

Cyanogenic phytochemicals are molecules present in plants that release hydrogen cyanide (HCN). Over 3,000 species of higher plants with diverse taxonomic designations are known to be cyanogenic including a significant number of food plants of global economic importance such as cassava, linseed, sorghum, peas, maize, barley, wheat, peanut, and rapeseed (Poulton, 1990; Jones, 1998; IPCS, 2004). The source of the HCN has been identified only in approximately 10% of the known cyanogenic plants (Poulton, 1990). Cyanogenic glycosides are the major source of HCN in cyanogenic plants. In certain sapindaceous seeds, cyanogenesis is a result of cyanolipid hydrolysis (Poulton, 1990).

In all higher plants including 'acyanogenic' species, biosynthesis of the hormone ethylene also generates HCN as a co-product in the 1-aminocyclopropane-1-carboxylic acid (ACC) pathway. This may account for the significant expression and activity of the cyanide detoxifying enzyme  $\beta$ -cyanoalanine synthase (syn: L-3-cyanoalanine synthase, EC 4.4.1.9) catalyzing the reaction in higher plants (Poulton, 1990):



Conditions of severe stress such as flooding and chilling may induce ethylene, and thus HCN, with possible consequences of cyanide phytotoxicosis and even plant death (Yip and Yang, 1998). Toxicity of the pesticide 2,4-dichlorophenoxyacetic acid is attributed to a similar mechanism of ethylene biosynthesis upregulation (Tittle et al., 1990).

HCN is thought to possess a number of physiological functions in the plant. At low, non-toxic concentrations, HCN may act as a signaling molecule involved in the control of some metabolic processes in plants while at high toxic levels it may be used for defense against herbivory (Siegien and Bogatek, 2006).

##### Structure, stability and solubility of cyanogenic compounds and hydrogen cyanide

###### *Cyanogenic glycosides*

Cyanogenic glycosides are secondary metabolites produced in over 2,600 plant species (Lechtenberg and Nahrstedt, 1999). Sorghum, cassava and linseed constitute the major sources of cyanogenic glycosides among the plants of worldwide economic importance. At least 60 cyanogenic glycosides are known (Lechtenberg and Nahrstedt, 1999) and all are *O*- $\beta$ -glycosidic derivatives of  $\alpha$ -hydroxynitriles (cyanohydrins) that, in turn, are mostly derived from any of five hydrophobic protein amino acids tyrosine, phenylalanine, valine, leucine and isoleucine or the non-protein amino acid cyclopentenylglycine. Based on these precursor amino acids, the cyanogenic glycosides can be aromatic, aliphatic or cyclopentenoid and the

majority involve a monosaccharide in the glycosidic linkage although di- and tri-saccharide derivatives also exist (Poulton, 1990). In plant tissues, the cyanogenic glycosides are stored intact separated from hydrolytic enzymes that degrade them to liberate hydrogen cyanide.

Plant tissue disruption following infection, technical processing or animal mastication removes the separation and the cyanogenic glycoside's carbohydrate moiety is cleaved off by  $\beta$ -glycosidases yielding the corresponding cyanohydrin intermediate (Poulton, 1990; EFSA, 2007). Glycosylation stabilizes the cyanohydrins, otherwise they decompose spontaneously and slowly and in plant tissues, the action of  $\alpha$ -hydroxynitrile lyase (EC 4.1.2.37) speeds up the process releasing HCN and an aldehyde or ketone. Hydrolysis to release HCN can also be accomplished by microorganisms in the digestive tract (EFSA, 2007).

### *Cyanolipids*

Cyanolipids mainly occur in the seeds of members of the Sapindaceae (Mustafa et al., 1986; Selmar et al., 1990; Avato et al., 2003 and 2005), and in some plants within the Hippocastanaceae, Boraginaceae, and Leguminosae (Acacia) plant families (Christie, 2004). None of the families include plants important for animal feed production at the moment.

Cyanolipids, like the cyanogenic glycosides are composed of a hydroxynitrile, which unlike conjugation to saccharides in the glycosides, obtains structural stabilization through esterification with a fatty acid (Johne, 1991). All the four known cyanolipid structures (Christie, 2004), Type I – IV are characterised by a branched five-carbon hydroxynitrile skeleton closely resembling leucine-derived cyanogenic glycosides (Poulton, 1983).

Only Type I (1-cyano-2-hydroxymethylprop-2-en-1-ol-diester) and Type IV (1-cyano-2-methylprop-2-en-1-ol ester) are cyanogenic. Activity of endogenous lipases removes the fatty acids from the cyanolipids and for Types I and IV, the released cyanohydrins are unstable and spontaneously decompose, liberating hydrogen cyanide and a carbonyl compound (Johne, 1991). In the intact plant tissue, subcellular localizations of the lipases and the cyanolipid must be different but currently these details are not reported nor are the substrate specificities of the lipases (Johne, 1991).

Cyanogenesis from both cyanogenic glycosides and cyanolipids serves a defensive role for the plant against herbivory from insects and animals. Both compounds are also thought to serve as storage compounds for reduced nitrogen as they can undergo metabolism in the plant into non-cyanogenic compounds (Selmar et al., 1990) in life stages such as seedling development.

### *Hydrogen cyanide*

Hydrogen cyanide (HCN) is a colourless, highly volatile and flammable liquid that is completely miscible in water, partly dissociating to liberate the cyanide ion (CN<sup>-</sup>) and forming a weak acidic solution. Major cyanophorous plants of relevance as animal and fish feed ingredients are listed in Table 4.2.18.1.

**Table 4.2.18.1.** Hydrogen cyanide concentrations in major cyanogenic plants important in food or feed production

<b>Plant</b>	<b>Parts with HCN</b>	<b>Total CN conc. (free and bound) mg/kg</b>
Cassava (Manioc)	Root (fresh weight) (sweet varieties)	10 – 20 25 – 33
	Root (fresh weight) (bitter varieties)	Up to 660 Up to 550
Lima bean	Seed (Burma variety)	2,000
	(white American variety)	100
Garden bean	Seed	20
Bitter almond	Seed, kernel	2,900 – 3,100
	Almond oil	800 – 40,000
Pea	Seed	20
Soyabean	Protein	0.03 – 0.07
	Hull seed	1.24 Up to 500

Adapted from (EFSA, 2007)

**Biological effects in fish**

Hydrogen cyanide is toxic to plants, animals and humans and its toxicity stems from its ability to link with the cofactors iron, manganese or copper of various metalloproteins, especially cytochrome c oxidase of the mitochondrial respiratory chain (EFSA, 2007). Uptake of HCN in fish can be through ingestion, the skin or the gill respiratory epithelium, following which it rapidly distributes throughout the body through binding to metalloproteins. The enzyme cytochrome c oxidase is the main site of rapid lethal cyanide action and the brain the major target organ of the HCN-induced cytotoxic hypoxia (Eisler, 1991).

HCN forms a stable complex with, and inhibits, the enzyme cytochrome c oxidase blocking the mitochondrial electron transfer chain. The result is cessation of cellular respiration causing cytotoxic hypoxia and tissue anoxia and a reactive shift from aerobic to anaerobic metabolism. Respiratory arrest due to the depression of the central nervous system by a combination of cytotoxic hypoxia and lactic acidosis is the cause of death (Eisler, 1991; Beasley and Glass, 1998). Effects on the circulatory system are also postulated as significant contributors to fatalities, especially in severe poisoning, manifesting as reduced cardiac output and cardiac shock in extreme cases, pulmonary oedema and possibly release and detrimental activity of biogenic amines (Beasley and Glass, 1998).

Acute cyanide toxicosis is a result of rapidly accumulating HCN levels in the body that overwhelm the cyanide detoxifying systems. The major HCN detoxifying pathway in animals is the enzymatic transsulphuration from thiosulphate to CN<sup>-</sup> forming thiocyanate (SCN<sup>-</sup>), a molecule 120 times less toxic that is then excreted in urine. The process is catalysed by the enzymes rhodanese (EC 2.8.1.1) and beta-mercaptopyruvate cyanide sulfurtransferase (EC 2.8.1.2; MST). Minor detoxification pathways include conjugation with cystine to form 2-iminothiazolidene-4-carboxylic acid or 2-aminothiazoline-4-carboxylic acid; combining

with hydroxocobalamin (B12) to form cyanocobalamin, which is excreted in urine and bile; and binding by haemoglobin to form methemoglobin (Eisler, 1991).

Long-term exposure to subclinical doses of HCN from food or feed results in chronic cyanide effects such as development of the human condition toxic ataxic neuropathy from eating insufficiently processed cyanogenic staples such as cassava or sudden death in livestock feeding on cyanogenic forage such as bracken fern (*Pteridium aquilinum*).

There are presently no publications on dietary effects HCN or on experiments involving other forms of oral dosing of HCN to fish. The reported toxicity of HCN to fish is in relation to cyanide in water, mainly in ecotoxicological experiments studying contamination of natural fish habitats by cyanide in industrial effluents. These exposures result in chronic cyanide effects in the fish that affect fecundity and other aspects of fish reproduction. For a detailed review refer to (Eisler, 1991; EFSA, 2007). The only report on exposure of fish to cyanide through the diet is a growth study conducted with Nile tilapia fed soaked and unsoaked sun-dried cassava meal with total cyanide levels of 9.9 and 71.1 mg/kg, respectively (Ng and Wee, 1989). The cyanide did not depress growth in the fish as the two diets showed similar growth at similar levels of inclusion.

No relevant information is available on maximum inclusion levels or interactions with gut microflora.

#### **4.2.19. UNKNOWN ANTINUTRIENTS**

The existence of as yet undiscovered antinutrients, toxins or antigens that may compromise the function of fishes' intestines or health cannot be ruled out. The complexity of plants used as feed ingredients and the difficulties often encountered when fractionating the plants as well as separating and analyzing the constituents may result in chemical changes or even destruction of certain compounds, precluding their identification and characterization. Limitations in the sensitivity of many methods of analysis may also prevent the identification of compounds present at very low levels.

#### **4.2.20. ANTINUTRIENTS - COMBINED EFFECTS**

More detailed knowledge 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. 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 antinutrients, preferably in proportions similar to those in plant-derived nutritional sources.

Tannins are known to interact with other antinutrients. For example, interaction between tannins and lectins removed the inhibitory action of tannins 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) reduced the deleterious effects of the latter.

Complex formation between saponins and other antinutrients could, however, lead to the inactivation of the toxic effects of both the substances (Makkar et al., 1995). Simultaneous consumption of saponin and tannin resulted in the loss of their individual toxicity to rats (Freeland et al., 1985). This is considered to be due to chemical reactions between them, leading to the formation of tannin–saponin complexes, inactivating the biological activity of both.

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.

Additive effects may, however, also occur. Alvarez and Torres-Pinedo (1982) suggested that soybean lectin bound to rabbit jejunal enterocyte apical membranes has a potentiating effect on saponin's detrimental influence on epithelial barrier function. Knudsen et al. (2008) recently demonstrated 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 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 soysaponins 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 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 to the different feed ingredients and an interaction between the saponins and microbiota could not be ruled out.

Bakke-McKellep et al. (2008) found that the combination of soybean lectin (SBA) and soybean trypsin inhibitor severely reduced intestinal epithelial function of Atlantic salmon, 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, PHA (phytohaemagglutinin, lectin) 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.

#### 4.2.21. CONCLUSIONS – ANTINUTRITIONAL FACTORS

Knowledge regarding responses to various qualities/processing methods of soybean meal in feeds for salmonids as well as to purified ANFs and/or various extracts thereof in soybeans has resulted in some insights in ANF effects and their possible role in the SBM-induced enteritis. Saponins, non-starch poly-/oligosaccharides, phytoestrogens, phytosterols 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. Table 4.2.21.1 indicates that various processing measures presently employed in feed manufacturing decrease activity/concentration of individual ANFs more or less effectively. However, recent findings suggest combinations of various ANFs, including the specific ANFs mentioned above, may have particular significance in causing detrimental effects to intestinal structure, function and defense mechanisms at lower levels than what the individual ANFs would elicit. More research is needed to investigate consequences of various ANFs, individually and in combinations, to nutrient digestibility, utilization and metabolism as well as to intestinal function, structure, defense mechanisms and microbiota. Long-term effects also merit investigation. This will aid in the ability to predict how a newly introduced plant ingredient as well as combinations of plant ingredients may affect the fish and identify steps needed to reduce/prevent detrimental health effects.

**Table 4.2.21.1.** Result of the authors' discussions regarding "damaging" potential of antinutrients and the type of processing that might reduce content of antinutrients in feed ingredients.

Antinutrient	Important sources	Potential to cause adverse effects <sup>1</sup>		
		In "raw" ingredients	In processed ingredients <sup>2</sup>	Type of processing
Proteinase inhibitors	Beans, peas	****	*	Heat, enzyme/fermentation
Amylase inhibitors	Peas	*	*?	Heat, enzyme/fermentation
Lipase inhibitor	Beans	**	*?	Heat, enzyme/fermentation
Lectins	All plants seeds	****	**	Heat, enzyme/fermentation
Saponins	Beans, peas	***	**	Alcohol extraction
Oestrogens	Beans	**	*	Alcohol/non-polar solvent extraction
Goitrogens	Rapeseed	**	**	?
Fibre	All plants	**	*	Dehulling; enzyme/fermentation
Oligosaccharides	Beans and peas	**	*	Aqueous extraction
Tannins	Rapeseed, beans	**	*	Dehulling
Phytic acid	All plants	*	*	Enzyme/fermentation
Allergens	Soy, unknown	**	*	Heat, enzyme
Gossypol	Cotton seed <sup>3</sup>	**	**	Non-polar solvent extraction
Glycoalkaloids	Potatoes	*	*	?
Arginase inhibitors	Sunflower, soy	*?	*?	Alcohol extraction
Quinolozidine alkaloids	Lupins <sup>4</sup>	**	*	Aqueous extraction
Cyclic fatty acids	Processed oils	*	*	?
Cyanide releasing compounds	Linseed, sorghum, peas	**	*	Heat, aqueous extraction

1) Increasing number of asterisks implies greater potential to cause adverse effects;

2) Following common processing procedures;

3) Glandless cotton seeds contain little or no gossypol;

4) Low alkaloid varieties exist

### 4.3. CURRENT AND FUTURE PLANT OILS IN AQUAFEEDS

Regardless of origin, plant oils are deficient in the typical marine long chain highly unsaturated fatty acids (HUFA) arachidonic acid (ARA, 20:4n-6), eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3). The composition of other fatty acids will vary with plant species and to some extent variants, soil and environmental

conditions. Some plant oils that are either used or considered having potential for use in aquafeeds are listed in Table 4.3.1.

Table 4.3.1. Annual oil production and fatty acid composition of some commonly used oils in aquafeeds.

Plant oil	Production*	10:0	12:0	14:0	16:0	18:0	18:1	18:2	18:3	20:1	22:1	HUFA <sup>a</sup>
Coconut	3.3	7	<b>48</b>	<b>19</b>	9	3	6	2				
Palm	37.3			1	<b>44</b>	4	<b>39</b>	10				
Olive	2.7				12	3	<b>83</b>	9				
Rapeseed (Canola)	17.3				5	2	<b>60</b>	22	10	1		
Rapeseed					4	1	15	14	9	10	<b>45</b>	
Safflower	0.2				6	2	13	<b>76</b>				
Safflower high 18:1					7	1	<b>81</b>	10				
Sunflower	10.7		1		6	5	22	<b>66</b>	1			
Corn	2.1				12	2	28	<b>56</b>	1			
Soybean	35.1				11	4	24	<b>52</b>	8			
Linseed	0.7				6	3	18	17	<b>55</b>			
Total plant oils	127.1											
Fish oil	0.9**			5	13	2	17	4	1	9	9	<b>18</b>

\*FAOSTAT, annual production for 2006 in million tons, \*\*FAOSTAT for 2003 in million tons.

<sup>a</sup>HUFA; ARA, EPA, DHA, typical composition is generally in the range ARA 1% of lipid, EPA and DHA 16-17% W with DHA at around 11%. But there are major differences.

Compiled from Ackman 1990; Mirza et al. 1998; Dubois et al. 2007; Gecgel et al. 2007

For more detailed information on these and other oils, the reader is referred to more comprehensive reviews (e.g. Guil-Guerrero et al., 2001; Dubois et al., 2007). There are several ways of classifying the suitability of plant oil for aquaculture. One obvious is the availability as high production generally means good availability and low price. An annual production of more than 127 million tons in 2006, plant oils have a significant advantage over fish oils, for which a declining production amounted to only 0.9 million tons in 2003 (FAOSTAT). However, of these oils, only a few are produced in large quantities. For 2006, palm and soy oils were predominating with an annual production of more than 72 million tons and contributed to 57% of the global production (Table 4.3.1). Other oils of significant production are rapeseed, sunflower, cottonseed, coconut, palm kernel and olive oils. Linseed oil, an oil of considerable potential to be used in fish diets, only has an annual production of 0.7 million tons (FAOSTAT 2006).

Another way to define plant oils is by their fatty acid contents. Using this classification, there are broadly speaking 3 or 4 groups of oilseeds. The most common, both in number of variants and production volumes, are those containing high levels of the polyunsaturated fatty acid (PUFA) linoleic acid (LA, 18:2n-6) ( $\geq 50\%$  of fatty acids). These include soybean, sunflower, safflower and corn oils. Another major group of plant oils are those with high levels of monoenoic fatty acids. In most cases these are high in oleic acid (18:1n-9), as in olive oil. More rarely, other monoenoic fatty acids predominate, as in rapeseed oil (now replaced by canola oil, see comments below), where the predominating fatty acid (>50%) is the very long chain erucic acid (22:1n-9). The third main group of seed oils is those having high levels of saturated fatty acids, such as coconut oil (mainly 12:0) and palm oil (mainly 16:0). Until recently, these oils were not considered as particularly

interesting in fish feeds. Mainly because it has been stated that saturated fatty acids with a corresponding high melting point will reduce lipid digestibility, particularly at lower water temperatures (NRC 1993; Olsen and Ringø 1998). However, some recent data do point to some use of palm oil in fish diets (Ng et al. 2007), particularly in diets containing high levels of other plant oils (Ng et al. 2004).

There are also some oils that do not fit into these broad categories. One that has gained significant interest in salmonid feeds is linseed oil. As it contains more than 50% of the fatty acids as linolenic acid (LNA, 18:3n-3) it has been explored as a potential for increasing the content of n-3 HUFA in salmonid fish flesh (Tocher et al. 2003a; Bendiksen and Jobling 2004).

It should be noted that the compositional data in Table 4.3.1 mainly covers the commonly used brands of plant oils. Within each plant species, many variants are available that differ in their fatty acid profiles, and more are being developed that can be produced in large quantities if required. One example is safflower, where the typical composition is 71-75% 18:2n-6 and 16-20% 18:1n-9 (Knowles 1989; Gecgel et al. 2007; Table 1). However, variants with almost 90% 18:2n-6 have been reported (Futehally and Knowles 1981) as have some other and very interesting variants with very high levels of 18:1n-9 (> 80%) (Fernandez-Martinez et al. 1993; Mirza et al. 1998, Table 1). Another popular oilseed is canola oil. It originated as rapeseed that contains large amounts of 22:1n-9. However some earlier data indicating that this fatty acid could cause heart problems (e.g. Clement and Renner 1977) prompted a selective breeding program that eventually succeeded in producing a very low 22:1n-9 variant in the early 1970s. The seed was named after **Canadian** (origin of the seed) **oil**, **low acid**, **Canola**, and has a typical composition of 60% 18:1n-9, 20% 18:2n-6 and 10% 18:3n-3 (Ackman 1990, Table 1). As an alternative to standard breeding procedures, molecular cloning is now gaining more ground as an alternative to tailor various oilseeds. These may soon be available on the market.

#### 4.3.1. FUNCTION OF PLANT OILS IN FISH

##### 4.3.1.1. Energy

The main incentive for including plant oils into fish diets is the provision of energy for growth and consequently deposition in adipose tissue (Henderson and Tocher 1987; Tocher 2003). As opposed to mammalian species, fish use fatty acids as substrates for energy requiring processes such as maintenance, growth and gonad development. In general, plant fatty acids seem as good substrates for  $\beta$ -oxidation as the major fatty acids in fish oils. An interesting exception is the medium chain triglycerides (MCT) with chain lengths ranging from around C<sub>6</sub> to C<sub>12</sub> (coconut oil has much 12:0). MCT are known to be very good substrates for  $\beta$ -oxidation (Bach et al. 1996; Piot et al. 1999) and dietary inclusions have been explored as readily available energy sources for adult and larval fish, to increase protein sparing and to sustain growth without increasing lipid deposition in larger fish (Tocher 2003). Although data differ between studies, species and sizes there is accumulating evidence that moderate inclusions of MCT will increase growth and feed conversion in several fish species including ayu (*Plecoglossus altivelis*) (2-6% MCT of diet) (Netatipour et al. 1990; Mustafa et al. 1991), red drum (*Sciaenops ocellatus*) (Davis et al. 1999) (1% of diet) and carp (*Cyprinus carpio*) (3% of diet) (Fontagne et al. 2000b). In most cases there is also a notable reduction in body lipid content.

Moderate inclusion levels of MCT (<10%) do not seem to affect growth rate but will enhance feed conversion, nitrogen retention, nutrient digestibility and intestinal enzyme activity in Atlantic salmon (Nordrum et al. 2000; 2003). They will also reduce muscle lipid content in a dose-dependent manner (Røsjø et al. 2000; Nordrum et al. 2003). A possible

impairment of absorption of some HUFA do however warrant more detailed studies if used extensively in aquafeeds (Røsjø et al. 2000). Further increases (>10%) in the level of MCT will have negative impacts on the fish. Adding 17% tricaprylin (C<sub>8</sub>) of the diet to rainbow trout depresses growth and nutrient digestibility compared to fish oil controls (Nielsen et al. 2005). In Atlantic salmon, including more than 10% MCT into the diet (mixture of C<sub>8</sub> and C<sub>10</sub>) appears to reduce growth, feed intake and energy retention (Nordrum et al. 2003).

The response of the fish to MCT depends on the type of MCT administered. In carp larvae, tricaprylin (C<sub>8</sub>) will reduce growth and survival at a 10% inclusion level (total lipid 24%) while this is not observed with coconut oil (mainly 12:0) or triolein (Fontagne et al. 1999). At a 3% inclusion level both tricaprylin and tricaproin will enhance growth of carp larvae for the first week of feeding when compared to triolein (Fontagne et al. 2000a). However further feeding with tricaprylin will subsequently reduce growth and survival. In European sea bass (*Dicentrarchus labrax*) larvae, adding 3% tricaprylin to the diet will double survival compared to tricaproin (C<sub>6</sub>) or tricaprln (C<sub>10</sub>), while growth of fish fed tricaprln and tricaprylin was better than those fed tricaprln or triolein (Fontagne et al. 2000 b).

An interesting use of MCT has been suggested in Atlantic cod where the development of fatty livers is a significant welfare problem in commercial aquaculture. Including MCT into these diets may thus help by lowering the lipid deposition. However, when Atlantic cod were fed up to 4% coconut oil or conjugated linoleic acid (CLA) of the lipid (lipid content 15%) over 55 days, the effects were rather marginal (Olsen, unpublished data, Table 4.3.1.1.1.). Growth rate (SGR) was similar. However, at 1% level both coconut oil and CLA actually increased liver size from 7.7% in control fish to more than 8.7%. At the highest level (4%), livers were similar to the control group. The only option of reducing liver size in this trial was to reduce the dietary lipid from 15% to 8%, causing liver size to be reduced from 7.7% to 6.0%.

Table 4.3.1.1.1 Growth, and liver data in Atlantic cod fed coconut oil or conjugated linoleic acid over 55 days.

Diet	Initial weight	Final weight	End length	K-index	SGR	HSI	Liver lipid
15% FO	340	411 ± 54	34.6 ± 1.9	1.02 ± 0.08	0.44 ± 0.02	7.69 ± 1.64 <sup>ab</sup>	72.8 ± 5.7
8% FO	340	392 ± 84	34.2 ± 2.5	1.04 ± 0.11	0.47 ± 0.01	5.99 ± 1.63 <sup>a</sup>	71.2 ± 5.3
11% FO + 4% CO	336	471 ± 171	35.0 ± 4.2	0.95 ± 0.15	0.46 ± 0.03	7.45 ± 2.14 <sup>ab</sup>	72.7 ± 5.5
11% FO + 4% CLA	329	418 ± 94	35.3 ± 2.4	1.09 ± 0.21	0.44 ± 0.01	7.10 ± 1.32 <sup>ab</sup>	72.5 ± 6.3
14% FO + 1% CO	331	484 ± 123	35.5 ± 2.4	0.95 ± 0.08	0.47 ± 0.04	8.74 ± 1.78 <sup>b</sup>	76.3 ± 3.6
14% FO + 1% CLA	337	475 ± 100	35.6 ± 2.4	0.97 ± 0.10	0.47 ± 0.08	8.78 ± 1.91 <sup>b</sup>	74.4 ± 4.2

FO, fish oil; CO, coconut oil; CLA, conjugated linoleic acid. Columns with superscripts not sharing the same letter are significantly different.

#### 4.3.1.2. Essential fatty acids

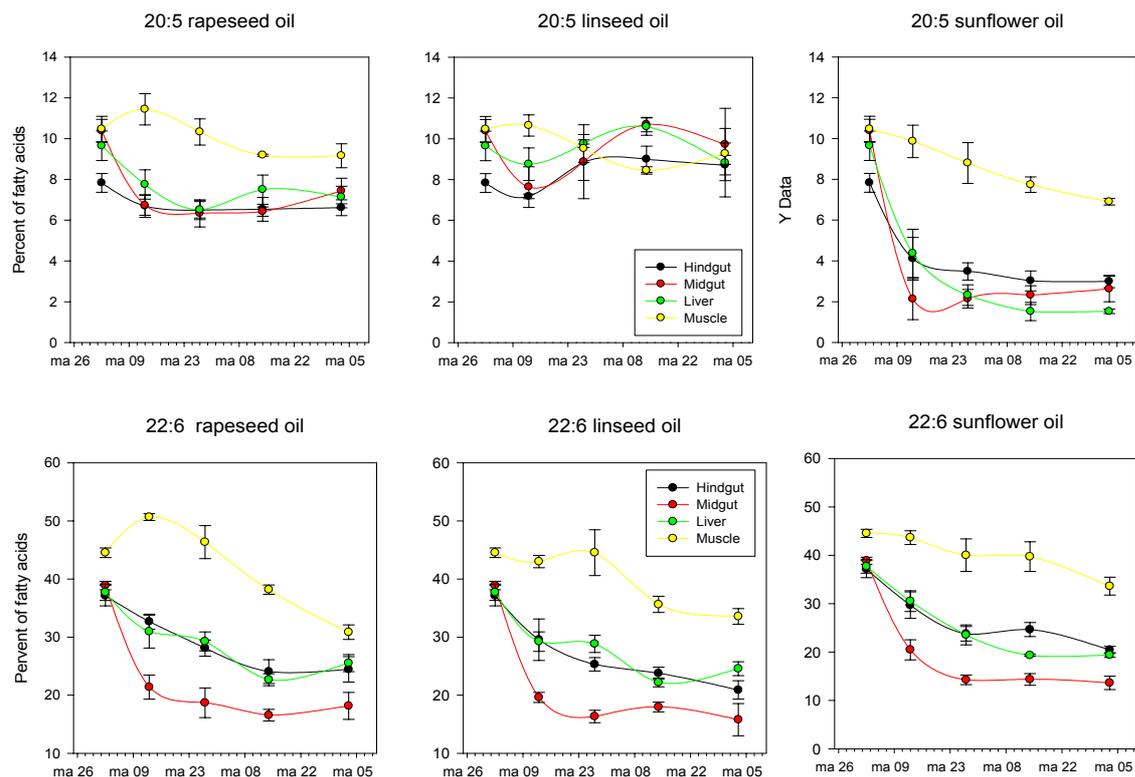
An inevitable consequence of replacing fish oil with plant oils in aquafeeds is that the level and composition of essential fatty acids (EFA) in the diets will change. EFA is the definition of a series of PUFA that the fish are incapable of producing, yet are required for the maintenance of various essential biological functions. Fish, as mammals, seem to require two series of EFA, termed n-6 and n-3 PUFA. They originate from 18:2n-6 and 18:3n-3 produced

mainly by photoautotrophic organisms in the marine and aquatic environment. But their biological functions are found in their elongated and desaturated products of the C<sub>20</sub> and C<sub>22</sub> series (Henderson and Tocher 1987; Tocher et al. 1998; Sargent et al. 1999a; 1999b; Tocher 2003). For the n-3 series, 20:5n-3 and 22:6n-3 are regarded as the most important, although the quantitatively minor fatty acids such as 20:3n-3, 20:4n-3, 22:4n-3 and 22:5n-3 may have some significance as they are found in virtually every tissue analyzed. For the n-6 series, 20:4n-6 is the predominant product, but smaller amounts of 20:3n-6 and 22:4n-6 and 22:5n-6 may also be found and may have some biological importance.

Which series of fatty acids are regarded as EFA does however vary with species. As mentioned above, the “biologically active EFA” are mainly 20:5n-3, 22:6n-3 and 20:4n-6. Of those fish that are the focus in the present report, both Atlantic salmon and rainbow trout have a significant capacity to elongate and desaturate 18:2n-6 and 18:3n-3 to their C<sub>20</sub> and C<sub>22</sub> homologs. There is one possible exception in that these salmonids have limited capacity to maintain a high level of 22:6n-3 if this series is supplied mostly as 18:3n-3 (Sargent et al. 2002; Tocher 2003). This may indicate an absolute requirement for 22:6n-3. But the significance of this is not known at present. As opposed to salmonids, marine fish seem incapable of producing any significant amounts of C<sub>20</sub> and C<sub>22</sub> PUFA from 18:2n-6 and 18:3n-3 (Sargent et al. 1999a; 2002). The mechanisms for this appear to differ between species; in turbot elongating activity appears down-regulated while  $\Delta 5$  desaturase seems to be down-regulated in gilthead sea bream (Tocher et al. 1998; Tocher 2003). In cod, both elongase and desaturase appear to have low activities (Bell et al. 2006). Although little data is available for Atlantic halibut, it is clear that there is very little or no metabolism of C<sub>18</sub> PUFA. Thus, in Atlantic cod, and Atlantic halibut, plant oils cannot be used as EFA supplements, making these fish to rely solely on fish oils as EFA sources.

The EFA in the organism has, broadly speaking, two main functions: maintaining membrane integrity and as precursors for hormone-like compounds, the eicosanoids. Optimum cell membrane functions are in many ways a prerequisite for normal functions of the organism. Originally, the membrane was assumed to form the borderline between watery exterior and cell interior. But accumulating research over many decades now draws a much more complex picture of membrane functions affecting and regulating areas such as protein-peptide binding, enzyme function, ion channeling and signal transduction (McIntosh and Simon 2006) to mention a few. With the relatively recently identified lipid rafts (membrane compartments enriched in cholesterol, sphingolipids and certain phospholipids) in cell membranes, new and interesting links have been established between lipid and cell proliferation, apoptosis, cell signaling and diseases such as Alzheimer’s disease and cancer (McIntosh and Simon 2006; Ma 2007).

Of the EFA in fish, 22:6n-3 seems to be in a class by itself. It is found in relatively high quantities in all membrane lipids and particularly in neural tissues, including eye and brain of all living animals (Sargent et al. 1999 a; Tocher 2003). The reason for this absolute requirement remains elusive. Originally, it was believed to be essential for lowering membrane melting point and thus maintaining membrane fluidity. But the main determinant for this is now known to be the ratio of monounsaturated to saturated fatty acids (Wodtke and Cossins 1991). Rather, it appears that 22:6n-3 with its particular helical twist and compact form in the membrane is what makes it unique and essential (Applegate and Glomset 1986). Other EFA, particularly 20:5n-3 and, 20:4n-6, are also found in membranes, but generally in lower amounts than 22:6n-3. Furthermore, the content of such fatty acids is more variable being under more direct dietary influence. For example, while 22:6n-3 appears to be selectively conserved at low dietary inclusion levels, the content of 20:5n-3 is quickly reduced if diets do not contain it (Tocher 2003). This is also illustrated in Figure 4.3.1.2.1. (Olsen and Ringø, 2004 unpublished data).



**Figure 1.** Changes in content of DHA and EPA in tissue total phospholipids in Atlantic salmon fed purified diets over 8 weeks.

The other main function for EFA of the  $C_{20}$  series is that they serve as precursors for eicosanoids. These are signaling molecules made from the  $C_{20}$  EFA. They are generally short-lived and mainly affect local sites of their production. In these areas, they control many bodily functions including inflammation and immune regulation (de Pablo and de Cienfuegos 2000). There are two main series of eicosanoids produced: those derived from n-3 (mainly from 20:5n-3 but also 20:3n-3 and 20:4n-3) and n-6 EFA (mainly from 20:4n-6, but also 20:3n-6 and 20:4n-6) (Smith, 1989; de Pablo and de Cienfuegos, 2000; Kim and Luster, 2007). It has been suggested that EFA of the  $C_{22}$  series may be used for the production of eicosanoids, and although some products have been shown the significance and biological effects remain to be verified. Within each series, prostaglandins (PG), prostacyclins and thromboxanes (TX) are formed through the initial activation through cyclooxygenases (COX) while leucotrienes (LT) and lipoxins are formed through the action of lipoxygenases. At present, several hundred eicosanoids have been identified, and with the increased focus of research, it is not unlikely that many new products will be discovered in the future. Although very simplified, it is a general consensus that eicosanoids of the n-6 series are generally more potent and pro-inflammatory than eicosanoids of the n-3 series that are assumed to have very low potency and efficiency. The severity of response through eicosanoid production is thus dependent on the ratio of these products used as substrates (Smith 1989; Suchner and Senftleben 1994; de Pablo and de Cienfuegos 2000; Balfry and Higgs 2001).

#### 4.3.2. QUANTITATIVE ESSENTIAL FATTY ACID (EFA) REQUIREMENT

The EFA requirement of fish is the sum of EFA required for maintaining growth, health, gonad development, cellular integrity and eicosanoid production (Sargent et al. 2002). Despite the fact that fish lipid nutrition has gained notably more focus over the past decades, the quantitative EFA requirement for many species is still rather obscure. Furthermore, for

many species, EFA requirements were obtained in the past using suboptimal diets that may have invalidated the precision of the results. In most studies, growth rates have been the standard method to assess EFA requirements. In freshwater fish, EFA requirements have been assessed through the quantification of 20:2n-9 and 20:3n-9. These two non-EFA PUFA are being produced in an attempt to compensate for the loss of EFA in cellular membranes during deficiency (Olsen et al. 1991a; NRC 1993). In these studies, EFA requirements are satisfied when these fatty acids disappear from the phospholipids. Whatever the procedure, current requirements can only be viewed as minimum values as other levels and mixtures of n-6 / n-3 ratio may be required for optimum immune competence and sexual maturation, to mention a few. A compilation of the EFA requirement of some selected species is given in Table 4.3.2.2.1.

#### 4.3.2.1. Salmonids

For larger freshwater fish (no figures for Atlantic salmon) EFA requirement is around 1-2% of diet each of 18:2n-6 and 18:3n-3, and generally half of that if supplied as C<sub>20</sub> and C<sub>22</sub> PUFA (NRC 1993; Sargent et al. 2002; Table 7.3). Salmonids generally seem to require around 1%, with a possible exception of Arctic charr, where requirements may be closer to 2% (Olsen et al. 1991a; Yang and Dick 1994). For smaller salmonid fish, the requirement is most likely higher due to higher rates of tissue growth (but data not available). It is also possible that the endogenous elongation and desaturation may not be capable of supplying all the HUFA required for normal growth. Hence, an absolute requirement for 22:6n-3 has been suggested in rainbow trout (Wirth et al. 1997) and it is not unlikely that this may be valid for other species as well, including Atlantic salmon.

It has also been suggested that EFA requirements should be expressed in percentage of lipid rather than as percentage of diet. This was suggested as far back as in 1977 when it was shown that growth of rainbow trout was reduced when the level of lipid increased and the level of 18:3n-3 was kept constant (Takeuchi and Watanabe 1977). Increasing the level of n-3 PUFA up to 20% of the lipid or n-3 HUFA to 10% restored growth.

With the high content of 18:2n-6 and 18:3n-3 in many plant oils, it seems highly unlikely that salmonids will experience classical deficiencies even with high inclusion levels of plant oils. Furthermore, at present, fish diets do still contain significant amounts of fishmeal that is a very good source of C<sub>20</sub> and C<sub>22</sub> HUFA. The challenges seem rather to be more related to the effects of alteration in the n-6/n-3 ratio that these diets will produce. Some of these effects will be covered in more detail below.

#### 4.3.2.2. Marine fish

As plant oils do not supply any EFA to marine fish, these oil sources can only be regarded as energy sources. The challenge under these circumstances is to avoid a dilution that may cause EFA deficiencies. The EFA requirements in Atlantic cod and Atlantic halibut have yet to be established. But based on knowledge from other marine species, a requirement of around 0.5-1.5% each 20:5n-3 and 22:6n-3 should be expected for juvenile and adult fish, and probably somewhat higher for larvae (Sargent et al. 2002; Table 7.2.2.1). It has also been suggested that the EFA requirement in marine fish should be calculated in percent of lipid rather than in percent of diet. In red sea bream and yellowtail the EFA requirement has been estimated at 20% n-3 HUFA of dietary lipid (Takeuchi et al. 1992a; 1992 b) while 10% seems sufficient in red drum (*Sciaenops ocellatus*) (Lochmann and Gatlin 1993). Regardless of species, 22:6n-3 appears to be the primary EFA followed by 20:5n-3. In small (larval) fish the ratio ranges between 4/1 to around 2/1 depending on species and study. In larger fish, the level appear to be more evened out, and ratios of around 1 have been reported in gilthead sea bream (Ibeas et al. 1997). Deficiency in 22:6n-3 has been reported in several studies and

appears to affect neural tissues in early stages. Indications of impaired vision and neural development has been reported in larval herring ( Navarro et al. 1993; Bell et al. 1995; ) and sea bass ( Bell et al. 1996b; Navarro et al. 1997) while good pigmentation in Japanese flounder required dietary supplements of 22:6n-3 (Estevez and Kanazawa 1996; Estevez et al. 1997).

The requirement of 20:4n-6 is probably much lower than for n-3 HUFA. Around 0.3% has been suggested in turbot (*Scophthalmus maximus*) (Castell et al. 1994). There are no clear figures for other species.

Much of current research into EFA requirements in marine fish focuses on larval development. And in particular on the relative requirement on the ratio of 20:5n-3/20:4n-6/22:6n-3 rather than the absolute quantitative levels (Sargent et al. 2002; Bell et al. 2003a; Bell and Sargent 2003; Sargent et al. 1999a; Sargent et al. 1999b). But as current plant oils do not contain these fatty acids, further discussions here are beyond the scope of this review.

Table 4.3.2.2.1. Essential fatty acid requirement in selected freshwater and marine fish (adapted from Sargent et al. 2003 and references therein).

	Larvae and early juvenile		Older juvenile and preadults	
	EFA	% of diet	EFA	% of diet
<b>Freshwater</b>				
Rainbow trout ( <i>O. mykiss</i> )	LNA	0.7-1.0	DHA	required
	n-3 HUFA	0.4-0.5		
Chum salmon ( <i>O. keta</i> )	LN & LNA	1.0 & 1.0		
Coho salmon ( <i>O. kisutch</i> )	LN & LNA	1.0 & 1.0		
Cherry salmon ( <i>O. masou</i> )	LNA & n-3 HUFA	1.0 & 1.0		
Arctic charr ( <i>S. alpinus</i> )	LNA	1.0-2.0		
Carp ( <i>C. carpio</i> )	LN	1.0	n-6PUFA	1% (0.25% LN)
	LNA	0.5-1.0	n-3 PUFA	ca. 0.05%
<b>Marine</b>				
Cod ( <i>G. morhua</i> )			EPA	required
			DHA	ca. 1%
Turbot ( <i>S. maximus</i> )	n-3 HUFA	0.8	DHA	required
	AA	ca. 0.3		
Red sea bream ( <i>P. major</i> )	n-3HUFA	0.9	n-3HUFA	2.1 (1%DHA)
	EPA	1.0	EPA	2.3
	DHA	0.5	DHA	1.0-1.6
Gilthead sea bream ( <i>S. aurata</i> )	n-3HUFA <sub>DHA:EPA=1.0</sub>	0.9	n-3HUFA	5.5 <sub>DHA:EPA=0.3</sub>
	n-3 HUFA <sub>DHA:EPA=0.5</sub>	1.9	n-3HUFA	1.5 <sub>DHA:EPA=2.0</sub>
			n-3HUFA	1.5 (in PL)
			DHA:EPA	ca 2
Yellowtail flounder ( <i>P. ferrugineus</i> )			n-3HUFA	2.5
Yellowtail ( <i>S. quinqueradiata</i> )		n-3HUFA	3.9 <sub>DHA:EPA=0.5</sub>	
			EPA	3.7
			DHA	1.4-2.6

#### 4.3.3. SPECIFIC EFFECTS OF PLANT OILS IN AQUAFEEDS

In marine fish, the most predominant effect of increasing plant oils in the diets is the reduction of cellular EFA content and some alteration in membrane composition. In salmonids, including plant oils will not only affect membrane composition, but will also alter

the ratio of n-6 / n-3 C<sub>20</sub> PUFA, which may have pronounced effects on immune functions and eicosanoid production.

#### 4.3.4. EFFECT OF THE OIL FRACTION ON IMMUNITY

From mammalian studies it is well known that dietary marine n-3 HUFA have the potential to modulate the immune system. The studies have been spurred by the considerable evidence that high incidences of cardiovascular and inflammatory conditions, and some cancers, are related to very high intakes of 18:2n-6 relative to 18:3n-3 with the consequence of elevated production of 20:4n-6 (Okuyama et al. 1997). In general, the effects of n-3 HUFA in mammals appear to be due to dampening of the immune responses. These include reductions of mitogen-induced lymphocyte proliferation, reductions in the production of pro-inflammatory cytokines such as interleukin 1, interleukin 2, tumor necrosis factor and reduced natural killer activity. The only area where n-3 HUFA appear to enhance activity are through increased activity in phagocytotic cells such as macrophages (de Pablo and de Cienfuegos 2000).

The mechanism for the immunomodulatory effect of n-3 HUFA is generally explained by two (or three) factors. Firstly, dietary n-3 HUFA will increase membrane fluidity that will affect enzymes and proteins involved in surface receptors and ion channels, thus altering cytokine binding (de Pablo and de Cienfuegos 2000). Increased phagocytosis of zymosan particles has also been related to increased incorporation of unsaturated fatty acids into macrophages (Calder et al. 1990). Secondly, altering dietary n-6/n-3 ratio will alter the ratio of membrane C<sub>20</sub> HUFA. Upon stimulation, leucocytes stimulate phospholipase A<sub>2</sub> to release C<sub>20</sub> PUFA, mainly 20:4n-6 and 20:5n-3 which are used as substrates for eicosanoid production. While LT and lipoxins have chemotactic activity attracting cells to sites of inflammation and stimulate lymphocyte proliferation, PG are vasodilative opening blood vessels enabling more migration of leucocytes to active sites (Balfry and Higgs 2001). Although eicosanoids derived from n-3 HUFA have many similarities to their n-6 counterparts, they are generally recognized as having lower activities and potencies, thus explaining their lowering effect on the immune response ( de Pablo and de Cienfuegos 2000; Balfry and Higgs 2001; Tocher 2003). As a consequence, treatment of patients with autoimmune disorders have found some use in humans, but with a drawback that fish oils impair resistance to some infectious diseases (de Pablo and de Cienfuegos 2000).

It is also generally accepted that dietary lipids will affect fish tissue membranes and cells of the immune system ( Henderson and Tocher 1987; Waagbø et al. 1993; Tocher 2003; Gjøen et al. 2004). However, fish membranes are relatively high in 20:5n-3 being tailed by 20:4n-6 by a factor of more than 10. This is completely the reverse of what is found in mammals. One would thus expect that eicosanoids of the n-3 HUFA series should predominate in fish. This does not appear to be the case, and there is considerable evidence suggesting that eicosanoids of the n-6 series predominate in fish too. There are probably many lines leading to this selectivity. Firstly, 20:4n-6 is particularly concentrated in the phosphatidylinositol (PI) phospholipids, suggesting a particular role of this phosphoglyceride in the supply of 20:4n-6 for the eicosanoid cascade (Tocher 2003). Secondly, there appears to be considerable selectivity of enzymes in the eicosanoid cascade favoring 20:4n-6 as substrate. One of these regulatory sites appears to reside at the level of prostaglandin H synthase (COX) which is a central branching point in the eicosanoid synthesis. While the human and ovine COX has a relatively broad substrate acceptance, the COX from brook trout (*Salvelinus fontinalis*) has a narrow substrate specificity discriminating against 20:5n-3 and 22:6n-3 (Liu et al. 2006). A natural consequence of this is that increases in n-6 HUFA in PI following increased feeding of 18:2n-6 should cause major increases in the production of this series of eicosanoids. Indeed most available literature do point to this in salmonids while

increases in n-3 HUFA or linseed oil (18:3n-3) will inhibit the production of n-6 eicosanoids (Ashton et al. 1994; Bell et al. 1996a; Balfry and Higgs 2001; Tocher et al. 2003b; Gjøen et al. 2004).

The question then remains if increasing the n-6 PUFA in plant oil supplemented diets will enhance the immune response as seen in mammals and reduce the likelihood of infections. The answer is perhaps, and as in mammalian literature, data are contradictory and inconclusive. On one hand, there is some literature supporting the notion of immunosuppression of n-3 HUFA and/or enhancement by n-6 PUFA. In channel catfish, *Ictalurus punctatus*, high dietary intake of n-3 HUFA has been associated with reduced immunocompetence, particularly at high temperatures. These include reduced phagocytic ability and intracellular superoxide production (Lingenfelter et al. 1995), reduced LTB production (Fracalossi and Lovell 1994) and decreased disease resistance upon challenge with *Edwardsiella ictaluri* (Fracalossi and Lovell 1994; Li et al. 1994). These differences generally seem to disappear at lower temperatures, suggesting an additional temperature-dependent role of n-3 HUFA. Furthermore, in Arctic charr fed diets supplemented with soybean oil or fish oil and challenged with *Aeromonas salmonicida* (the causative agent of furunculosis), increased mortality (48%) was observed in the fish oil group, compared to the soybean oil group (20%) (Lødemel et al. 2001). One group of fish supplemented with a linseed oil diet was intermediate between the two. In Atlantic salmon, decreased antibody titers and reduced likelihood of survival was related to high dietary n-3 HUFA content (Erdal et al. 1991).

There is also considerable data supporting immunostimulatory effects of n-3 HUFA, or immune suppression by n-6 PUFA. In channel catfish these include increased phagocytic activity (Sheldon and Blazer 1991; Lingenfelter et al. 1995), which, as suggested in mammals (de Pablo and de Cienfuegos 2000), is related to increased membrane fluidity. Lower haematocrit, higher thrombocyte counts, and increased resistance to erythrocyte fragility has been noted (Klinger et al. 1996). A possible functional disability was observed in rainbow trout when head kidney supernatants from sunflower oil fed fish were less chemo-attractant to neutrophils compared to those fed fish oils (Ashton et al. 1994). In Atlantic salmon parr, exchanging fish oil to sunflower oil reduced the number of B cells (kidney and spleen) in vaccinated fish responding to *A. salmonicida* and made them more susceptible to disease when non-vaccinated fish were challenged with *A. salmonicida* and *Vibrio anguillarum* (Thompson et al. 1996). In the latter case, no effects were seen on non-specific immune parameters. The same was found in Atlantic salmon fed diets supplemented with up to 60% of the lipid as a mixture of canola oil and poultry fat (Balfry et al. 2006). While the non-specific immunity was unaffected, antibody titers against *V. anguillarum* decreased in the canola: poultry group as did peripheral blood leukocyte respiratory burst. Similarly, in a long-term feeding study using soybean oil and fish oils, Waagbø et al. (1993) showed that fish fed high levels of fish oils had better survival when infected with *A. salmonicida*, despite the fact that head kidney macrophage phagocytosis and bacterial killing was reduced in these fish (IL-1 and serum hemolytic activity unaffected).

There are also data showing no or little effect of diet on some immunological parameters. Many of these have already been mentioned above. Additionally, in Atlantic salmon substituting up to 100% of the added fish oil (fish oil still added in the fishmeal) with soybean oil did not increase LTB<sub>4</sub> or PGE<sub>2</sub> production from head kidney macrophages when 20:4n-6 was added to the medium, except at very low environmental temperatures (Gjøen et al. 2004). Phagocytic activity was also unaffected as was susceptibility to infection by *A. salmonicida*. In a relative extreme feeding trial (purified diets), rainbow trout were fed diets supplemented with only soybean oil, linseed oil and fish oil over 9 weeks (Kiron et al. 2004). They found no differences in serum complement or lysozyme activity or serum

immunoglobulin activity. Head kidney leukocyte phagocytosis and superoxide anion production were only marginally affected by the diets. Furthermore, Puangkaew et al. (2004) fed diets containing DHA concentrated fish oil (48% n-3 PUFA, n-3/n-6 6.3) to rainbow trout and compared immune parameters with fish fed standard fish oil (20% n-3 PUFA, n-3/n-6 8.9). Provided that the fish were fed 100 mg/kg vitamin E, no effects were seen in plasma immunoglobulins, lysozyme, complement, or head kidney leukocyte superoxide anion production. Some reduction in head kidney leukocyte phagocytic index was observed (and phagocytic index) at increased n-3 PUFA content. The latter study illustrates a considerable plasticity in fish responses to dietary alterations. It is particularly interesting to note that the high levels of PUFA supplied by the linseed and DHA enhanced oil diets would probably cause significant oxidative stress. However, the effects of high PUFA may be attenuated at lower temperatures, as indicated in Arctic charr (Olsen and Henderson 1997; Olsen et al. 1999a;).

Much of the above relates to fish capable of metabolizing plant PUFA to C<sub>20</sub> and C<sub>22</sub> products. In marine fish, replacement of fish oil with plant oils will to a large extent reduce the content of long chain HUFA eventually induce EFA deficiency. This may eventually impair membrane functions, immunity and the eicosanoid cascade but will not alter the n-6 / n-3 HUFA ratio. In gilthead sea bream (*Sparus auratus*) for example, stepwise increase in various plant oils to 80% of lipid caused reductions in both alternative complement pathway and head kidney phagocytic activity (Montero et al. 2003) while moderate replacements have very few immunologic effects (Montero et al. 2003; Wassef et al. 2007). The most extreme groups of fish had a content of 22:6n-3 and 20:5n-3 amounting to 7% of lipid or 0.6 and 1.1% of diet respectively, which is very close to EFA deficiency in this species (Kalogeropoulos et al. 1992; Ibeas et al. 1994;2000). Reductions in the number of circulating leucocytes and macrophages have been noted in European sea bass (*Dicentrarchus labrax*) fed diets with 60% replacements of fish oils (Mourente et al. 2005a).

There is however some data that deserve extra note. As previously noted, the ability of marine fish to elongate and desaturate C<sub>18</sub> PUFA may reside in the lack of only one enzyme, not the whole chain of reactions. Some fish such as gilthead sea bream appear to have very active C<sub>18</sub> to C<sub>20</sub> elongase activity but low  $\Delta 5$  desaturase (Tocher 2003). Similar findings have been made in turbot (*Scophthalmus maximus* L.) (Bell et al. 1994). Thus, feeding 18:2n-6 and 18:3n-3 will, in these fish, cause an accumulation of elongation products such as 20:2n-6 and 20:3n-3 in cellular membranes, and consequently reductions in the production of TX and PG from 20:4n-6 (Bell et al. 1994). Although the effects are generally marginal, they still hold some intriguing possibilities for manipulating marine fish immunology by using some plant oils in diets for marine fish. This is particularly interesting with some new brands of oils that are being evaluated as alternatives to standard plant oils. One of these is echium oil, which is very rich in 18:3n-6 and 18:4n-3 and could potentially elevate the content of 20:3n-6 and 20:4n-3 if included into the diets (Bell et al. 2006). This possibility was explored when supplemental fish oil (11% of diet) was replaced with echium oil in diets to Atlantic cod (Bell et al. 2006). The results showed that echium oil would, albeit at a low degree, increase tissue levels of 20:3n-6 (but not 20:4n-3). However, the levels of 20:5n-3 and 20:4n-6 were significantly reduced due to lower dietary input. Furthermore, echium oil did decrease PG production in gill cells and reduced head kidney macrophage activity but not lysozyme or other hematological parameters. Whether these effects were due to lowered levels of 20:4n-6 or an inhibitory effect of 20:3n-6 remains unknown. However, it is not unlikely that these results should be taken into effect, further limiting inclusion levels of some plant oils.

#### 4.3.5. OXIDATIVE STRESS

Feeding high levels of HUFA will inevitably increase oxidative stress in any organism. This has been shown in fish such as Arctic charr (Olsen and Henderson 1997; Olsen et al. 1999a), Atlantic salmon (Waagbø et al. 1993) and red drum, *Sciaenops ocellatus*, (Craig et al. 1999) and is probably the main reason for the resulting increased vitamin E requirement in fish fed such diets (Cowey et al. 1983; Hamre et al. 1994). The reduced growth of juvenile gilthead sea bream with increased n-3 HUFA (from 2 to 5% of diet, 19 to 39% of lipid) probably relates to increased oxidative stress although this was not tested (Ibeas et al. 2000). In some cases, consequences of subclinical oxidative stress become evident when fish are subjected to additional stressors. In rainbow trout for example, increasing the n-3 HUFA level from 13 to 31 g/kg diet led to increased oxidative stress during high density stress, particularly at low dietary vitamin E levels (Trenzado et al. 2007). Some impairment of immune functions has been reported at high HUFA levels that may relate to these mechanisms (Puangkaew et al. 2004). Temperature is another stressor that may affect the progress of oxidative stress, i.e. lowered temperatures will reduce antioxidant enzyme activities but the membrane fluidity will be maintained (Olsen and Henderson 1997; Olsen et al. 1999a). However, as most plant oils have a relatively low level of unsaturation and fish oils high, supplementing plant oils in fish diets would generally reduce the potential for oxidative stress and in this respect be advantageous to the fish. One of few exceptions is probably linseed oil, where high levels of 18:3n-3 seem sufficient to induce oxidative stress under some circumstances (Olsen and Henderson 1997).

#### 4.3.6. STRESS RESPONSE

Several reports support a link between lipid nutrition and the stress response in fish. Most of these appear to be mediated through eicosanoids. In rainbow trout Gupta et al. (1985) showed that PGE<sub>1</sub> increased cortisol production in head kidneys in a dose-dependent manner, and in hagfish (*Myxine glutinosa*) both 20:4n-6 and PGE<sub>2</sub> (but not PGE<sub>1</sub> or PGE<sub>2</sub>) increased plasma cortisol output (Wales 1988). These effects can be mediated through many pathways, including direct influencing secretion of the adrenocorticotrophic hormone (ACTH) or corticotrophin releasing factor. Moreover, administration of the COX inhibitor acetylsalicylic acid has been reported to reduce plasma levels of both PGE<sub>2</sub> and cortisol in Mozambique tilapia (*Oreochromis mossambicus*), in addition to reducing the cortisol response following acute stress (Van Anholt et al. 2003). In line with this, feeding sunflower oil to Atlantic salmon during parr-smolt transformation led to consistently higher plasma cortisol levels compared to those fed fish oil (Jutfelt et al. 2007). Furthermore, in Chinook salmon (*Oncorhynchus tshawytscha*), dietary soybean oil increased plasma cortisol levels following stress compared to fish oil controls (although not higher basal levels before stress) (Welker and Congleton 2003) and in Atlantic salmon, supplementing 10% sunflower oil to a basal diet led to increased mortality following transport-induced stress (Bell et al. 1991). It thus appears that dietary increasing levels of n-6 HUFA in membranes will enhance the severity of the stress response.

Results are however not uniform. In the cyprinid golden shiner (*Notemigonus crysoleucas*) exchanging fish oil with soybean or cottonseed oil had no effect on pre or post stress (2h) cortisol levels (Lochmann et al. 2004). In Mozambique tilapia feeding 20:4n-6 for 18 days did not affect plasma cortisol levels, nor did co-feeding with acetylsalicylic acid (Van Anholt et al. 2004c). Furthermore, acetylsalicylic acid did not depress the cortisol response when the fish were subjected to acute stress, but rather increased it compared to the controls. This suggested a marginal effect of eicosanoids on the stress response in this species. In Atlantic salmon substituting up to 100% of the added fish oil (fish oil still constituted 5-9% of fishmeal) did not affect mortalities during a 12 h transport stress (Gjøen et al. 2004).

However, data on cortisol were not presented. In rainbow trout, increasing the n-3 HUFA level from 13 to 31 g/kg increased plasma cortisol levels during chronic crowding stress (Trenzado et al. 2007). As mentioned above, this observation may relate to oxidative stress and not a direct effect of eicosanoids.

In marine larvae and juveniles, low content or imbalances between 20:5n-3, 20:4n-6 and particularly 22:6n-3 have been reported to decrease stress resistance (Weirich and Reigh 2001). Increased cortisol response has also been seen in larger gilthead sea bream fed 60 and 80% linseed oil of the lipid (Montero et al. 2003). Strangely, this was not seen in fish fed diets supplemented with soybean or rapeseed oil and may again relate to increased oxidative stress. Furthermore, with the high level of plant oils used, fish were fairly close to EFA deficiency that may cause other responses not necessarily related to eicosanoids per se. In an interesting set of studies, Van Anholt and colleagues tested the effect of 20:4n-6 and acetylsalicylic acid in gilthead sea bream. When adult fish were fed diets supplemented with 20:4n-6, the stress response was reduced when subjected to confinement stress, and partially counteracted by acetylsalicylic acid (Van Anholt et al. 2004b). When similar studies were carried out in larval sea bream, 20:4n-6 again reduced the cortisol response and increased survival of fish subjected to air exposure stress. But in this case, feeding 20:4n-6 increased the stress response when subjected to salinity stress, thus reducing growth and survival (Koven et al. 2003; Van Anholt et al. 2004a). It thus seems that different stressors initiate differential responses, and that the responses depend on the species under study, plus the fatty acid composition of their diet.

#### 4.3.7. PHOSPHOGLYCERIDE REQUIREMENT

Fish probably have an absolute requirement for phospholipids (PL), mainly phosphatidylcholine (PC) and possibly phosphatidylinositol (PI) (Coutteau et al. 1997). The most striking example of this is seen in some marine larvae where lipid droplets tend to accumulate, sometimes in livers (Salhi et al. 1999), but most commonly in enterocytes upon feeding with artificial or supplemented live feeds (Deplano et al. 1989; Geurden et al. 1997; Salhi et al. 1999; Morais et al. 2006; 2007). The cause appears to be linked to impaired enterocyte lipoprotein synthesis where PC (and apolipoproteins) is needed to form a coat around the absorbed TAG enabling it to be transported out of the cell. If PC is lacking, no coat will be formed, and TAG droplets will fuse, eventually forming large droplets that cannot be used for lipoprotein synthesis and thus accumulate in the cells. In most cases, addition of PL (sometimes up to more than 7%) will not only alleviate these problems, but in some cases also enhance growth (Fontagne et al. 2000a; Weirich and Reigh 2001; Jingle et al. 2002; Chen and Liu 2004; Morais et al. 2007). Although most of the growth-enhancing effects in larval fish may be ascribed to increased lipid transport, soy lecithin does in many cases appear as efficient as marine phospholipids in supporting growth and removal of lipid droplets. However, PL may also be required for emulgating properties in the gastrointestinal tract, increasing the efficacy of intestinal lipases (Olsen et al. 1991a,b; Coutteau et al. 1997; Izquierdo et al. 2001b). Positive effect of PL is not limited to juvenile marine fish, and PL has been shown both to remove lipid droplets and enhance growth in for example carp (Geurden et al. 1995; Fontagne et al. 1998; Geurden et al. 1998). There is less information for salmonid fish. However, in early feeding fry of rainbow trout, 4% soybean lecithin has been reported to enhance growth and survival compared to non-supplemented fish (Poston 1990a), which is fairly similar to that found for Atlantic salmon less than 2g of weight (3-4%) (Poston 1990b; Hung et al. 1997).

As the fish grow to larger sizes, growth enhancement by PL is less pronounced. Supplementation of PL had little effect on growth and feed efficiency in both flounder (*Paralichthys olivaceus*) (5 g and 2% lecithin) (Kim et al. 2006) and hybrid striped bass (5g

and 6% lecithin) (Sealey et al. 2001). Similar findings were made in rainbow trout where PL have no effect on fish larger than 2g (Poston 1990a). The reason is probably that livers and digestive capacities develop to full strength enabling for sufficient bile and possibly enterocyte PL synthesis to cover these basic needs.

In recent years however, reports have surfaced of large fish effects resembling PL deficiencies. Using rainbow trout, Rincharde et al. (2007) showed that both growth and survival of rainbow trout with initial weight 182g was superior when soy lecithin (14%) was used as lipid source compared to soybean oil, linseed oil and fish oil. Likewise, large amounts of lipid droplets have been reported to accumulate in livers of European sea bass (Mourete et al. 2005b) and gilthead sea bream (Wassef et al. 2007), in intestinal enterocytes of gilthead sea bream (Caballero et al. 2003), Arctic charr (Olsen et al. 1999b; 2000), rainbow trout (Olsen et al. 2003) and Atlantic salmon (Olsen and Ringø unpublished data). Many of these experiments do seem to have at least two factors in common. Firstly, the protein sources are either highly purified containing very little or no PL, or they use small amounts of fishmeal that is diluted with gluten and wheat to lower the contribution of PL from fishmeal. As for marine larvae, lipid droplets, at least in enterocytes, are removed by adding exogenous phospholipids like soybean lecithin (Olsen et al. 1999b; Caballero et al. 2003; Olsen et al. 2003). Secondly, there is often a direct correlation between lipid droplet accumulation and increased content of plant oils such as linseed and soybean in the diets. If fish oils are used, the problem is either less pronounced or totally absent. This has led to the hypothesis that lipid droplet accumulation is not necessarily due to PL deficiency, but an inhibition of enterocyte endogenous PL synthesis. The argument is based on the fact that PC synthesis requires 16:0 in 1 position to proceed at optimum rates (Morimoto and Kanoh 1978; Oxley et al. 2005). Plant oils are however very low in this fatty acid. Addition of 16:0 to a level similar to that of most fish oils (around 20-25%) to Arctic charr fed a linseed oil based diet completely remove lipid droplets (Olsen et al. 2000; Oxley et al. 2005), suggesting that 16:0 may be regarded as an EFA-like compound under these circumstances. Optimal intestinal lipid synthesis may accordingly be the reason that larval gilthead sea bream appear more resistant to stress when fed fish oils and palmitic acid rather than DHA oils and soybean lecithin (Liu et al. 2002). It thus appears that there is an interrelationship between PL requirement and dietary levels of 16:0. The extent of this relationship remains to be elucidated.

#### **4.3.8. BROODSTOCK NUTRITION**

Lipids are thus well known to affect all elements of broodstock development, including maturation, hatching rate, egg and larval development (Izquierdo et al. 1997). In addition to being essential for energy and membrane integrity, a direct link between eicosanoid production and oocyte maturation and steroidogenesis has been suggested (Izquierdo et al. 2001a). Again, a distinction is made between fish (mainly marine) that do not have the capacity to elongate and desaturate C<sub>18</sub> PUFA, and those that can. In the first case, addition of plant oils will mainly provide energy, and the main cause here is to make sure that the broodstocks are supplied with sufficient amounts of HUFA. For fish capable of metabolizing C<sub>18</sub> PUFA, the situation is more complicated. In these cases, plant oils may significantly alter the content and ratio of 20:4n-6 and 20:5n-3, and in large amounts also the availability of 22:6n-3.

For marine fish, replacements of n-3 HUFA for plant oils containing for example 18:3n-3 are obviously not sufficient for good egg quality (Almansa et al. 1999; Izquierdo et al. 2001a). As a general rule, increasing the level of n-3 HUFA in broodstock diets will increase fecundity, fertilization, embryo development and larval quality and survival (Izquierdo et al. 2001a). However, at very high levels, excess n-3 HUFA appear to be

detrimental (Izquierdo et al. 2001a) probably through the induction of oxidative stress. The level required in broodstocks appears to depend on species, but 1.5-2.0% of diet to sparids has been suggested. In salmonids the level has been estimated to be around 1% (Izquierdo et al. 2001a).

Although much of previous research has focused on the level of n-3 PUFA, more studies on n-6 PUFA requirement are also required. Any mismatch between the two will cause altered ratios of cellular 20:4n-6 and 20:5n-3 (and 22:6n-3), which have the potential to affect gonad development even when there is little sign of EFA deficiency (Mokoginta et al. 1998; Bruce et al. 1999). Low levels of 20:4n-6 and a high 20:5n-3/20:4n-6 ratio of farmed cod has also been suggested as a cause of observed lower fertilization rates, cell symmetry and survival compared to eggs from wild broodstocks (Pickova et al. 1997; Salze et al. 2005). In Japanese eel (*Anguilla japonica*) feeding graded levels of diets enriched with up to 7% of the lipid as corn oil (17% total lipid) left the egg content of 22:6n-3 unchanged, but increased 20:4n-6 and decreased 20:5n-3. At highest level of corn oil, fertilization rate was higher but hatching rate lower compared to the other groups. Feeding lower levels of corn oil did not affect these parameters compared to a fish oil control (Furuita et al. 2007).

In many freshwater fish, it has been suggested that broodstock diets should contain more n-6 HUFA than what is normally found in standard broodstock diets based on marine type lipids. The rationale is that these fatty acids are more common in the freshwater environment. In the salmonid Arctic charr, Pickova et al. (2007) compared lipid composition and hatching rate of broodstocks of wild and farmed populations. The results showed that the n-3/n-6 ratio was much higher in farmed (13.5) than wild populations (3.5). This again was correlated to a higher hatching rate of wild (>80%) compared to farmed eggs (between 20% to 70% hatching rate). It was suggested that 20:4n-6 or 18:2n-6 should be included into charr diets to reduce the n-3/n-6 ratio. This may also have been the case in another freshwater fish, swordtail (*Xiphophorus helleri*), where mixtures of squid oil and linseed oil produced more fry than squid oil alone. In these diets, the n-3/n-6 ratio was decreased from 11 to 5.5 in the linseed oil group. However the level of 20:4n-6 in the eggs did not change but 22:6n-3 was reduced. Linseed oil alone produced similar hatching as squid oil (Ling et al. 2006). When Atlantic salmon were fed a fish oil based diet or a 1:1 mixture of fish oil : rapeseed oil for almost 1 year, no effect of diet was observed on rates of fertilization, hatching or survival to first feeding (Rennie et al. 2005). In this case, the n-3/n-6 ratio was already fairly low (3.6) only to decrease to 1.6 in the rapeseed oil diet. In the freshwater fish white bass (*Morone chrysops*), hatchability was inversely related to decreases in the content of corn oil and increases in fish oil (0-100% of a 15% lipid diet) (Lane and Kohler 2006). This was in part explained by limited capacity to elongate and desaturate C<sub>18</sub> PUFA in this species. However, in Nile tilapia (*Oreochromis niloticus*), which is clearly capable of metabolizing C<sub>18</sub> PUFA (Olsen et al. 1990), earlier studies have indicated that general performance (spawning frequency, fry per spawning etc) was higher when fed diets supplemented with soybean oil (Izquierdo et al. 2001a). However, recent findings seem to contradict this as high levels of soybean oil in broodstock diets was negatively correlated with fecundity, eggs per spawn and larval quality (El-Sayed et al. 2005). However, the soybean diet was rather deficient in n-3 HUFA and would cause EFA deficiency if fed for a very long period. Accordingly, no difference in quality was observed when fed fish oil only or a 1:1 mixture of soybean oil and fish oil. Interestingly, the soybean oil deterioration of egg quality was only noticeable at 7 and 14 ppt salinity, and not that much in freshwater (0 ppt).

Moderate amounts of plant oils do not appear to affect reproductive performance in cod. Coating standard diets with 9% soybean oil for 24 months did cause small changes in egg fatty acid composition, but had no effect on egg production, mean fertilization or egg size (Lie et al. 1993). In turbot (*Scophthalmus maximus*) optimum egg quality and fry survival was

observed when diets contained 14% lipid with at least 20% n-3 HUFA (Ma et al. 2005). Increasing the level of 20:4n-6 in broodstock diets to Pollock (*Pollachius pollachius*) did not increase egg production or fertilization rate (Omnes et al. 2004). However, increasing the level of 22:6n-3 by replacing 6% capelin oil with tuna oil did enhance fertilization rate. In Japanese flounder (*Paralichthys olivaceus*), egg production was highest when the fish were maintained on 6% n-3 HUFA of the diet. However, egg quality parameters including hatching rate and percentage of normal larvae were highest in the group fed 2% HUFA of diet (Furuita et al. 2002). A possible relation to 20:4n-6 deficiency was also suggested. Rainbow trout broodstock performance appears to be very resistant to dietary manipulations. Feeding the fish for over one year on diets containing corn oil as lipid source did not appear to affect fecundity of egg viability when compared to the control group fed cod liver oil (Corraze et al. 1993).

#### 4.3.9. CYCLIC FATTY ACIDS

##### General characteristics

Cyclic fatty acid monomers (CFAMs) are formed from the unsaturated 18 carbon chain length fatty acids of the edible oils as a result of domestic frying and industrial refining. CFAMs occurring from 18:1n-9 are composed of at least 8 different saturated cyclic fatty acids with a C5- or a C6-membered ring. Thirteen different monoenoic CFAMs are formed from 18:2n-6, containing mostly a C5-membered ring. Finally, 18:3n-3 gives rise to 16 identified dienoic CFAMs, with a mixture of C5- and C6-membered rings with some bicyclic acids.

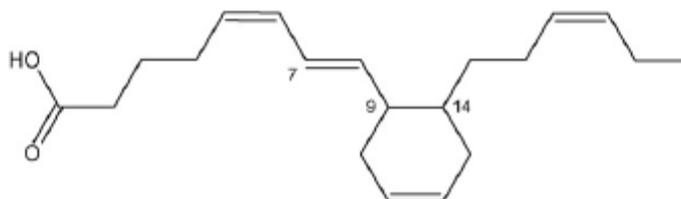


Figure 4.3.9.1. Cyclic form of EPA and DHA formed during prolonged heating (Berdeaux et al., 2007).

##### Biological effects in fish

Cyclic fatty acids are usually present at low levels in many oils (from 0.01 to 0.66% of the total fatty acids), and they have in experiments demonstrated adverse effects in various animal models. For instance, they caused a higher death rate when administered orally to mice. They decreased the weight gain of weaning rats, increased the liver weight and the death rate of rat pups from mothers fed CFAMs, as well as reduced the number of pups per litter. They are also incorporated into heart cells in culture where they altered the electrophysiological properties. It is not known whether the toxicity is related more to the C5- or to the C6-membered ring CFAMs or both equally (reviewed by Martin et al., 1997).

Martin et al. (1997) conducted an investigation of intestinal digestion of CFAMs isolated from heated linseed oil and their effects upon fatty acid lymphatic transport and lipoprotein profile in lymph in rats. The oil contained cyclic fatty acid monomers acylated in specific positions in the glycerol backbone of triacylglycerols and was administered intragastrically to lymph-cannulated animals. Their luminal digestibility was also assessed *in vitro* using a pancreatic lipase assay. The lipase activity towards cyclic fatty acids was

reduced by 50 to 85% compared to the triacylglycerols acylated control. In the hydrolytic products, the cyclic fatty acid contents were similar between the experimental groups. The authors concluded that the effects of cyclic fatty acid monomers upon the intestinal metabolism are greatly influenced by their positioning within the triacylglycerol and that the structure of the cyclic fatty acids influences their lymphatic recovery only when they are absorbed as free fatty acid.

Relevant information regarding effects in fish is limited. Cyclopropenoic acid is found in the lipid fraction of cottonseed products. Cyclopropenoic acid has been shown to reduce growth rate in rainbow trout (Lee et al, 1972; Hendricks et al., 1980) and act as a potent synergist for the carcinogenicity of aflatoxins (Hendricks et al., 1980). Other pathologies observed with trout include extreme liver damage (liver is pale in colour) with increased glycogen deposition and decreased protein content, and a decrease in activity of several key enzymes.

No relevant information is available on maximum inclusion levels or interactions with gut microflora.

#### **4.3.10. PLANT OILS - CONCLUSIONS**

In fish larvae, it appears that inclusions from 1-3% of diet is safe and may also increase growth and survival. There are however no data in cod and halibut and that situation may be different. MCT may also improve growth and feed conversion and reduce adipose tissue in larger fish but the level depends on size and species. In salmonids, up to 10% of diet seems relatively safe. Growth is however not affected. Moderate inclusions of MCT to cod do not appear to reduce liver lipid deposition. The level of inclusion may also depend on type of MCT. Shorter chain MCT (C<sub>6</sub> or C<sub>8</sub>) appears to cause more problems than longer chain MCT such as coconut oil.

In marine fish, addition of plant oils must not reduce the level of HUFA to less than the required minimum. For juveniles and adults the requirement of EPA and DHA is probably in the region 0.5-1.5% each, and somewhat higher for larvae. It is also possible that the requirement should be estimated to around 10-20% of the lipid. The requirement of AA may be in the region 0.3-0.5%. Existing data are however from other species than cod and halibut. For rainbow trout, the EFA requirement is around 1-2% if supplied as C<sub>18</sub>, and half that if given as HUFA. It has also been suggested that the level should be 10-20% of lipid depending on what form of delivery is used. In rainbow trout, it has been suggested that DHA should be regarded as an EFA due to a too low conversion ratio from 18:3n-3. Similar mechanisms seem likely in Atlantic salmon.

If the EFA requirements are covered, plant oils do not seem to cause any major harm to marine fish. Some elongase activity of C<sub>18</sub> PUFA to their C<sub>20</sub> counterparts has been noted, although activity appears very low in Atlantic cod and remains unknown in halibut. If produced, these compounds may compete with AA and EPA for active sites for eicosanoids production. But the significance of this is unknown.

Fish probably have a high requirement of PL. If fishmeals are used as protein source, this is only a problem in larval fish where addition of soy lecithin is advised (in the range 2-6% of total diet). However, as other protein sources are now introduced, addition of some soybean lecithin (or marine PL) should be considered for larger fish as well (1-3% of total diet).

In freshwater fish, the effect of adding plant oils on fish immunity is inconclusive. Cases of increased disease resistance or immunocompetence in fish added high n-6 PUFA oils have been attributed to production of more eicosanoids from AA with more potent activity in inflammatory processes than those from EPA. However, fish are different to mammals, and

there are a considerable number of studies suggesting that n-6 PUFA-rich oils will cause a reduction of fish immunocompetence. This may be related to altered eicosanoid cascade in addition to membrane fluidity. Furthermore, other studies do not seem to have any effect on immune competence despite cases where all supplementary fish oil is exchanged with high n-6 PUFA oils. Some of the differences may be due to parameters studied or, in case of disease challenge, the delivery of disease. In cohabitant experiments, primary barriers are important and affected by diet, while in injection studies this barrier is bypassed. At present however, exchanging up to 50% of the fish oil does not seem to be excessively harmful to the fish. However, increased level of AA and its related eicosanoids may also enhance the stress-response and may also cause levels of subclinical stress that increase responsiveness to environmental stress or noxious substances and may affect fish health in the long term. At present it would therefore be safe not to use very high levels of such oils.

Adding high levels of linseed oil to fish diets is a potential problem as it may contribute to oxidative stress. Although no concrete figures are available for salmonids, cod and halibut, 25% addition of lipid appears to be relatively safe.

The requirement for EFA in broodstock nutrition seems to be in the same range as that for fish EFA requirement. For sparids, 1-2% and for salmonids 1% has been suggested, for turbot 20% n-3 HUFA of lipid. There are also reports suggesting that even higher levels of PUFA or HUFA should be used. But at very high levels of n-3 HUFA (6%) negative effects are sometimes observed. It has also been argued in both cod and some salmonids that the level of n-6 (AA) in broodstock diets could increase compared to standard diets used today. And some cases of increased egg quality following this recommendation have been published. In rainbow trout no effect was found on fecundity or egg viability despite being fed corn oil as supplemental oil source.

As many of the potential disadvantages of using plant oils in salmonid diets is related to either very high levels of n-6 PUFA (most available oils), or very high levels of linseed oil, it would be recommended that mixtures of plant oils be used as feed inclusions. By adjusting the ratio of n-6 and n-3 the level of eicosanoids can be controlled. By including palm oil, potential problems of intestinal lipid droplets can be controlled. A standard inclusion of soybean lecithin may also be advisable. These and other variants of mixtures of oil sources have been explored in recent years with some success in salmonid fish (Torstensen et al. 2005; Bell et al. 2003b; Tocher et al. 2003a). Such mixtures do not seem to be necessary for marine fish.

With plant alternatives, care should be taken in selecting both types and qualities to prevent nutrition-related diseases such as skeletal deformities, cataracts, heart conditions, and other, unspecific symptoms.

#### **4.4. UNDESIRABLE SUBSTANCES**

Feed ingredients, of plant and animal origin, may contain compounds not inherent to the plant or animal, for example pesticides, mycotoxins, environmental pollutants or substances that occur during processing of the feed ingredients. Further, feed ingredients may contain elevated levels of inherent substances, e.g. feed material of plant origin may contain elevated levels of heavy metals due to geological characteristics of the soil. To ensure that animal feed does not pose any threat to animal and human health or to the environment, statutory limits for maximum content have been set for a number of undesirable substances in feed materials and complete feedingstuffs (Norway; Forskrift 7. November 2002 nr. 1290 om fôrvarer, and European Union; Directive 2002/32/EC). An undesirable substance is defined as “any substance or product, with the exception of pathogenic agents, which is present in and/or on the product intended for animal feed and which presents a potential danger to animal or human health or to the environment or could adversely affect livestock production”

(2002/32/EC). The current European feed legislation (2002/32/EC and amendments) includes 40 undesirable substances, such as the heavy metals mercury, lead and cadmium, several pesticides and other organohalogenated compounds such as dioxins, furans and dioxin-like PCBs, and the mycotoxin aflatoxin B<sub>1</sub> (Table 4.4.1).

Table 4.4.1. List of the undesirable substances included in the Norwegian and European feed legislation. For levels of maximum content in feed materials and feed, please refer to Forskrift 7. November 2002 nr. 1290 om fôrvarer and Directive 2002/32/EC, and amendments.

Ions or Elements	Products	Organohalogenated compounds	Botanical impurities
Arsenic	Aflatoxin B <sub>1</sub>	Aldrin	Apricots - <i>Prunus armeniaca</i> L.
Lead	Hydrocyanic acid	Dieldrin	Bitter almonds - <i>Prunus dulcis</i> (Mill.) D.A. Webb var. <i>amara</i> (DC.) Focke (= <i>Prunus amygdalus</i> Batsch var. <i>amara</i> (DC.) Focke)
Fluorine	Free gossypol	Camphechlor (toxaphene)	Unhusked beech mast - <i>Fagus silvatica</i> L.
Mercury	Theobromine	Chlordane	Camelina - <i>Camelina sativa</i> (L.) Crantz
Nitrites	Volatile mustard oil	DDT	<i>Mowrah</i> , <i>Bassia</i> , <i>Madhuca</i> - <i>Madhuca longifolia</i> (L.) Macbr. (= <i>Bassia longifolia</i> L. = <i>Illipe malabrorum</i> Engl.) <i>Madhuca indica</i> Gmelin (= <i>Bassia latifolia</i> Roxb.) = <i>Illipe latifolia</i> (Roscb.) F. Mueller)
Cadmium	Vinyl thiooxazolidone	Endosulfan	Purghera - <i>Jatropha curcas</i> L.
	Rye ergot ( <i>Claviceps purpurea</i> )	Endrin	Croton - <i>Croton tiglium</i> L.
	Weed seeds and unground and uncrushed fruits containing alkaloids, glucosides or other toxic substances separately or in combination, including a. <i>Lolium temulentum</i> L. b. <i>Lolium remotum</i> Schrank c. <i>Datura stramonium</i> L.	Heptachlor	Indian mustard - <i>Brassica juncea</i> (L.) Czern. and Coss. ssp. <i>intergrifolia</i> (West.) Thell.
	Castor oil plant - <i>Ricinus communis</i> L.	Hexachlorobenzene (HCB)	Sareptian mustard - <i>Brassica juncea</i> (L.) Czern. and Coss. ssp. <i>juncea</i>
	Crotalaria - <i>Crotalaria</i> ssp.	Hexachlorocyclo-hexane (HCH)	Chinese mustard - <i>Brassica juncea</i> (L.) Czern. and Coss. ssp. <i>juncea</i> var. <i>lutea</i> Batalin
		Dioxins and dioxin-like PCBs	Black mustard - <i>Brassica nigra</i> (L.) Koch
			Ethiopian mustard - <i>Brassica carinata</i> A. Braun

#### 4.4.1. PLANT INGREDIENTS AND UNDESIRABLE SUBSTANCES

Although plant ingredients are different from marine ingredients with regard to the presence of undesirable substances, e.g. the levels of dioxins and PCBs are lower in plant oil

than in marine oils, there may be challenges related to the use of plant ingredients in fish feed. The plant ingredients may contain elevated levels of undesirable substances such as pesticides, mycotoxins and phytotoxins.

#### 4.4.2. PESTICIDES

The European feed legislation currently includes 10 different pesticides (aldrin and dieldrin, chlordane, DDT, endosulfan, endrin, heptachlor, HCB (hexachlorobenzene), HCH (hexachlorocyclo-hexane), and toxaphene; 2002/32/EC), and all have recently been assessed as undesirable substances in animal feed by the European Food Safety Authority (EFSA; [www.efsa.eu.int](http://www.efsa.eu.int)). Of the 10 pesticides only endosulfan is still in use; the use of the rest has been banned by the European Union. Feed ingredients are, however, traded on the global market. Hence, one may find elevated levels of pesticides which are banned in Norway and in the European Union in ingredients originating from countries where the pesticides are still in use or have only recently been banned. The inclusion of these pesticides in the legislation is therefore still justified. There is little or limited data available on the occurrence of pesticides in feed materials and feeds (available data are compiled in scientific opinions by EFSA; Aldrin and dieldrin, EFSA, 2005a; chlordane, EFSA, 2007a; DDT, EFSA, 2006a; endosulfan, EFSA, 2005b; endrin, EFSA, 2005c; heptachlor, EFSA, 2007b; HCB (hexachlorobenzene), EFSA, 2006b; HCH (hexachlorocyclo-hexane), EFSA, 2005d; and toxaphene (camphechlor), EFSA, 2005e). In general, organochlorine pesticides are not often found in plant ingredients. When pesticides are found in plant ingredients it is generally in low levels and within the current limits for maximum content. However, there is still a risk that some plant ingredients accidentally may be polluted. The generally low levels of pesticides and other chlorinated compounds in plant ingredients is in contrast to the higher contents of these compounds reported in ingredients of animal origin, and especially in fish oil and fishmeal. This is in agreement with data from the Norwegian Food Safety Authorities (Mattilsynet) surveillance programme on feed for fish and other aquatic animals (Maage et al., 2007); data (for selected pesticides) from 2006 are shown in Table 4.4.2.1.

The adverse toxic effects of pesticides (those included in the current legislation) in fish have been evaluated by EFSA (Aldrin and dieldrin, EFSA, 2005a; chlordane, EFSA, 2007a; DDT, EFSA, 2006a; endosulfan, EFSA, 2005b; endrin, EFSA, 2005c; heptachlor, EFSA, 2007b; HCB (hexachlorobenzene), EFSA, 2006b; HCH (hexachlorocyclo-hexane), EFSA, 2005d; and toxaphene (camphechlor), EFSA, 2005e). The number of studies of the dietary toxicity of pesticides in fish is limited. Fish are generally highly sensitive to waterborne exposure to organochlorine pesticides, and some studies indicate that fish are also sensitive to oral exposure. Of the 10 pesticides included in the European feed legislation, endosulfan is the only one not banned by the European Union. Atlantic salmon appear to be less sensitive to oral exposure to endosulfan than to exposure via water. High dietary level of endosulfan (710 µg/kg feed) elicited haematological effects (elevated levels of haematocrit and haemoglobin) and elevated hepatic EROD activity. Hepatic histological changes were seen in Atlantic salmon from all exposure groups (4, 18, 30, 50 and 710 µg/kg feed) (Petri et al., 2007; Glover et al., 2007).

In addition to endosulfan, a wide range of pesticides are in use in today's agriculture. Feed materials of plant origin can potentially contain residues of these pesticides. They are, however, not included in the current feed legislation, and hence not monitored. There is a need for thorough survey of their presence in feed ingredients of plant origin, and, if present, studies of their potential toxic effects in fish. Further, if potential hazardous pesticides are identified, they ought to be included in the feed legislation.

Table 4.4.2.1. Levels of pesticides in plant and fish oils. Data from the Norwegian Food Safety Authority surveillance programme on feed for fish and other aquatic animals (Maage et al., 2007). Levels for maximum content apply to feedingstuffs ('products of vegetable or animal origin, in their natural state, fresh or preserved, and products derived from the industrial processing thereof, and organic or inorganic substances, used singly or in mixtures, for oral animal feeding', 2002/32/EC and amendments).

		Plant oil	Fish oil	Maximum content	Unit
Sum DDT	2005	5.4 (1.6-12, n=12)	95 (27-201, n=10)	500	µg/kg
	2006	15.6 (4.9-37, n=5)	87 (36-162, n=10)	500	µg/kg
Sum endosulfan	2006	1.6 (<1.1-4.1, n=10)	1.6 (<1.1-1.9, n=10)	100	µg/kg
HCB (hexachlorobenzene)	2006	0.14 (<0.07-0.43, n=10)	8.6 (0.40-20, n=10)	200	µg/kg
Sum chlordane	2006	<3.7 (n=10)	24 (<3.7-80, n=10)	50	µg/kg
Sum toxaphene	2006	<5.0 (n=10)	28 (5-92, n=10)	200	µg/kg

#### 4.4.3. MYCOTOXINS

Mycotoxins are toxic compounds produced by fungus, e.g. *Aspergillus* spp., *Penicillium* spp. and *Fusarium* spp. Relevant mycotoxins with regard to feed ingredients and feed are aflatoxins B<sub>1</sub>, deoxynivalenol and other trichothecenes, fumonisins, ochratoxin A and zearalenone. They have all been recently assessed by EFSA (aflatoxins B<sub>1</sub>, EFSA, 2004a; deoxynivalenol, EFSA, 2004b; fumonisins, EFSA, 2005f; ochratoxin A, EFSA, 2004c; and zearalenone, EFSA, 2004d). Mycotoxins are more commonly found in feed materials of plant origin, especially in maize and small grains and products thereof, than in feed materials of animal origin. Of the mentioned mycotoxins only aflatoxin B<sub>1</sub> is included in the European feed legislation (2002/32/EC), but recommendations for other mycotoxins are made by the European Union (2006/576/EC). Based on those recommendations many countries, including Norway, have set upper limits for one or more mycotoxins in addition to aflatoxin B<sub>1</sub>. The Norwegian Food Safety Authority makes recommendations for maximum levels of both fungi and mycotoxins in feed ingredients (Norway, 2007).

Data for the occurrence of mycotoxins in feed and feed materials are limited. For aflatoxin B<sub>1</sub>, products coming from regions with subtropical or tropical climate have been identified as being of high risk with regard to contamination (EFSA, 2004a) and the following products are presumed to have a potential for contamination with aflatoxin B<sub>1</sub>; groundnut, copra, palm-kernel, cotton seed, babssu, maize and their products (2002/32/EC). Deoxynivalenol and ochratoxin A are usually found in cereals, such as wheat, rye, barley and maize and their products (EFSA, 2004b; EFSA, 2004c), while fumonisins and zearalenone are common in maize and maize products (EFSA, 2005f; EFSA, 2004d). In Norway in 2006 various mycotoxins were detected in samples of feed materials of plant origin (a total of 21 samples were included in the surveillance programme on feed for fish and other aquatic animals; Maage et al., 2007).

Toxicological studies of various mycotoxins in animals have shown a broad range of effects and some may be carcinogenic, mutagenic or immunotoxic. The knowledge of the toxic effects and toxicokinetics of mycotoxins in fish is limited, and studies of dietary exposure to mycotoxins are scarce. In channel catfish exposed to graded levels of dietary fumonisin B<sub>1</sub> (FB<sub>1</sub>; 0, 3, 20, 80, 320 and 720 mg/kg feed) for 10 weeks, high mortality (70%) was observed in the two highest exposure groups, with the first mortalities occurring after 2 weeks of exposure (Lumlertdacha et al., 1995). Hepatic histological changes were seen in channel catfish exposed to 20 or more mg FB<sub>1</sub>/kg feed, and the occurrence and severity of the

changes appeared to be dose-dependent (Lumlertdacha et al., 1995). Indications of liver damage have been seen in carp (Pepeljnjak et al., 2002), while no hepatic lesions were found in Nile tilapia after dietary exposure to fumonisin B<sub>1</sub> (Tuan et al., 2003).

#### 4.4.4 PHYTOTOXINS

Phytotoxins are a group of toxic substances produced by plants. The current feed legislation includes botanical impurities and natural plant products (hydrocyanic acid, free gossypol, theobromine and glucosinolates). However, SCAN (the Scientific Committee on Animal Nutrition) concluded in a scientific opinion that the plant species and products included in the legislation "... does not represent the present taxonomic status of potential botanical contaminants or the real risk represented by such impurities in modern animal feeding practice" and recommends that the list be re-evaluated (SCAN, 2003). With the increased use of plant ingredients in fish feed, and feed for other animals, a re-evaluation is still relevant. Some of the natural products in feed materials of plant origin (plant protein) are antinutritional factors, and should be given focus in a re-evaluation. The presence of antinutritional factors in feeds and their effects in fish are discussed in Chapter 4.2.

#### 4.4.5. POLYAROMATIC HYDROCARBONS (PAHs)

Polycyclic aromatic hydrocarbons (PAHs) are a large group of organic compounds containing two or more fused aromatic rings. PAHs are not included in the European feed legislation, only in the food legislation (2006/1881/EC). Many of the PAHs are carcinogenic and immunotoxic, and benzo[*a*]pyrene (BaP) has been selected as an indicator for the occurrence and effect of PAHs in food (SCF, 2002). A range of PAHs, including BaP, are included in the Norwegian surveillance program on feed for fish and other aquatic animals, and results from 2006 show that the level of PAHs, contrary to other organic pollutants, is higher in plant oils than in marine oils with mean concentrations of BaP of 3.1 (n = 5) and 0.6 (n = 9) µg/kg feed, respectively (Maage et al. 2007).

In feral fish hepatic tumours have been linked to PAHs in the sediments (e.g. Hylland, 2006). However, only a few studies have investigated the toxicity of dietary PAHs to fish when fed contaminated feed over a longer period. One study reported a reduction in weight in juvenile Chinook salmon (*Oncorhynchus tshawytscha*) fed a mixture of 21 PAHs (22 µg/g fish ww/day) for 54 days (Meador et al., 2006), while no effect on growth was seen in juvenile grouper (*Epinephelus aerolatus*) fed BaP (12.5 µg/g fish ww/day) for 4 weeks (Wu et al., 2003).

#### 4.4.6. NITROSAMINES

Nitrosamines, or N-nitroso compounds (NOC) are carcinogenic, and toxic effects have been reported for fish. There are a limited number of dietary studies, but the development of liver tumours has been reported for Rainbow trout after dietary exposure to dimethylnitrosamine (Ashley and Halver, 1968; Grieco et al., 1978). In rainbow trout exposed to graded levels of dimethylnitrosamine (0, 3, 50, 200, 400 and 800 mg/kg feed) no hepatic changes were observed after 26 weeks of exposure, while after 52 weeks tumours had developed in liver in rainbow trout in the three highest exposure groups (Grieco et al., 1978).

Nitrosamines may be formed during the processing of feeds. However, the formation of nitrosamines from processing of plant ingredients has not been identified (Please refer to Chapter 11).

#### 4.4.7. ELEMENTS

The current European feed legislation includes the heavy metals cadmium, lead and mercury, the metalloid arsenic, and fluorine (2002/32/EC and amendments). They have all been assessed by EFSA (arsenic, *The EFSA Journal* (2005) 180, 1-35; cadmium, *The EFSA Journal* (2004) 72, 1-24; fluorine, *The EFSA Journal* (2004) 100, 1-22; lead, *The EFSA Journal* (2004) 71, 1-20; and mercury, *The EFSA Journal* (2008) 654, 1-74). As for pesticides and mycotoxins, there are little or limited data available on the occurrence of elements in feed materials and feeds. Data compiled in the abovementioned assessment suggest that, in general, the levels of arsenic, cadmium, fluorine and lead in feed materials of plant origin are low.

#### 4.4.8. CONCLUSIONS

It is a premise for the use of feed ingredients of plant origin that the use complies with current Norwegian and European feed legislation on the presence of undesirable substances. Available data on the occurrence and levels of undesirable substances in feed material of plant origin is, however, limited. It is important to continue the surveillance of undesirable substances in feed materials and feed, with special focus on pesticides, mycotoxins and PAHs in feed materials of plant origin. With regard to pesticides, many of the pesticides in use in today's agriculture are not included in the current feed legislation. Their presence in feed material of plant origin must be investigated, and their potential toxicity to fish should be studied. Further, if potential hazardous pesticides are identified, they ought to be included in the feed legislation.

The list of undesirable substances included in the feed legislation is, in general, sufficient, but it should be considered to include pesticides in use today and more of the mycotoxins. Currently only aflatoxin B<sub>1</sub> is included, while only recommendations (of maximum content) exist for other mycotoxins. Based on the recommendations, some European countries, including Norway, have national legislation for deoxynivalenol, fumonisins, ochratoxin A and zearalenone.

Studies are scarce of dietary exposure to undesirable substances, e.g. pesticides and mycotoxins, and their toxic effects in fish. More studies, and especially long-term feeding trials, are needed for improved risk assessments.

### 4.5. THE USE OF GENETICALLY MODIFIED PLANT INGREDIENTS IN FISH DIETS

One question which often arises is the safe use of feed ingredients derived from genetically modified (GM) plants, especially soybeans and maize, which are the largest grown GM plants of interest to the feed industry today, constituting 64% and 24% of global GM-cultivated area, respectively (James 2005).

Since 2004, GM food and feed applications are regulated in the European Community, requiring labelling of products containing more than 0.9% of GM ingredients. The European Food Safety Authority (EFSA) is responsible for the science-based assessment of GM food and GM feed in Europe. The term "Substantial equivalence" was formulated by the Organisation for Economic Cooperation and Development as a guiding tool for safety assessment of genetically modified foods, and is based on the idea that an existing plant with known feed value and with a history of safe use can serve as comparison when assessing the safety and nutritional value of a genetically modified plant (OECD 1993). Substantial equivalence is the starting point for safety assessment of a GM variety, with comparisons of the GM variety with its closest traditional counterpart, and can be described as a comparative approach to the assessment of safety (EFSA 2004). Compositional equivalence is based on

compositional analysis, which is a part of the safety assessment, and in studies with fish is defined as experimental diets being equal in macro- and micronutrients, and as equal as possible in anti nutrient factors (ANFs). However, the latter is difficult to obtain as the modification of plants very often leads to alterations in ANF amount and profile between the GM variety and its near-isogenic parental line (Flachowsky et al. 2005). Safety assessments include toxicity testing of the newly expressed proteins, potential occurrence of secondary effects, potential for horizontal gene transfer to other species, the potential allergic effects of newly inserted traits, and the role of the new food in the diet (Kuiper et al., 2001; Herrero et al., 2007).

Random integration of DNA into the plant genome can result in DNA rearrangements of the transgenic construct and of the insertion site, and cause alteration or disruption of DNA/genes (Cellini et al. 2004). The presence of rearrangements is often associated with instability in transgenes expression (Fladung 1999; Kumar and Fladung 2000). Complex integration patterns have been seen with *Agrobacterium*-mediated integration, such as directed and inverted repeats (Krizkova and Hroudá 1998) and integration of vector 'backbone' sequences from outside the left and right borders of the transgenic DNA (De Buck et al. 2000). Evidence exists that integration of transgenic DNA causes mutations resulting in loss of gene function. Gene-rich regions are known to be hotspots for recombination of transgenic DNA (Gill et al. 1996; Cellini et al. 2004), which increases the risk of altering the gene expression profiles of plants in the process of transgenic DNA insertion. The potential risk of unintended effects are one of the concerns in genetic engineering (Kuiper et al. 2001; Cellini et al. 2004). Unintended effects might be the silencing or activation of present genes or origin of new genes, which might lead to the formation of new proteins or altered levels of the existing ones (Kuiper et al. 2001), such as new anti-nutritional factors. Another concern is the risk of horizontal gene transfer (HGT) of novel DNA to prokaryotic and eukaryotic organisms. The latter often deals with possible transfer of antibiotic-resistance genes, which are constituents of the transgenic DNA, inserted to be able to sort out the successfully modified organisms, and which eventually might result in an increase in antibiotic-resistant pathogens as a secondary effect (Gay and Gillespie 2005).

Concerns arise regarding possible toxicity or secondary effects of intended or unintended transgenic proteins expressed in the GM organism. Force-feeding and intraperitoneal injection of Atlantic salmon with pellets spiked with round-up ready soybean (RRS) DNA sequences of various sizes from 100 to 1,000 base-pairs showed that all sizes were absorbed and entered blood, kidney and liver tissue, with maximums found 6 to 8 hrs after feeding (Nielsen et al. 2005). By means of *in situ* hybridization techniques, DNA from RRS soya were identified in the epithelial cells in salmon intestine (Sanden et al. 2006). Whether or not the inclusion of GM soybean or GM maize affects the fish in any possible manner has been investigated in several studies with fish, and with various results (Hammond et al. 1996, Sanden 2004, Sagstad 2006). A major conclusion is that growth, digestibilities, feed utilization and general health parameters are more influenced by the plant material as such than the plant being genetically modified. Chainark et al. (2006) investigated the use of GM soybean meal in the diets of rainbow trout. A GM defatted soybean meal was compared to a regular soybean meal fed at two levels (15 and 30%) in a 42% protein diet fed to juvenile trout (48.3 g) for 12 weeks. The results showed no significant differences in growth or feed performance between the two soybean meal sources. Promoter fragments were detected in muscle of fish fed the GM soybean meal initially, but not after five days on a non-GM based diet. Some indications of alterations of the immune system were found by Sagstad et al. (2007). Further studies on how metabolic pathways are influenced are under way in order to identify eventually which pathways are altered (M. Sanden 2007, unpublished; Séralini et al. 2007). In Atlantic salmon, no major differences in organ sizes, except for distal intestine, are

reported due to feed containing GM. Organs investigated were liver, heart, brain, kidney, head kidney, spleen, and the different sections of the gastrointestinal tract. Whole body or liver proximate compositions were not altered due to GM, and only minor effects were identified on the stress-response system; parameters used in the evaluations were heat-shock protein 70, catalase, superoxide dismutase, and leakage of organ-specific enzymes to the plasma compartment (Sanden 2004, Sagstad 2006). The transport mechanism for glucose in the anterior gut was however found to be altered by the plant being GM (Hemre et al. 2007).

#### **4.5.1. CONCLUSIONS ON THE USE OF GENETICALLY MODIFIED INGREDIENTS IN FISH DIETS**

Results reported on GM maize and soy bean confirm the need for a case by case assessment on each new GM variety. The studies performed only partly support each other's findings. There are clear batch and variety differences. The conclusions made could not have been drawn without feeding studies, as the compositional equivalent and/or substantially equivalent focus would not be able to reveal the registered biological findings on e.g. absorption patterns of glucose.

## **4.6. IMMUNOSTIMULANTS, PREBIOTICS AND NUCLEOTIDES**

### **4.6.1. IMMUNOSTIMULANTS**

A number of immunostimulants have a molecular architecture consisting of repeating units of a certain moiety such as glucose in  $\beta$ -glucans and (deoxy) riboses in DNA/RNA, fatty acid chains in bacterial lipopolysaccharides (LPS) and certain lipoproteins. Such patterns are abundant in microbial communities of prokaryotes, and can be termed pathogen associated molecular patterns (PAMPs) if they initiate inflammatory responses.

According to Sakai (1999) immunostimulants can be divided into several groups depending on their sources: bacterial, algae-derived, animal-derived, nutritional factors as immunostimulants, and hormones/cytokines. This sub-grouping is independent of their mode of action. A former definition of immunostimulants that restricted the target cells to being only of a mononuclear phagocyte system should be redefined in view of recent discoveries of the pattern recognition receptors (PRRs) (Seljelid, 1990). Different leucocytes may possess different PRRs and may bring about different immunological responses dependent on the binding receptor and intracellular signalling events. As such, a new definition must include all elements of the immune system. That definition could be: "*An immunostimulant is a naturally occurring compound that modulates the immune system by increasing the host's resistance against diseases that in most circumstances are caused by pathogens*". Immunostimulants have been used as feed additives for many years in aquaculture, and yeast  $\beta$ -glucan may be the one with the longest track record. In nature,  $\beta$ -glucans are widespread and have been characterized in microorganisms, algae, fungi and plants (Volman et al., 2008). The chemical structure of  $\beta$ -glucan varies with respect to molecular weight and degree of branching. For example,  $\beta$ -glucan from yeast contain a particular carbohydrate that consists of glucose and mannose residues and is a major constituent in the cell membrane.

Nucleotide-supplemented diets are not strictly immunostimulants by definition but provide a dietary supplement that allows improved resistance to a pathogen insult (for review see Table 10.3.1). Although such diets have been reported not to induce measurable immunostimulatory effects (Burrells et al., 2001 a; 2001 b; Low et al., 2003), there seems to be an up-regulation of several immune genes in turbot fed these diets, which contradicts the earlier claims that these diets are not immunostimulatory (Low et al., 2003).

The biological effects of immunostimulants are highly dependent on the receptors on the target cells recognising them as potential high-risk molecules and triggering defence pathways. Thus, it is important to increase the knowledge base concerning receptor specificity and the inflammatory processes the different receptors, upon antigen binding, induce. However, many mammalian receptors reported to bind immunostimulants have yet to be reported in fish. Nevertheless, assuming that fish and mammalian cells share many similar receptors, one may predict the biological outcome of immunostimulants in fish.

Probiotics appear to modulate immune activity of the host by improving the barrier properties of mucosa and modulating production of cytokines. Viable live probiotics are better than the non-viable heat-killed probiotics in inducing higher immune responses in rainbow trout, especially elevating head kidney leucocyte phagocytosis, serum complement activity etc. (Panigrahi et al., 2005). In recent years, a number of *in vivo* and *in vitro* studies have investigated the interaction between dietary probiotics and immunocompetence. By increasing the host's adaptive and innate immune mechanisms, lactic acid bacteria (LAB) can protect the host against infection by enteric pathogens and tumor development. Immunological mechanisms behind the probiotic action may include:

- Stimulation of antibody secreting cell response (Kaila et al., 1992)
- Enhancement of phagocytosis of pathogens (Panigrahi et al., 2004; 2005)
- Modification of cytokine production (Panigrahi et al., 2007)

Consequently, probiotic bacteria may influence both adaptive and innate immune responses. Probiotics may reverse the increased intestinal permeability induced by antigens, but no information is available about long-term effects.

#### 4.6.1.1. $\beta$ -1,3/1,6-glucans

Glucans are high molecular-weight substances composed of glucose as building blocks. Glucans in general comprise a great variety of substances common in nature (such as cellulose, glycogen, and starch), most of which do not interact with the immune system. The common feature of immunomodulatory glucans is a chain of glucose residues linked together in  $\beta$ -1,3-linkages, also called beta-glucans. Of the different  $\beta$ -glucans, the products known as  $\beta$ -1,3/1,6-glucans (read as "beta-one-three-one-six-glucans"), derived from baker's yeast, are suggested to be the most potent immune-system enhancers.  $\beta$ -1,3/1,6-glucans are characterized by side-chains attached to the backbone that radiate outward like branches on a tree (Figure 4.6.1.1.1).

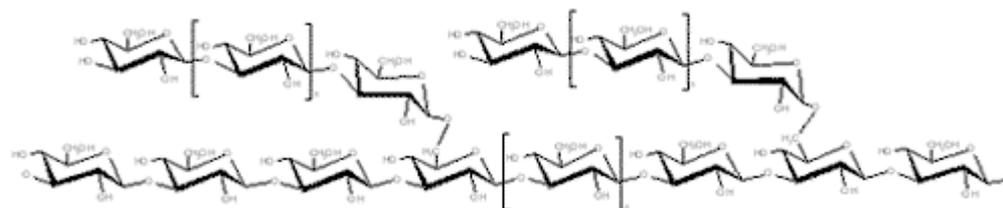


Figure 4.6.1.1.1 The primary structure of  $\beta$ -1,3/1,6-glucans, characterised by side-chains attached to the backbone.

The primary structure of the  $\beta$ -1,3/1,6-glucan is determinant for its immune-enhancing ability. The frequency and nature of side-chains strongly affect the ability of the glucan to mediate binding to surface receptors on the target cells influencing the effectiveness of the

glucan as an immunostimulant. Table 4.6.1.1.1 presents an overview of relevant papers using glucans as immunostimulants in fish.

Table 4.6.1.1.1 Glucans as immunostimulants in fish.

Immunostimulant	Fish	Administration and dose	Length of administration	Mechanism of action/results	References
$\beta$ -1,3 glucans	Carp	i.p; 2-10 mg kg <sup>-1</sup> BW	12 days	↑ phagocytic activity of kidney leucocytes	Yano et al. (1989)
Yeast glucan	Salmon	i.p	20 days	↑ resistance to <i>V. anguillarum</i> , <i>V. salmonicida</i> and <i>Y. ruckeri</i>	Robertsen et al. (1990)
$\beta$ -glucan	Coho	i.p (5 and 15 mg kg <sup>-1</sup> )	36 days	No immune response	Nikl et al. (1991)
$\beta$ -1,3 glucans	Channel catfish	Injected; 50 and 70 $\mu$ g fish <sup>-1</sup> (100 g BW) suspended in 0.2 ml PBS	2 weeks	↑ phagocytic and bacterial activity ↑ serum antibody concentration	Chen and Ainsworth (1992)
Yeast glucan	Salmon	i.p	4 weeks	↑ complement and lysozyme activity	Engstad et al. (1992)
Glucan (barley Extract (Sigma)	Trout	Injected i.p. 100 $\mu$ g glucan dissolved in PBS. Immersed the concentration of 100 $\mu$ g ml <sup>-1</sup> glucan for 30 min	10 days	↑ phagocytic activity ↑ numbers of circulatory glass-adherent cells	Jeney and Anderson (1993)
$\beta$ -1,3 and $\beta$ -1,6 inked yeast glucan (M-glucan)	Salmon	Injected i.p 1 ml of 0.5% (w/v) M-glucan in 0.9% saline	22 days	↑ macrophage and neutrophil numbers, head kidney macrophages ↑ ability to kill <i>A. salmonicida</i>	Jørgensen et al. (1993 a)
$\beta$ -1,3 and $\beta$ -1,6 inked yeast glucan (M-glucan)	Trout	Injected i.p 1 ml of 1% M-glucan suspension	3 weeks	Macrophages increased ability to kill <i>A. salmonicida</i> ↑ serum lysozyme activity	Jørgensen et al. (1993 b)
Yeast glucan	Salmon	i.p (0.5 mg/fish)	43 weeks	↑ survival against <i>A. salmonicida</i> infection	Rørstad et al. (1993)
Yeast glucan	Salmon	i.p	7 weeks	↑ antibody → resistance to <i>A. salmonicida</i>	Aakre et al. (1994)
Yeast glucan	Shrimp	immersion	43 days	↑ growth at 0.5; 1 and 2 mg/ml but not at 0.25 mg/ml ↑ phenoloxidase activity ↑ resistance to <i>V. vulnificus</i>	Sung et al. (1994)
Yeast glucan	Trout	i.p	n.i	↑ lysozyme activity	Thompson et al. (1995)
Yeast glucan	Salmon	Oral and anal (150 mg/	2 days	↑ acid phosphatase	Dalmo et al. (1996)

Immunostimulant	Fish	Administration and dose	Length of administration	Mechanism of action/results	References
		kg)			
β-glucan	Turbot	Oral (2 g kg <sup>-1</sup> )	5 weeks	↑ leukocyte number	Ogier de Baulny et al. (1996)
Yeast glucan + Vitamin C	Trout	Diets containing yeast Glucan and vitamin C at 150, 1,000 and 4,000 p.p.m	4 weeks	↑ alternative pathway of complement – and macrophage activity. ↑ specific Ab response following vaccination with <i>Y. ruckeri</i>	Verlhac et al. (1996)
β-glucan	Trout	Oral	4 weeks	↑ stress resistance	Jeney et al. (1997)
β-glucan	Sea bass	Oral (2% wet body weight)	2 weeks	↑ humoral activation	Bagni et al. (2000)
β-glucan + mannose	Snapper	Oral (0.1-1.0% w/w)	n.i <i>in vitro</i> study	↑ macrophage activation	Cook et al. (2001)
β-glucan + LPS	Salmon	10 µg ml <sup>-1</sup>	n.i <i>in vitro</i> study	↑ lysozyme activity	Paulsen et al. (2001)
β-glucan	Flounder	Oral (3 g kg <sup>-1</sup> )	n.i	↑ respiratory burst activity	Galindo-Villegas et al. (2002)
β-glucan	Nile tilapia	Oral	6 weeks	↑ stress resistance	Cain et al. (2003)
β-glucan	Sea bass	Oral (0.1%)	60 days	↑ serum complement activity ↑ serum lysozyme, gill and liver HSP	Bagni et al. (2005)
β-glucan	Red drum	Oral (2% of diet)	6 weeks	→ on stress resistance	Li et al. (2005)
β-glucan	Sea bass	Oral (250, 500, 1,000 ppm)	25 days	↑ respiratory burst activity of head kidney macrophages	Bonaldo et al. (2007)
β-glucan	Channel catfish	Oral (1g/kg)	4 and 6 weeks	→ growth performance hematology or immune function Some improvement is stress resistance	Welker et al. (2007)
β-glucan	Trout	i.p	18 days	↑ resistance to IHNV	Sealey et al. (2008)

Symbols represent an increase (↑) in the specified response; no change (→). n.i – no information given.

Salmon – Atlantic salmon; Trout – rainbow trout; Coho – Coho salmon (*Oncorhynchus kisutch*), Turbot – *Scophthalmus maximus*; Sea bass - *Dicentrarchus labrax*, Snapper – pink snapper (*Pagrus auratus*); Flounder – Japanese flounder (*Paralichthis olivaceus*), Nile tilapia - *Oreochromis niloticus*; Red drum - *Sciaenops ocellatus*; Channel catfish - *Ictalurus punctatus*

ROS – reactive oxygen species

IHNV – infectious hematopoietic necrosis virus

#### 4.6.1.2. Bioactive alginate (high-M alginate)

The adaptive immune system is poorly developed in early developmental stages of fish (Schröder et al., 1998; Grøntvedt and Espelid, 2003), which is why immunostimulants and probiotics have been used in an attempt to increase survival of larvae against microbial pathogens (Gildberg et al. 1995; Gildberg and Mikkelsen, 1998; Nikoskelainen et al., 2001). In this respect, alginate has been proposed as a potential candidate. Alginate is a polysaccharide composed of  $\beta$ -1,4-D-mannuronic acid (M) and  $\alpha$ -L-glucuronic acid (G). The monomers are usually arranged in M-blocks, G-blocks and alternating MG-blocks (Haug et al., 1967). Alginate also binds various cations found in the seawater such as  $Mg^{2+}$ ,  $Si^{2+}$ ,  $Ba^{2+}$ , and  $Na^+$ . Commercial alginates are extracted from three species of brown algae. These include *Laminaria hyperborean*, *Ascophyllum nodosum* and *Macrocystis pyrifera*, in which alginate comprises up to 40% of the dry weight (Smidsrød and Skjåk-Bræk, 1990). Furthermore, bacterial alginates have also been isolated from *Azotobacter vinelandii* and several *Pseudomonas* species (Reemminghorst and Rehm, 2006).

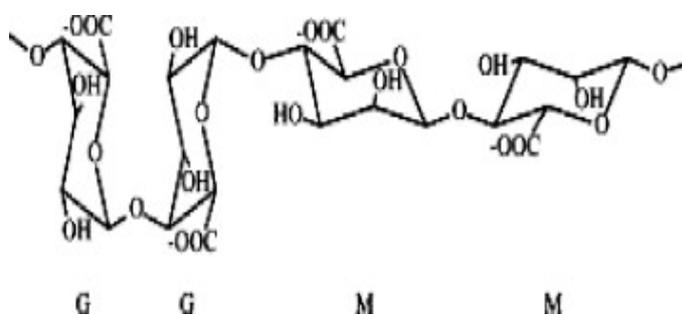


Figure 4.6.1.2.1 Chemical structure of alginate. Shown is a polymer chain of 2 guluronic acid (G) monomers and 2 mannuronic acid (M) monomers, with (1–4) linkages. According to George and Abraham (2006).

Commercially available alginates today have M-content ranging between 30 and 70%. Alginates with even higher M-content, typically higher than 80%, have also been shown to be potent stimulators of immune cells such as human monocytes (Skjåk-Bræk et al. 2000). High-M alginate has also been used as an immunostimulant for enhancement of innate immune resistance in fish larvae and fry (Skjermo et al., 1995; Vadstein, 1997; Skjermo and Vadstein, 1999; Vollstad et al., 2006). In a study with rainbow trout, Peddie et al. (2002) reported that a single injection of 1 mg of Ergosan significantly augmented the proportion of neutrophils in the peritoneal wall, increased the degree of phagocytosis, respiratory burst activity and expression of interleukin-1 $\beta$  (IL-1 $\beta$ ), interleukin-8 (IL-8) and one of the two known isoforms of tumour necrosis factor-alpha (TNF- $\alpha$ ) in peritoneal leucocytes one day post-injection. However, humoral immune parameters were less responsive to intraperitoneal alginate administration with complement stimulation only evident in the 1 mg treated group at 2 days post-injection. Table 4.6.1.2.1 presents an overview of reports using alginates and Ergosan in fish studies.

Table 4.6.1.2.1 Bioactive alginate (high-M alginate) and Ergosan as immunostimulants in fish.

Immunostimulant	Fish	Administration	Length of administration	Mechanism of action/results	References
High-M alginate	Atlantic halibut	Immersion	n.i	↑ survival	Vadstein et al. (1993)
High-M alginate	Turbot	Enriched in <i>Artemia</i>	1 week	↑ survival	Skjermo et al. (1995)
Alginate	Atlantic salmon	Macrophage culture	n.i	↑ phagocytic activity and respiratory burst	Rokstad et al. (unpublished data) cited in Vadstein (1997)
Alginate	Atlantic salmon	Diet	Six months	→ survival → colour, taste consistency and grading ↑ lysozyme activity	Gabrielsen and Austrheim (1998)
High-M alginate	Atlantic halibut	Bioencapsulated in <i>Artemia</i>	Different periods during initial feeding	↑ growth	Skjermo and Bergh (2000)
High-M alginate	Atlantic cod Spotted wolffish	Diet	59 days 55 days	↑ growth Uptake of 125I-labelled molecule in the stomach and intestine	Vollstad et al. (2006)
Alginate	Turbot	Diet	13 days	↑ protein synthesis ↑ protein turnover → survival and larval size	Conceicao et al. (2001)
Ergosan	Rainbow trout	Injection	1 day	↑ neutrophils ↑ respiratory burst	Peddie et al. (2002)
Ergosan	Sea bass	Diet (0.5%)	60 days	↑ serum complement activity ↑ serum lysozyme, gill and liver HSP	Bagni et al. (2005)

Symbols represent an increase (↑) in the specified response; no change (→).  
n.i – no information given

#### 4.6.1.3. Plant extracts

Some immunostimulants cannot be used because of various disadvantages, such as high cost and limited effectiveness upon parenteral administration. On the other hand, a large number of plants have been used in traditional medicine for the treatment and control of several diseases (Duke, 1987). Three of such plants are mistletoe (*Viscum album*), nettle (*Urtica dioica*), and ginger (*Zingiber officinale*). Some medicinal plants have also been used as phyto-genic-based immunostimulatory preparations, and preparations have been used as adjuvant therapy, in cancer and AIDS (Verporte et al., 1999; Mentle et al., 2000; Zarkovic et al., 2001). Especially mistletoe and nettle have been asserted to possess immunomodulatory activity. As no information was available about the use of plant extracts in fish, Dugenci et al. (2003) investigated the effects of mistletoe, nettle and ginger on dietary intake of rainbow

trout. Food contained lyophilized extracts of these plants at two levels of inclusion, 0.1 and 1% in a three-week experiment. At the end of the experimental period, various parameters of innate defence mechanisms, including extracellular and intracellular respiratory burst activities, phagocytosis in blood leukocytes, total plasma protein level, specific growth rates and condition factors were examined. The plant materials tested increased the extracellular respiratory burst activity ( $P < 0.001$ ) compared to the control group. Furthermore, fish fed the diet containing 1% ginger roots exhibited a significant innate immune response. Phagocytosis and extracellular burst activity of blood leukocytes were significantly higher in this group than those in the control group. All plant extracts increased plasma protein level except for the 0.1% ginger supplemented diet. The highest level of plasma proteins was observed in the group fed with 1% ginger extract.

Lectins are proteins or glycoprotein substances, usually of plant origin, and they are sugar-binding proteins which are highly specific for their sugar moieties. Lectins are also known to play important roles in the immune system by recognizing carbohydrates that are found exclusively on pathogenic bacteria, or that are inaccessible to host cells. Examples are the lectin complement activation pathway and mannose binding lectin (MBL), also named mannose- or mannan-binding protein (MBP). MBL recognizes carbohydrate patterns, found on the surface of a large number of pathogenic micro-organisms, including bacteria, viruses, protozoa and fungi. Readers with special interest in lectins and immune response are referred to the recent comprehensive reviews of Boshra et al. (2006) and van Kooyk and Rabinovich (2008). See also Section 3.5.

#### 4.6.1.4. Do immunostimulants raise the level of protection against disease?

A large number of reviews have been published concerning the advantages of immunostimulants in fish (Anderson, 1992; Robertsen et al., 1994; Raa, 1996; Gannam and Schrock, 1999; Robertsen, 1999; Sahoo and Mukherjee, 1999; Sakai, 1999; Philip et al., 2003; Bricknell and Dalmo, 2005; Galindo-Villegas and Hosokawa, 2005; Peddie and Secombes, 2005; Tassakka and Sakai, 2005). In this chapter an attempt has been made to compile the recent developments in the field, mainly of the last five years, besides the earlier work done in the field of disease resistance in fish with exposure to immunostimulants.

An overview of the effect of immunostimulatory substances in enhancing the degree of protection in fish is presented in Table 4.6.1.4.1. As most pathogens used in challenge experiments are bacteria found in commercial aquaculture, very little information is available regarding the roles of immunomodulatory substances on viral or parasite infections. The pathogens used in such studies are; *Aeromonas hydrophila*, *Aeromonas salmonicida*, *Aphanomyces invadans*, *Edwardsiella ictaluri*, *Edwardsiella tarda*, *Photobacterium damsela* ssp. *piscicida*, *Vibrio anguillarum*, *Vibrio harvei*, *Yersinia ruckeri*, *Lactococcus garvieae*, *Streptococcus* sp. and *Streptococcus iniae* as well as infectious hematopoietic necrosis virus (IHNV) and parasites.

Table 4.6.1.4.1. Immunostimulants and disease susceptibility in fish.

Resistance to pathogen	Agent	Fish	Route of exposure	Length of administration	Mechanism of action/results	References
<i>Aeromonas hydrophila</i>	Yeast glucan	Tilapia and grass carp	i.p	14 days	↑ protection in both species ↑ number of NTB-positive staining cells	Wang and Wang (1997)
	Glucan	Rohu	Oral	7 days	↑ phagocytotic activity ↑ bactericidal activity	Sahoo and Mukherjee (2001)

Resistance to pathogen	Agent	Fish	Route of exposure	Length of administration	Mechanism of action/results	References
					↑ antibody titre ↑ agglutinin level	
	Glucan	Catla	i.p	30 days	↑ antibody titre ↑ macrophage activating factor	Kamiyla et al. (2006)
	Glucan	Asian catfish	Oral	30 days	↑ antibody titre ↑ protection	Kumari and Sahoo (2006)
	Glucan	Rohu	i.p	28 days	↑ phagocytotic activity ↑ lysozyme activity ↑ bactericidal activity ↑ complement activity ↑ resistance	Misra et al. (2006 a)
	Glucan - injections	Rohu	i.p	28 days	↑ phagocytotic activity ↑ lysozyme activity ↑ bactericidal activity ↑ complement activity ↑ resistance	Misra et al. (2006 b)
	Garlic	Rohu	i.p	10 days	↑ superoxide production ↑ lysozyme activity ↑ resistance	Sahu et al. (2007)
	Glucan	Indian major carp	i.p	1 week	↑ superoxide production ↑ phagocytic activity resistance unclear	Kamiyla et al. (2008)
<i>Aeromonas salmonicida</i>	Glucan	Brook trout	i.p and 30 min immersion	4 weeks	↑ resistance (up to 7 days) By day 14 resistance was reduced	Anderson and Siwicki (1994)
	Macro gard	Trout	Oral	1 week	↑ MPO activity ↑ phagocytic activity ↑ superoxide production ↑ Ig level	Siwicki et al. (1994)
	<i>Saccharomyces cerevisiae</i>	Trout	Oral	1 week	↑ MPO activity ↑ phagocytic activity ↑ superoxide production ↑ Ig level	Siwicki et al. (1994)
<i>Aphanomyces invadans</i>	Ergosan	Striped snakehead	Injected intramuscularly	2 weeks	↑ inhibitory effect of serum ↑ macrophage activating factor	Miles et al. (2001)
<i>Edwardsiella ictaluri</i>	Yeast glucan	Channel catfish	i.p	2 weeks	↑ resistance ↑ phagocytic activity	Chen and Ainsworth (1992)
	Glucan	Channel catfish	Oral	2 weeks	→ resistance ↑ macrophage and neutrophil migration	Duncan and Klesius (1996)

Resistance to pathogen	Agent	Fish	Route of exposure	Length of administration	Mechanism of action/results	References
<i>Edwardsiella tarda</i>	Glucan	Carp	i.p	12 days	↑ resistance	Yano et al. (1989)
	Yeast glucan	Tilapia and grass carp	i.p	14 days	↑ protection in both species ↑ number of NTB-positive staining cells	Wang and Wang (1997)
	Glucan	Rohu	i.p	10 days	↑ bactericidal activity ↑ resistance	Sahoo and Mukherjee (2002)
	Glucan	Rohu	i.p	28 days	↑ phagocytotic activity ↑ lysozyme activity ↑ bactericidal activity ↑ complement activity ↑ resistance	Misra et al. (2006 a)
	Glucan - injections	Rohu	i.p	28 days	↑ phagocytotic activity ↑ lysozyme activity ↑ bactericidal activity ↑ complement activity ↑ resistance	Misra et al. (2006 b)
<i>Pasteurella piscicida</i>	Glucan	Yellowtail	i.p	10 days	→ resistance	Matsuyama et al. (1992)
<i>Photobacterium damsela</i> ssp. <i>piscicida</i>	Glucan	Gilthead sea bream	Immerse	10 days	→ phagocytic activity ↑ resistance (10 g kg <sup>-1</sup> ) → resistance (5 g kg <sup>-1</sup> )	Couso et al. (2003)
<i>Vibrio anguillarum</i>	Yeast glucan	Salmon	i.p	20 days	↑ resistance	Robertson et al. (1990)
	High-M alginate	Turbot	Oral	1 week	↑ resistance	Skjermo et al. (1995)
	Cationic cod milt protein	Atlantic cod	Bath challenge	4 weeks	↑ resistance	Pedersen et al. (2004)
	High-M alginate	Atlantic halibut	Oral	Different periods during initial feeding	↑ resistance	Skjermo and Bergh (2004)
<i>Vibrio harvei</i>	Glucan	Croaker	i.p	8 weeks	↑ lysozyme activity ↑ phagocytic activity ↑ resistance (0.09%) → resistance (0.18%)	Ai et al. (2007)
<i>Vibrio vulnificus</i>	Glucan	Tiger shrimp	Immerse	43 days	↑ resistance in shrimp treated with 0.5 and 1 mg/ ml glucan but no in groups treated with 0.25 and 2 mg/ ml glucan	Sung et al. (1994)

Resistance to pathogen	Agent	Fish	Route of exposure	Length of administration	Mechanism of action/results	References
<i>Yersinia ruckeri</i>	Yeast glucan	Salmon	i.p	20 days	↑ resistance	Robertson et al. (1990)
<i>Lactococcus garviae</i>	Alginate micro-particles	Trout	i.p	3 weeks	→ resistance	Romalde et al. (2004)
<i>Streptococcus</i> sp.	Glucan	Yellowtail	i.p	10 days	↑ resistance ↑ serum complement ↑ lysozyme activity	Matsuyama et al. (1992)
	Sodium alginate	Grouper	i.p	6 days	↑ lysozyme activity ↑ respiratory bursts ↑ phagocytic activity ↑ resistance	Chiu et al. (2008)
<i>Streptococcus iniae</i>	Glucan	Hybrid striped bass	Oral	3 weeks	→ resistance	Jaramillo and Gatlin (2004)
	Glucan	Nile tilapia	Oral	14 weeks	↑ resistance when fish were fed 100 and 200 mg glucan	Whittington et al. (2005)
	Glucan	Shark	Oral	2 weeks	↑ resistance	Russo et al. (2006)
<b>Virus</b>						
IHN	Glucan	Rainbow trout	i.p	18 days	→ respiratory bursts → TNF- $\alpha$ -expression ↑ resistance	Sealy et al. (2008)
<b>Parasite</b>						
<i>Loma morhua</i>	Glucan	Atlantic cod	Oral	n.i	↑ lymphocyte density	Jenkins and Barker (2004)

Symbols represent an increase (↑) in the specified response; no change (→).

i.p – intraperitoneal injection

n.i – no information given

IHN – infectious hematopoietic necrosis virus

Rohu – *Labeo rohita*, Catla – *Catla catla* (Hamilton), Asian catfish – *Clarias batrachus*, Striped snakehead – *Channa striata*, Croaker – large yellow croaker (*Pseudosciaena crocea*), Tiger shrimp – *Penaeus monodon*, Yellowtail – *Seriola quinqueradiata*, Hybrid striped bass – *Morone chrysops* x *Morone saxatilis*, Shark – red tail black shark (*Epalzeorhynchus bicolor*), Grouper – *Epinephelus fuscoguttatus*

#### 4.6.1.5. Effect of immunostimulants on gut microbiota

Immunostimulants seem to be valuable for the control of fish diseases. However, knowledge regarding the ability of immunostimulants to stabilize the gut microbiota in a healthier way and decrease the infection pressure by improving the gut function is scarce. To our knowledge only two studies, Gildberg and Mikkelsen (1998) and Skjeremo et al. (2006) have been done on the topic of immunostimulants and the gut microbiota. In their study on Atlantic cod fry, Gildberg and Mikkelsen (1998) evaluated the effect of supplementation of lactic acid bacteria either alone or in combination with immunostimulating peptides. After

three weeks of feeding, the fish were challenged by bath exposure to *V. anguillarum* ( $10^7$ /ml, 1 h). Twelve days after infection significantly ( $p < 0.05$ ) reduced cumulative mortality was recorded in fish fed diet supplemented with lactic acid bacteria, originally isolated from Atlantic salmon, and with immunostimulating peptides. No synergistic or cumulative effects were achieved by combining lactic acid bacteria and immunostimulating peptides. Four weeks after infection similar cumulative mortality (80–84%) was observed in all groups. Lactic acid bacteria could colonize the internal mucus layer of the cod fry pyloric caeca, and a significant number of the bacteria survived the passage of the whole gastrointestinal tract.

In a study with Atlantic cod larvae, Skjermo et al. (2006) evaluated the effect of  $\beta$ -(1→3, 1→6)-glucans (chrysolaminaran) from the marine diatom *Chaetoceros mülleri*, a commercial yeast-glucan product and high-M alginate (high content of mannuronic acid isolated from *Durvillaea antarctica*), on the microbial conditions in larval gut and water with respect to total colony forming units (CFU) on marine agar, and *Vibrio*- and *Pseudomonas*-like species on selective agars (TCBS and marine *Pseudomonas* agar with CFC-supplement). The larvae were rapidly colonised after hatching, but no or weak effects of the stimulants were observed on the colonisation rates or the composition. The total CFU varied from  $10^1$  to  $10^2$  CFU per  $\mu\text{g}$  larva after initiation of the first feeding. Bacteria belonging to *Pseudomonas* seem to increase throughout the period, whereas the level of *Vibrio*-like bacteria was low and stable. However, in this investigation the authors only focused on characterisation of *Pseudomonas*- and *Vibrio*-like bacteria and did not use molecular methods to evaluate the entire bacterial community.

Based on the fact that less information is available about the effect of immunostimulants on the “good” gut microbiota in fish with antagonistic activity against fish pathogenic bacteria, this should be a topic of further research as the gastrointestinal tract is a potential port of entry for pathogenic bacteria (Ringø et al., 2004; Birkbeck and Ringø, 2005; Ringø et al., 2007 a; 2007 b; Salinas et al., 2008; Sugita et al., 2008). The importance of the topic is illustrated by a recent study showing that intraperitoneal injection of immunostimulatory substances affects the allochthonous (transit) gut microbiota including LAB of Atlantic salmon (Liu et al., 2008).

#### 4.6.1.6. Nutritional factors and the immune response

It is well known that nutrition influences the state of health of fish and may have a great impact on immune response in fish. In contrast to immunostimulants, the nutritional factors are essential for normal growth and development of fish, and thus they are added to the ration not solely with the aim of acting as active substances for the immune system. The nutritional factors which undoubtedly have primary roles in influencing the immune response are vitamins and lipids. Vitamins C, B6, E and A and the minerals iron and fluoride have been identified as micronutrients that can affect disease resistance. With respect to the strict definition of immunostimulants, vitamins and minerals are not considered immunostimulants since these compounds enhance the immune system by providing substrates and serving as cofactors necessary for the immune system to work properly.

Readers with special interest in nutritional factors on immune response of fish are referred to the comprehensive reviews of Landolt (1989), Anderson (1992), Blazer (1992), Waagbø (1994), Raa (1996), Galeotti (1998), Gannam and Schrock (1999), Sakai (1999), Sohn et al. (2000), Galindo and Hosokawa (2004), Trushenski et al. (2006), Panigrahi and Azad (2007) and Sahoo (2007). Information on the effect of lipids on immunity is presented in Chapter 7: *Current and future plant oils in aquafeeds*. As no information is available about nutritional balance and the immune response, this should be given high priority in future studies.

#### 4.6.2. PREBIOTICS

Prebiotics have been defined as non-digestible components that are metabolised by specific microorganisms beneficial to the health and growth of the host (Gibson and Roberfroid 1995; Manning and Gibson 2004). Carbohydrates can be classified according to their molecular size or degree of polymerization (number of monosaccharide units combined), into monosaccharides, oligosaccharides or polysaccharides. According to IUB-IUPAC nomenclature, oligosaccharides are defined as saccharides containing between 3 and 10 sugar moieties. Other authorities classify saccharides including polymers from 3 to 19 monosaccharide units in this group. However, there is not a rational physiological or chemical reason for setting these limits (Voragen, 1998). Consequently, oligosaccharides are low molecular weight carbohydrates. At the same time, based on the physiological properties, the carbohydrates can be classified as digestible or non-digestible. The concept of non-digestible oligosaccharide (NDO) originates from the observation that the anomeric C atom (C1 or C2) of the monosaccharide units of some dietary oligosaccharides has a configuration that makes their glycosidic bonds indigestible to the hydrolytic activity of the human digestive enzymes (Roberfroid and Slavin, 2000). The main categories of NDOs presently available or in development as food ingredients include carbohydrates in which the monosaccharide unit is fructose, galactose, glucose and/or xylose. See Figure 4.6.2.1.

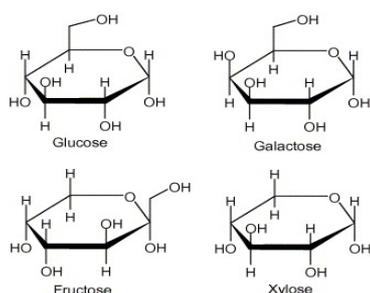


Figure 4.6.2.1. Monosaccharides components of non-digestible oligosaccharides. According to Mussatto and Mancilha (2007).

Dietary fibres belong to the broad category of carbohydrates. Burkitt and co-authors (1972) defined dietary fibre as the sum of polysaccharides and lignin that are not digested by the endogenous secretions of the human gastrointestinal tract. They can be classified as soluble (e.g., inulin and oligofructose), insoluble (e.g., cellulose) or mixed (e.g., bran). It is well known from endothermic animals that dietary fibres are fermented by the anaerobic intestinal microbiota, primarily bifidobacteria and lactobacilli colonizing the large intestine and thus improving the host's intestinal balance (Roberfroid, 1993; Gibson, 1998). However, prebiotics is in its infancy with fishes, compared to the progress that has been made in the development of prebiotics for poultry (Patterson and Burkholder, 2003).

Traditional uses of antibiotics have been criticized due to the potential development of antibiotic-resistant bacteria, the presence of antibiotic residues in seafood, destruction of microbial populations in the aquacultural environment and suppression of the aquatic animal's immune system. As an alternative strategy to antibiotics, probiotics have recently attracted extensive attention in aquaculture. Many reports have been published regarding application of probiotics in the aquatic environment (for review see, Ringø and Gatesoupe, 1998; Gatesoupe, 1999; Verschuere et al., 2000; Irianto and Austin, 2002; Burr et al., 2005; Gram and Ringø, 2005; Farzanfar, 2006; Tinh et al., 2008; Wang et al., 2008). Due to high cost,

potential impact on the environment, regulatory issues, and product safety, large-scale application of probiotics in the water has been limited. Alternatively, it appears more economically efficient to manipulate the gastrointestinal tract microbiota in aquatic animals by application of dietary prebiotics that alter the conditions of the gastrointestinal tract to favour certain bacterial species that may enhance growth efficiency and disease resistance of the host organism (Gatlin, 2002; Burr et al., 2005).

The first study on prebiotics in aquaculture was reported in 1995 by Hanley and co-authors. Since then several studies have been carried out, and an overview of the studies carried out using prebiotics in aquaculture is presented in Table 4.6.2.1.

Table 4.6.2.1. Use of prebiotics in aquatic animals.

Prebiotic	Dose and length of administration	Fish	Results	References
Inulin	i.p (10mg kg <sup>-1</sup> ) – 14 days	Grass carp ( <i>Ctenopharyngodon idellus</i> )	→ resistance against <i>A. hydrophila</i> and <i>E. tarda</i>	Wang and Wang (1997)
	15% - 4 weeks	Arctic charr ( <i>Salvelinus alpinus</i> L.)	Intestinal cell damage	Olsen et al. (2001)
	2% - 1 month	Turbot ( <i>Psetta maxima</i> ) larvae	→ growth No effect on gut microbiota ( <i>Bacillus</i> and <i>Vibrio</i> )	Mahious et al. (2006)
	15% - 4 weeks	Arctic charr	↓ TVC Microbiota – control: <i>Pseudomonas</i> , <i>P. glacincola</i> , <i>C. divergens</i> , <i>Micrococcus</i> , <i>Staphylococcus</i> , <i>Streptococcus</i> Microbiota – inulin: <i>Bacillus</i> , <i>C. maltaromaticum</i> , <i>Staphylococcus</i> , <i>Streptococcus</i> Different colonization pattern on enterocytes surface	Ringø et al. (2006)
	7.5% - 3 weeks	Atlantic salmon ( <i>Salmo salar</i> L.)	→ TVC Control ↓ <i>M. psychrotolerans</i> , <i>C. maltaromaticum</i> , <i>E. faecalis</i> Inulin ↓ <i>Pseudoalteromonas</i> , <i>Micrococcus</i> → intestinal cell damage	Bakke-McKellep et al. (2007)
FOS	0, 0.2 and 0.6% - 58 days	Hybrid tilapia ( <i>Oreochromis niloticus</i> ♀ × <i>O. aureus</i> ♂)	↑ growth rate ↑ survival ↑ non-specific immunity	He et al. (2003)
	0, 0.15, 0.25% -100 days	Soft-shell turtle ( <i>Triortyx sinensis</i> )	↑ growth rate at 0.25% inclusion ↑ SOD activity at 0.25% inclusion ↓ lysozyme activity	Ji et al. (2004)
	2% - 1 month	Turbot larvae	↑ growth rate Effects on gut microbiota ( <i>Bacillus</i> and <i>Vibrio</i> )	Mahious et al. (2006)
scFOS	Dose 0.8 or 1.2 g kg <sup>-1</sup> - 8 weeks	Hybrid tilapia	↑ growth rate, feed intake, feed conversion → survival and condition factor ↑ <i>V. parahemolyticus</i> , <i>A. hydrophila</i> , <i>Lactobacillus</i> spp. <i>S. faecalis</i>	Hui-Yuan et al. (2007)
	0.025, 0.05, 0.075, 0.1, 0.2,	White shrimp ( <i>Litopenaeus vannamei</i> )	→ weight gain, feed conversion and survival	Li et al. (2007)

	0,4 and 0.8% - 6 week		scFOS affected gut microbiota	
	0.04 – 0.16% - 8 weeks	White shrimp	↑ growth rate, feed intake, feed conversion scFOS affected gut microbiota	Zhou et al. (2007)
	1 g kg <sup>-1</sup> – 56 days	Hybrid tilapia	↑ uncultured bacterium clones and <i>T. eikelboomii</i>	Zhou et al. (2008)
MOS	0, 0.2 and 0.6% - 58 days	Hybrid tilapia	→ growth rate ↑ survival ↑ non-specific immunity	He et al. (2003)
MOS	0, 1.5, 3 and 4.5 g kg <sup>-1</sup> – 48 days	Tiger shrimp ( <i>Penaeus semisulcatus</i> )	3 g kg <sup>-1</sup> ↑ growth, feed conversion and survival No detrimental effect was noted on hepatopancreas tissue	Genc et al. (2007)
	2 g kg <sup>-1</sup> – 90 days	Rainbow trout ( <i>Oncorhynchus mykiss</i> )	↑ growth and survival ↑ antibody titre and lysozyme activity in one trial → bactericidal activity	Staykov et al. (2007)
	2 or 4% - 67 days	Sea bass ( <i>Dicentrarchus labrax</i> )	↑ growth → feed conversion ↓ lipid vacuolization ↓ presence of <i>Vibrio alginolyticus</i> on head kidney	Torreccillas et al. (2007)
	0, 1.5, 3 or 4.5 g kg <sup>-1</sup> - weeks	Rainbow trout	1.5 g kg <sup>-1</sup> ↑ growth rate 1.5 g and 3 g kg <sup>-1</sup> ↑ intestinal villi → feed conversion, hepatosomic index, intestinal morphology	Yilmaz et al. (2007)
GroBiotic®-AE	1 and 2% - 2 and 4 weeks	Hybrid striped bass ( <i>Morone chrysops</i> × <i>M. saxatilis</i> )	↑ feed efficiency ↑ respiratory bursts ↑ resistance against <i>S. iniae</i>	Li and Gatlin (2004)

FOS – fructooligosaccharides

scFOS – short-chain fructooligosaccharides

MOS – mannanoligosaccharides

Symbols represent an increase (↑), decrease (↓) or no change (→) in the specified response.

i.p – intraperitoneal injection; SOD – superoxide dismutase

Total viable counts – TVC, *Psychrobacter glacincola* – *P. glacincola*, *Carnobacterium divergens* - *C. divergens*, *Carnobacterium maltaromaticum* - *C. maltaromaticum*, *Marinilactibacillus psychrotolerans* – *M. psychrotolerans*, *Enterococcus faecalis* - *E. faecalis*, *Vibrio parahemolyticus* - *V. parahemolyticus*, *Aeromonas hydrophila* - *A. hydrophila*, *Streptococcus faecalis* - *S. faecalis*, *Streptococcus iniae* – *S. iniae*, *Thiothrix eikelboomii*. – *T. eikelboomii*.

#### 4.6.2.1. Inulin

Some of the more commonly used prebiotics include inulin, fructooligosaccharides (FOS) and transgalactooligosaccharides (TOS) (Vulevic et al., 2004). Inulin-type fructans are composed of β-D-fructofuranoses attached by β-2–1 linkages. The first monomer of the chain is either a β-D-glucopyranosyl or β-D-fructopyranosyl residue. They constitute a group of oligosaccharides derived from sucrose that are isolated from natural plant sources. Inulin is found in a variety of edible fruits and plants such as wheat, onions, leeks, garlic, asparagus, artichokes and bananas (Roberfroid, 1993). Although inulin is not a natural fibre in fish diets, the prebiotic potential of inulin and other dietary fibres may have interesting application in aquaculture by stimulating beneficial gut bacteria and suppressing the potentially deleterious bacteria.

One recent study, Mahious et al. (2006) evaluated the effect of dietary inulin on fish gut bacteria, but the study focused mainly on *Vibrio* and *Bacillus* and did not provide any information about other gut bacteria.

Lactic acid bacteria (LAB) have been suggested as potential probiotic candidates in future aquaculture, and some information is available about fermentation of inulin by LAB isolated from fish gut, notably *Carnobacterium piscicola*, *Carnobacterium mobile* and *Carnobacterium* spp. (Ringø, 2004). In a more recent study, Ringø et al. (2006) investigated the effect of dietary inulin on aerobic and facultative aerobic bacteria associated with the hindgut (distal intestine) of Arctic charr determined using classical microbiology and molecular bacteriology (16S rRNA) and electron microscopy. The general effect of inulin compared to that of fish fed dextrin is shown in Table 4.6.2.1. Electron microscopical analysis of hindgut regions confirmed traditional culture-based microbial analysis as fewer bacterial cells were observed between microvilli and associated with the surfaces of enterocytes of fish fed inulin rather than dextrin.

In a recent study, Bakke-McKellep et al. (2007) investigated the dietary effect of fishmeal, extracted soy bean meal or inulin, with or without oxytetracycline on the intestinal microbiota of Atlantic salmon. Interestingly, Bakke-McKellep et al. (2007) clearly showed that inclusion of 7.5% inulin in the diet led to less diverse gut microbiota. These results are in accordance with that reported for Arctic charr (Ringø et al., 2006) but contradict findings in mammalian studies (Gibson and Roberfroid, 1995; Pool-Zobel et al., 2002; Xu et al., 2002). The reason for this has not been elucidated, but Bakke-McKellep et al. (2007) suggested that inulin has a selective effect on Atlantic salmon and Arctic charr gastrointestinal microbiota. Whether this effect is valid for other fish species and whether it has a beneficial or detrimental effect on fish health remains to be examined in future studies. The dietary effect of inulin on fish gut bacteria might have some relevance to aquaculture, as it is well documented that the indigenous microbiota, particularly in the colon of endothermic animals, play an important role in providing natural resistance to pathogenic microorganisms (Havenaar et al., 1999).

#### 4.6.2.2. Fructooligosaccharides (FOS)

One of the most common prebiotics studied in humans and terrestrial animals is FOS, a general term that includes all nondigestible oligosaccharides composed of fructose and glucose units (Swanson et al., 2002 a). FOS refers to short and medium chains of  $\beta$ -D-fructans in which fructosyl units are bound by  $\beta$ -(2-1) glycosidic linkages and attached to a terminal glucose unit. Because of a lack of  $\beta$ -fructosidases, mammalian digestive systems cannot hydrolyze the  $\beta$ -(2-1) glycosidic linkages (Teitelbaum and Walker, 2002). However, FOS can be fermented by certain bacteria expressing this enzyme, such as lactobacilli and bifidobacterial species (Sghir et al., 1998; Manning and Gibson, 2004) and will thus selectively support the growth and survival of such bacteria in the GI tract of animals. Despite occasional inconsistent results in terrestrial species, some studies have shown that FOS influenced protein digestion and intestinal morphology (Teitelbaum and Walker, 2002; Swanson et al., 2002 b). These modifications might contribute to improved growth, feed efficiency, and disease resistance. Dietary supplementation of FOS has been shown to enhance growth rate of some aquatic animals such as soft-shell turtle (*Triortyx sinensis*) (Ji et al., 2004) and turbot larvae (*Psetta maxima*) (Mahious et al., 2006).

#### 4.6.2.3. Short-chain fructooligosaccharides (scFOS)

Supplementation of prebiotic compounds, including scFOS, has been shown to confer benefits on nutrient utilization, growth, and disease resistance of various endothermic animal species through improved gastrointestinal (GI) microbiota. From an aquaculture point of view this is important as during the last decade, there has been an improved understanding of the importance of intestinal microbiota in fish. However, scientific validation is lacking regarding the use of scFOS on any important Norwegian fish species. On the other hand, Chinese scientists have recently published new and vital information on the effect of prebiotics and

scFOS on the intestinal fish microbiota in hybrid tilapia (Hui-Yuan et al., 2007; Zhou et al., 2008) and white shrimp (*Litopenaeus vannamei*) (Li et al., 2007 a; Zhou et al., 2007).

In the study by Hui-Yuan et al. (2007) an 8-week feeding trial of hybrid tilapia showed that specific growth rate and daily feed intake were significantly improved with increasing level of scFOS while feed conversion ratio and hepatopancreas somatic index decreased. Survival rate and condition factor were however not affected. Furthermore, gut microbiota was also investigated. However, the authors focus only on culturable counts of *Vibrio parahemolyticus*, *Aeromonas hydrophila*, *Lactobacillus* sp. and *Streptococcus faecalis*. The results showed increasing trend in the population level of the investigated bacteria with increasing level of dietary scFOS, but no significant differences were observed.

In a more recent study by Zhou et al. (2008) the authors evaluated the autochthonous (adherent) gut microbiota by denaturing gradient gel electrophoresis (DGGE) in fish fed either yeast culture or scFOS. There were clear differences in the bacterial community by feeding the fish yeast culture and scFOS. When the fish were fed scFOS some unique bands occurred, and these belong to uncultured bacterium clones and *Thiothrix eikelboomii*. Whether these bacterial strains are beneficial to the host has not been elucidated and needs to be investigated in future studies.

During the last two years, two studies have investigated the effect of dietary supplementation of scFOS on intestinal microbiota of white shrimp (Li et al., 2007 a; Zhou et al., 2007). Li and co-authors conducted a 6-week trial in a recirculating system and showed that dietary scFOS did not improve weight gain, feed conversion or survival of shrimp. However, DGGE analysis suggested that the gut microbiota was affected by scFOS compared to shrimp fed the basal diet. However, the gut microbial community from shrimp fed the scFOS-supplemented diets was similar. In this study, most of the bacteria were either uncultured or unidentified to genus and species level, and most of the bacteria were related to those associated with marine sediments and biofilms or similar to intestinal bacteria previously reported in humans and rats.

In the recent report of Zhou et al. (2007) the authors showed that scFOS supplementation at concentrations from 0.04 to 0.16% improved specific growth rate and feed conversion of white shrimp cultured in a recirculating system, although survival was relatively low (42 – 61%) for all treatments. In this study, weight gain and survival were not significantly enhanced by scFOS supplementation. But concerning intestinal microbiota, does scFOS supplementation affect the gut bacterial community? Significant differences were observed in the counts of *V. parahemolyticus*, *A. hydrophila*, *Lactobacillus* sp. and *S. faecalis*. The count of *V. parahemolyticus*, was highest in the gut of fish fed 0.04 and 0.08% scFOS, while the population level of *S. faecalis* was highest when fish were fed the 0.12 and 0.16% scFOS.

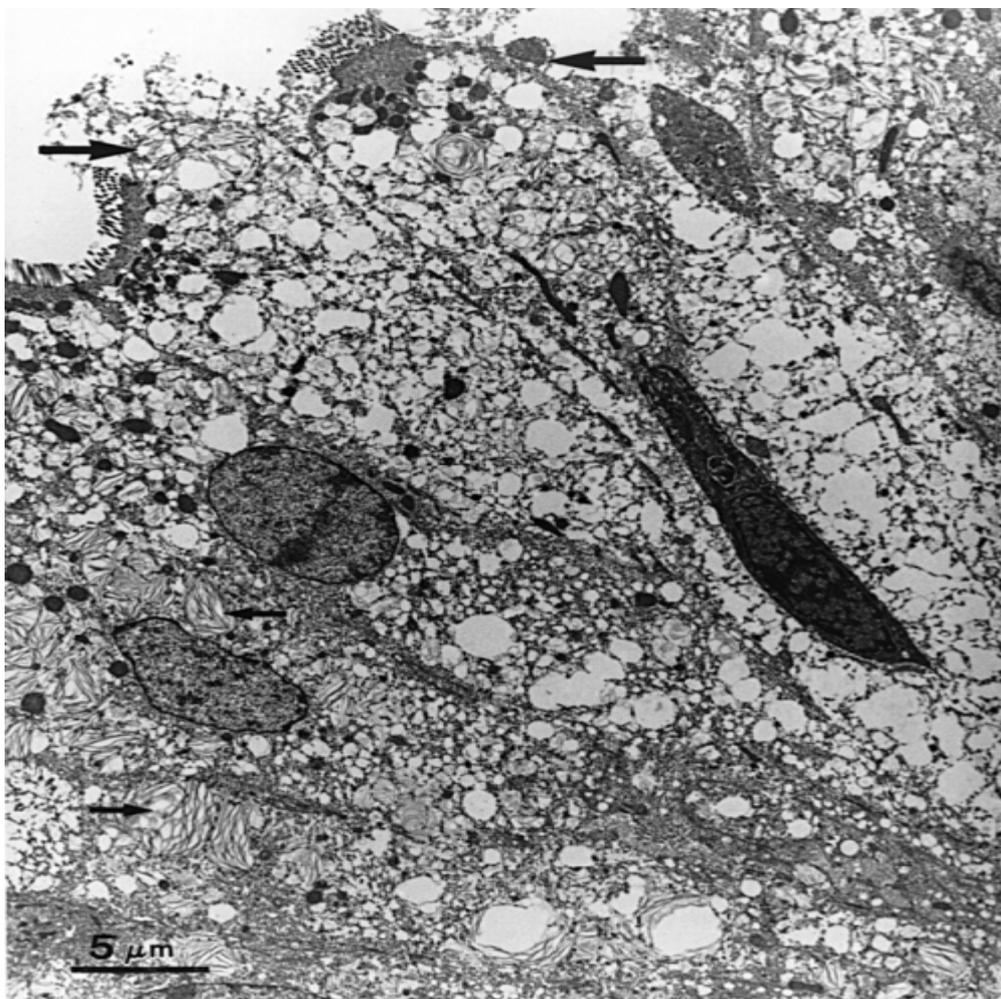
With regard to the latter investigations this illustrates the increased effort of the government of P.R. of China to seek new growth promoters and alternatives to antibiotics. However, from a bacteriological point of view it is of importance to evaluate whether the microbial shift has any positive effect on fish health. This has not been investigated directly by disease challenge trials.

#### **4.6.2.4. Use of light and electron microscopy**

In addition to the information available on prebiotics of the gut microbiota, papers have also been published investigating the effect of prebiotics on intestinal morphology in fish (Olsen et al. 2001; Bakke-McKellep et al., 2007; Yilmaz et al., 2007). In their early study on Arctic charr, Olsen and co-authors (2001) demonstrated that dietary inulin, a 15% supplementation, had a destructive effect on microvillous organization in the hindgut compared to fish fed control diet. The microvilli were often in disarray, lacking in some areas

and less straight than in the control animals. The presence of inulin-induced lamellar bodies (2.3% of cellular volume) dominated much of the cell interior, and inulin also caused an increase in vacuoles from 14.3% to 22.1% of cell volume in dextrin- and inulin-fed fish respectively (Figure 10.2.4.1). This study clearly showed the potential harmful effects of feeding high levels of inulin to Arctic charr. The damages to the enterocytes appeared to be linked to the accumulation of lamellar structures which may have been absorbed inulin. Based on their results the authors stated that inulin that cannot be degraded by the cells would accumulate to an extent that cell function would become impaired, and that the increase in lysosome-like structures in pyloric caeca probably reflects a cellular response to the accumulation of this indigestible component.

Figure 4.6.2.4.1 The epithelium in the hindgut of Arctic charr (*Salvelinus alpinus* L.) fish on an inulin diet. The cells are highly vacuolated and many of the vacuoles have a lamellar content (small arrows) which may be inulin. The apical surface of these cells shows signs of damage including loss of membrane and microvilli (large arrows). According to Olsen et al. (2001).



In the study by Bakke-McKellep et al. (2007), statistical analysis did not reveal significant effects of inulin inclusion in the diet on histological scores. However, the mid intestine of six of twelve inulin-fed fish showed moderate leucocytic cell infiltration of the muscular layers. Eleven of twelve fish fed the inulin diet showed normal morphology of the distal intestine, characterized by the presence of well-differentiated enterocytes with many absorptive vacuoles, though there appeared to be increased vacuolization in fish fed inulin. Inulin did not induce damage to the intestinal mucosa of the salmon as reported in Arctic

charr (Olsen et al. 2001), although Bakke-McKellep et al. (2007) noted a qualitative increase in the vacuolization of inulin-fed fish. The contrasting findings in Arctic charr may be due to different dietary levels of inulin, 15% vs. 7.5%, or differing analytic methods (transmission electron microscopy). Olsen et al. (2001) reported that 15% inclusion level of inulin did not cause histomorphological changes in the organization of microvilli and the presence of intracellular lamellar bodies in distal intestine enterocytes. Light microscopy does not allow sufficient resolution to evaluate these structures. However, Olsen et al. (2001) also reported increased vacuolization of distal intestine enterocytes (measured as percentage of cell volume). Therefore, the effect of inulin, including possible beneficial effects, in Atlantic salmon diets merits further study. The increased distal intestine somatic indices in inulin-fed fish appeared to be the result of hypertrophy of the muscularis externa. Greger (1999) reported similar hypertrophy of the caecal wall in rats fed inulin, possibly caused, as assessed by increased peristaltic activity light microscopy (Bakke-McKellep et al., 2007).

Yilmaz et al. (2007) investigated the effect of dietary mannan oligosaccharides (MOS) on growth, body composition and liver and foregut intestine histology of rainbow trout. The fish were fed four experimental diets supplemented with 0 (control), 1.5, 3.0, or 4.5 g MOS per kg. Improved growth performance was generally observed in fish fed the diet supplemented with 1.5 parts per thousand MOS. Intestinal villi of fish fed diets supplemented with 1.5 or 3.0 parts per thousand MOS were higher than those of fish fed 4.5 parts per thousand or no dietary MOS ( $p < 0.05$ ). The authors reported no significant differences in feed conversion ratio, protein efficiency ratio, or hepatosomatic index ( $p > 0.05$ ) and MOS had no detrimental effects on the intestinal morphology.

#### 4.6.2.5. Combined effect

Li and Gatlin (2004) evaluated a mixture of partially autolyzed brewers yeast, dairy ingredient components and dried fermentation products, and observed significantly enhanced feed efficiency of juvenile hybrid striped bass, although the dynamics of the intestinal microbiota was not defined in that study. Supplementation of this prebiotic also enhanced respiratory burst of head kidney leucocytes and resistance against *Streptococcus iniae* infection; however, the interpretation of these beneficial influences was complicated by the presence of brewers yeast, which is generally considered to be an immunostimulant for fishes (Siwicki et al., 1994; Ortuno et al., 2002; Li and Gatlin, 2003; Rodriguez et al., 2003). In 2005, Li and Gatlin reported that enhanced growth performance was generally observed in sub-adult hybrid striped bass fed diets supplemented with prebiotics or brewers yeast compared to fish fed the basal diet throughout the feeding trial with significantly ( $P < 0.05$ ) enhanced weight gain observed after 12 weeks of feeding. At the end of the feeding trial, fish fed 2% brewers yeast had significantly higher feed efficiency than fish fed the other diets. The *in situ* mycobacterial challenge employed in this experiment resulted in overall cumulative mortality of approximately 25%. Fish fed 2% prebiotics had a significantly ( $P < 0.05$ ) enhanced survival (80%) compared to the other treatments (72–73%) at the end of 21 weeks. It is concluded that dietary supplementation of 2% GroBiotic®-A showed moderate but significant ( $P < 0.05$ ) protection against mycobacterial infection. Dietary supplementation of partially autolyzed brewers yeast also may enhance growth performance under chronic infection of mycobacteria.

#### 4.6.2.6 Pro- and synbiotics

Microbial products, which are considered as alternatives to the prophylactic use of chemicals, are good candidates (Gatesupe, 2008). Probiotics are live microorganisms added to feed or rearing water that when administered to fish in adequate amounts, confer increase of viability, enhance immuno and digestive system, promote growth and general welfare. In

addition, the usage of microorganisms working on the nitrogen cycle will be beneficial for the environment as well.

Moreover, it is well established that the action of probiotics may be improved by specific prebiotics, nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of the microflora. A mixture of prebiotics and probiotics that beneficially affects the host by improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract by selectively stimulating the growth and/or by activating the metabolism of one or a limited number of health-promoting bacteria, and thus improving host welfare” was defined as synbiotics by Gibson and Roberfroid (1995).

#### 4.6.3. NUCLEOTIDES

Dietary nucleotides have attracted attention as a key ingredient missing from nutritional formulae for many years. They are the building blocks of tissue RNA and DNA and of ATP, and their presence in breast milk has stimulated research in babies which has indicated that supplementation of infant formula milk leads to improved growth and reduced susceptibility to infection (Gimble, 1996; Gimble and Westwood, 2001; Gutierrez-Castrellon et al., 2007). Nucleotide fortification of breast milk substitutes has been recommended to the U.S. Food and Drug Administration for approval (Aggett et al., 2003). There is increasing evidence that nucleotides administered intravenously or in the diet are capable of modifying immune responsiveness and recovery of organs that have undergone a metabolic or inflammatory insult.

It is generally accepted that nucleotides have essential physiological and biochemical functions including encoding and deciphering genetic information, mediating energy metabolism and cell signalling as well as serving as components of coenzymes, allosteric effectors and cellular agonists (Carver and Walker, 1995; Cosgrove, 1998). However, controversy has existed for many years over the roles of nucleotides administered exogenously. As neither overriding biochemical malfunctions nor classical signs of deficiency are developed in endothermic animal models, nucleotides have traditionally been considered to be non-essential nutrients. However, this opinion has been challenged by several research publications during the last decade which suggest that dietary nucleotide deficiency may impair liver, heart, intestine and immune functions (Gimble and Westwood, 2000). The modulatory effects of dietary nucleotides on lymphocyte maturation, activation and proliferation, macrophage phagocytosis, immunoglobulin responses, gut microbiota as well as genetic expression of certain cytokines have been reported in endothermic animals (Gil, 2002; Singhal et al., 2008). Nucleotide supplementation has been one important aspect of research on clinical nutrition and functional food development for humans (Gimble and Westwood, 2001; Gutierrez-Castrellon et al., 2007).

Although initial efforts in evaluation of dietary supplementation of nucleotides for fishes could be traced to the early 1970s, research at that time mainly focused on the possible chemo-attractive effects of these compounds (Mackie, 1973; Kiyohara et al., 1975; Mackie and Adron, 1978). However, the pioneer investigations by Burrels et al. (2001 a; 2001 b) resulted in increased attention on nucleotide supplementation for fishes as their studies indicated that dietary supplementation of nucleotides enhanced resistance of salmonids to viral, bacterial and parasitic infections as well as improved efficacy of vaccination and osmoregulation capacity. To date, research related to nucleotide nutrition in fishes has to some extent showed consistent and encouraging beneficial results in fish health management (Table 4.6.3.1), although most of the suggested explanations put forward by the authors remain hypothetical. Systematic research on fishes is therefore needed.

Table 4.6.3.1. Research on dietary supplementation of nucleotides on fishes.

Authors	Nucleotide form	Dose and/or feeding regime	Length of administration	Species	Initial size	Effect <sup>a</sup>
Ramadan and Atef (1991)	Ascogen S	2 and 5 g kg <sup>-1</sup> diet	16 weeks	Hybrid tilapia	21 days old	↑ Growth ↑ Survival
Ramadan et al. (1994)	Ascogen	5 g kg <sup>-1</sup> diet	120 days	Hybrid tilapia	30 days old	↑ Antibody titer after vaccination ↑ Mitogenic response of lymphocyte
Adamek et al. (1996)	Ascogen	0.62, 2.5 and 5 g kg <sup>-1</sup> diet at 1% bw day <sup>-1</sup>	37 days	Rainbow trout	163.4–169.7 g fish <sup>-1</sup>	↑ Growth
Burells et al. (2001a)	Optimûn	2 g kg <sup>-1</sup> diet, containing 0.03% NT, 2% bw day <sup>-1</sup>	3 weeks	Rainbow trout	217 ± 62 g	↑ Survival after challenge with <i>V. anguillarum</i>
		2 g kg <sup>-1</sup> diet, containing 0.03% NT, 1% bw day <sup>-1</sup>	2 weeks	Rainbow trout	53–55 g	↑ Survival after challenge with infectious salmon anaemia virus
		2 g kg <sup>-1</sup> diet, containing 0.03% NT, 2% bw day <sup>-1</sup>	3 weeks	Coho salmon	100 g	↑ Survival after challenge with <i>Piscirickettsia salmonis</i>
		2 g kg <sup>-1</sup> diet, containing 0.03% NT, 2% bw day <sup>-1</sup>	3 weeks	Atlantic salmon	60 g	↓ Sea lice infection
Burells et al. (2001 b)	Optimûn	2 g kg <sup>-1</sup> diet, containing 0.03% NT at 1.5% bw day <sup>-1</sup>	3 weeks before vaccination and 5 weeks post-vaccination	Atlantic salmon	34.7 ± 9.6 g	↑ Antibody titer ↓ Mortality
		2 g kg <sup>-1</sup> diet, containing 0.03% NT at 1.5% bw day <sup>-1</sup>	8 weeks	Atlantic salmon	43 ± 3.0 g	↓ Plasma chloride ↑ Growth
		2 g kg <sup>-1</sup> diet, containing 0.03% NT	10 weeks	Atlantic salmon	205 g	↑ Intestinal fold
Sakai et al. (2001)	Ribonuclease-digested yeast RNA	15 mg fish <sup>-1</sup> , by intubation	3 days	Common carp	100 g	↑ Phagocytosis ↑ Respiratory burst ↑ Complement ↑ lysozyme ↓ <i>A. hydrophila</i> infection
Lionardi	Optimûn	NA	120 days	“all-	80–100 g	↑ B lymphocytes

Authors	Nucleotide form	Dose and/or feeding regime	Length of administration	Species	Initial size	Effect <sup>a</sup>
et al. (2003)				female <sup>27</sup> rainbow trout		↑ Resistance to IPN virus ↓ Plasma cortisol
Low et al. (2003)	Optimûn	2 g kg <sup>-1</sup> diet, containing 0.03% NT to hand satiation daily	15 weeks	Turbot	120.9 ± 5.1 g	Altered immunogene expression in various tissues
Li et al. (2004 a; 2004 b)	Ascogen P	5 g kg <sup>-1</sup> diet, fixed ration approaching satiation daily	7 weeks	Hybrid striped bass	7.1; 9.1 g	↑ Neutrophil oxidative radical production. ↑ Survival after challenge with <i>S. iniae</i>
Li et al. (2005)	Optimûn	2 g kg <sup>-1</sup> diet	6 weeks	Red drum	1 g	Growth → Whole body composition → <i>In situ</i> challenge with <i>Amyloodinium ocellatum</i> →
Russo et al. (2006)	Aquagen	Oral	2 weeks	Shark	1.4 ± 0.2 g	↑ Resistance after challenge with <i>S. iniae</i>
Li et al. (2007 b)	Nucleotide mixture	4 g kg <sup>-1</sup> diet	5 weeks	Pacific white shrimp	ca. 0.83 g	↑ Growth
Li et al. (2007 c)	Nucleotide mixture	4 g kg <sup>-1</sup> diet	4 weeks	Red drum	10.2 ± 0.2 g	↑ Growth ↑ Neutrophil oxidative radical production ↑ Survival after challenge with <i>V. harveyi</i>

<sup>a</sup> - symbols represent an increase (↑), decrease (↓) or no change (→) in the specified response.

Because increasing concerns of antibiotic use have resulted in a ban on subtherapeutic antibiotic usage in Europe and the potential for a ban in the US and other countries (Patterson and Burkholder 2003), research on immunonutrition for aquatic animals is becoming increasingly important (Gatlin 2002). Research on nucleotide nutrition in fish and shrimp is needed to provide insights concerning interactions between nutrition and physiological responses as well as provide practical solutions to reduce basic risks from infectious diseases for the aquaculture industry. Devresse (2000) hypothesized that nucleotides are a key nutrient for the shrimp immune system and supplementation of nucleotides or other nucleic acid-rich ingredients such as yeast or yeast extract may enhance disease resistance and growth of shrimp. Although yeast products have been used in shrimp diet formulations, the role of yeast nucleotides remains largely unanswered. In their review, Li and Gatlin (2006) summarized and evaluated knowledge of nucleotide nutrition in fishes as compared with that of terrestrial animals.

The roles of nucleotides and metabolites in fish diets have been sparingly studied for nearly 20 years. Beside possible involvement in diet palatability, fish feeding behaviour and

biosynthesis of non-essential amino acids, exogenous nucleotides have shown promise most recently as dietary supplements to enhance immunity and disease resistance of fish produced in aquaculture. Research on dietary nucleotides in fishes has shown they may improve growth in early stages of development, enhance larval quality via broodstock fortification, alter intestinal structure, increase stress tolerance as well as modulate innate and adaptive immune responses. Fishes fed nucleotide-supplemented diets generally have shown enhanced resistance to viral, bacterial and parasitic infection. Despite occasional inconsistency in physiological responses, dietary supplementation of nucleotides has shown rather consistent beneficial influences on various fish species. Although nucleotide nutrition research in fishes is in its infancy and many fundamental questions remain unanswered, observations thus far support the contention that nucleotides are conditionally or semi-essential nutrients for fishes, and further exploration of dietary supplementation of nucleotides for application in fish culture is warranted. Hypothesized reasons associated with these beneficial effects include dietary provision of physiologically required levels of nucleotides due to limited synthetic capacity of certain tissues (e.g. lymphoid), inadequate energetic expenditure for *de novo* synthesis, immunoendocrine interactions and modulation of gene expression patterns.

#### 4.6.4. SUMMARY IMMUNOLOGY, PREBIOTICS AND NUCLEOTIDES

Use of immunostimulants is a potentially important approach for fish culturists as they assess various methods for controlling disease losses in their facilities. The immune response can be modulated by  $\beta$ -glucans and high-M-alginate.  $\beta$ -glucans are glucose polymers that are major structural components of the cell wall of yeast, fungi, and bacteria, but also of cereals like oat and barley. There is much structural variation in the  $\beta$ -glucans from these different sources, which may influence their physiological functions. Alginate is a polysaccharide composed of  $\beta$ -1,4-D-mannuronic acid (M) and  $\alpha$ -L-glucuronic acid (G). *In vitro* as well as *in vivo* studies in fish show that especially  $\beta$ -glucans derived from fungi and yeast and alginate have immune modulating properties. Most frequently evaluated are effects on macrophage activation and on lysozyme, respiratory burst and leukocyte activity, which have been suggested to contribute to the increased resistance against infections observed after immunostimulant exposure. Although more fish studies are needed, it is tempting to suggest that dietary  $\beta$ -glucans and alginate may be useful tools to prime the host immune system and increase resistance against invading pathogens. As no knowledge is available regarding short versus long-term effects and efficiency, more knowledge is needed on this topic.

To date, the application of pre- and probiotics for the improvement of aquatic environmental quality and for disease control in aquaculture seems to be promising; unfortunately, the information is limited and sometimes contradictory. Owing to these uncertain and incomplete results, there are still no standardized protocols to test the beneficial effects of these products and their impact on farmed fish welfare, growth and health status.

Currently there are numerous gaps in existing knowledge about exogenous nucleotide application to fish including various aspects of digestion, absorption, metabolism, and influences on various physiological responses, especially expression of immunogenes and modulation of immunoglobulin production. Additional information is also needed in regard to age/size-related responses and appropriate doses and timing of administration. Thus further research in these areas should be pursued.

#### 4.6.5. CONCLUSIONS AND RECOMMENDATIONS, IMMUNOLOGY, PREBIOTICS AND NUCLEOTIDES

##### Immunostimulants

It is generally accepted that immunostimulants used in fish experiments induce beneficial effects such as disease protection due to increased cellular and humoral responses. However, precautions have to be taken regarding issues such as tolerance, non-wanted side effects such as immunosuppression using too high doses of immunostimulants or non-desirable effects caused by a prolonged use of such compounds. In future, it is hoped that following the development of genomic and proteomic tools for several fish species, many issues with special attention to immune response polarisation after receptor binding of immunostimulants will be unveiled.

Possible effect of immunostimulants on potential probiotics ability to adhere to intestinal mucus should be given high priority in future studies. New and vital information on this topic is needed as the gastrointestinal tract is a potential port of entry for pathogenic bacteria.

##### Prebiotics

As there is limited knowledge of the anaerobic microbial community in the GI tract of various fish species, questions are raised: Is it possible to increase the population level of beneficial gut bacteria by prebiotic supplement, are the lactic acid bacteria beneficial to the fish and are *Bifidobacterium* present in the GI tract? Prebiotics have been reported to have numerous beneficial effects in terrestrial animals such as increased disease resistance and improved nutrient availability. If these types of responses are manifested in fishes, then prebiotics have much potential to increase the efficiency and sustainability of aquaculture production.

To fill the gaps in knowledge, future research needs to be performed in order to provide the aquaculture industry; the scientific community, the regulatory bodies and the general public with the necessary information and tools.

The concepts of synbiotics will provide scientific and technical support to implement productivity and quality of the farmed organisms, be beneficial to the consumers of farmed aquatic organisms and implement European policies for a sustainable and competitive development of aquaculture.

##### Nucleotides

Based on available knowledge and experience within nucleotides, demonstrating that nucleotide supplementation improves the composition of the gut microbiota in formula-fed infants should encourage bacteriologists working with fish to investigate interaction between nucleotides and gut microbiota. Furthermore, we recommend that the following topics should be given high priority in future; (1) in broodstock during oogenesis to improve fecundity, egg and larval quality, (2) during sea water transfer: feeding pre- and post- seawater transfer provides fish with a better osmoregulation and adaptation to marine environment, (3) combination with vaccines: feeding nucleotides during (pre- and post-) the vaccination period modulates the immune response and reduces the negative side effects caused by vaccines (e.g. growth suppression), (4) improved immune response and protection against pathogens such as; *V. anguillarum*, *A. salmonicida*, IPN, ISA, rickettsia-like bacteria, *Flavobacterium*, *Moritella viscosus* and (5) protection against sea lice and improved (higher) efficacy of pyrethroids when used combined with nucleotide feed.

#### 4.7. EFFECTS OF PROCESSING ON PLANT INGREDIENTS USED IN FISH FEEDS

Extrusion processing is the predominant method used for shaping fish feeds into particles with suitable shape and handling properties. The different feed ingredients except the oil fraction are ground in a hammer mill through a 1-mm sieve, and are then mixed. After mixing, the feed mash is subjected to a process termed conditioning. During conditioning, steam and hot water is added such that temperature of the mash increases to around 95 °C as it leaves the conditioner and enters the extruder (Strahm, 2000). The steam and water addition increases water content to around 25 to 30%. Retention time in the conditioner usually varies from 30 to 180 seconds. The conditioner may sometimes be pressurized. Immediately following the conditioning process, the feed mix is transported to an extruder. In the extruder, the feed material is pushed towards a die by one or two screws. Due to the small opening of the die, the internal pressure increases, and the friction and the heat applied causes temperature to increase to 115 to over 130°C (Aslaksen et al., 2007). Retention time in the extruder is usually between 15 and 20 seconds, but the time where maximum temperature is achieved may be less than 2 seconds. A knife will cut the feed into small pieces as it passes the die. Due to the sudden drop in pressure which causes the water to boil, the pellet will expand. Upon drying and cooling the pellet will obtain its hard and porous structure. Drying is performed using air with a temperature not exceeding 80-90°C, but with a target temperature close to that temperature level. The oil in the diet is added as warm oil with a temperature not exceeding 70 °C is applied to the pellets after extrusion. In this process, the fat is sprayed onto the pellet under vacuum, followed by slow release of air into the vacuum coater such that fat is pushed into the interior of the pellet.

Due to the heat and moisture applied, this process has a potential to affect chemical components in the feed. These changes may be beneficial in terms of inactivating substances which would otherwise be harmful, or they may be detrimental in terms of forming new compounds with harmful effects. Thus, the interaction between specific components in plant ingredients and processing may influence the physiological effects of these plant ingredients in the fish. The quantitatively largest plant component in feed for carnivorous fish is proteins. Functional effects of protein are usually associated with the tri-dimensional structure. Long chains of one or several protein molecules are folded into specific structures by a mixture of covalent and non-covalent bonds between different parts of the molecule and between molecules. At temperatures above 55 °C, the tri-dimensional structure of proteins will be altered through breakage of non-covalent bonds. Heat may therefore affect the functional properties of the proteins. Hence, harmful proteins such as enzyme inhibitors may be eliminated through feed processing. It has been shown that trypsin inhibitors in soy will to a large extent be eliminated through extrusion processing (Romarheim et al., 2005). Also, heat processing will potentially increase protein digestibility by rendering the proteins more available for enzyme hydrolysis. The temperature range for protein denaturation has been reported to vary between 55 and 108 °C (Damodaran, 2008). Thus, most pure proteins will be denatured during feed processing. Lectins are proteins with a carbohydrate residue covalently associated with the protein. Although the carbohydrate residue will render the protein more heat-stable, lectins will to a large extent become denatured during extrusion processing (McNaughton and Reece, 1980). Amino acid racemisation and protein cross-links may take place during processing. These heat-induced chemical changes may reduce protein digestibility, but will mainly be formed under conditions of high pH (Damodaran, 2008).

Fat is another component where plant ingredients may be a large source. Although the fatty acid composition will differ between plant fat and fish fat, the fundamental effect of processing of the fat will be similar. Processing is not considered to affect fat digestibility to

any large degree, since fats are not easily chemically altered by heat. However, small amounts of potentially harmful substances, such as cyclic fatty acids, may be formed during prolonged heating as mentioned earlier. It is uncertain whether extrusion processing will be able to form such substances, although this seems unlikely given the moderate temperatures and the short retention time.

Fibre is a component strictly associated with plant material. The use of plant ingredients will therefore inevitably result in fibre being introduced into fish feeds. There is a vast number of different fibre types with varying physiochemical properties which may influence physiological effects. Fibres will mainly affect the gut environment, since these fibres are not digestible and are too large to pass the cell barrier of the gut. One of the most significant effects of fibre is the increased viscosity of gut contents with some types of soluble fibres. The increased viscosity will potentially reduce nutrient digestion and absorption, resulting in reduced digestibility of nutrients. This may cause digestive disturbances, and although the significance is not fully revealed, may cause imbalance in the gut microflora of fish. It has been shown that solubility of fibres may increase with processing (Zimonja et al., 2008). Thus, plant fibres may increase their antinutritive properties as a consequence of extrusion processing. Soluble fibres with antinutritive properties are mainly found in fibre-rich cereals such as rye, oats and barley, although wheat may also exhibit antinutritive properties caused by soluble fibres. Since small quantities of these cereals are used in fish feeds, soluble fibre does not appear to be a major problem.

Processing aids may be used to enhance physical quality of the feed. These aids are not very commonly used in extruded feeds for fish, but may include fibrous viscous components and enzymes. Fibrous components may have detrimental effects as described for fibres above, while the enzymes added to aid flow are not likely to have any negative effects due to the fact that they do not hydrolyse feed components into harmful substances, e.g. amylase.

Plant ingredients may contain a number of novel components. As discussed above, some of these may be harmful for fish. The question then arises whether extrusion processing may eliminate these substances in a similar way as discussed above for proteins. Most of these components are rather small substances where the physiological effect is not associated with tri-dimensional structure caused non-covalent bonds. Thus, extrusion processing will generally have only small effects on substances such as tannins, phytic acids, gossypols and glucosinolates.

Harmful substances may be formed due to processing. Among the most common are covalent bonds formed between proteins and other substances in the feed. As the protein is denatured, hydrophobic groups are rendered uncovered, resulting in a decreased aquatic solubility of the protein (Camire et al., 1990). Even though extrusion is considered a lenient process, due to the high moisture content and short retention time, it may induce a great deal of protein modification. Such modifications can be covalent (such as fragmentation, hydrolysis, deamidation, imide formation, isomerization, racemization, transpeptidation, disulfide scission/reduction/formation, thiol-disulfide exchange, oxidation and aggregation) as well as non-covalent (such as denaturation, misfolding, adsorption, aggregation and precipitation). Certain amino acids may also react with aldehydic (reducing) sugars, producing a various range of different Maillard browning reactions (Dworschak, 1980; Friedman et al., 1984). Several of these abundantly occurring modifications are detrimental for protein digestibility, especially if the protein sources have been through previous heat treatments before extrusion. The Maillard reaction is a reaction between an amino acid, typically lysine, and a reducing sugar. Maillard product formation is not considered to be a major problem during extrusion processing, but may be formed as a result of pretreatments of plant ingredients under conditions of high temperature and moderate to low water content

(Sikorski et al., 2008). Maillard products will mainly be a problem due to their negative effect on protein digestibility. However, Maillard products may be involved in further reactions which produce other harmful substances. In plant products, formation of acrylamide appears to be the main concern (Ho et al., 2008). Other covalent bonds may also be formed. These include isopeptide bonds formed between different amino acids. The extent to which these are formed in common feed processes has not been extensively studied, but it is likely that the negative effects are mainly associated with reduced protein digestibility.

Apart from extrusion processing when mixing and shaping complete diets, plant feed ingredients may also be processed prior to this step. This usually occurs before the ingredient is purchased by the fish feed producer. Thus, properties of these products may be considered a property of the commercial product. The most relevant example is proteins derived from oil-rich plants. These products are usually heated as a part of the oil extraction process, but the temperature is usually kept below levels known to result in significant production of harmful substances. The process is however known to affect the quality of the product. Enzyme inhibitors in soy beans, for example, are to a large extent eliminated.

In conclusion, heat processing of raw materials and of the complete fish diets may potentially alter nutritional properties of plant materials. However, the negative effects appear to be modest under practical conditions.

#### **4.7.1. PROCESSING AND ANTINUTRITIONAL FACTORS**

The utilization of many plant feedstuffs is limited due to the presence of antinutritional factors. Among these are phytates, polyphenols, and enzyme inhibitors (Abd El-Hady and Habiba, 2003). Therefore, manipulation of processing conditions may be required to remove or reduce certain unwanted components (Alonso et al., 2000). Attempts to increase the utilization of plant feedstuffs have employed a wide range of processing techniques such as soaking, boiling, autoclaving, radiation, cooking, roasting, dehulling, germination, fermentation, supplementation with various chemicals and enzymes and recently extrusion cooking (Van der Poel, 1990; Gujska & Khan, 1991; Bishnoi & Khetarpaul, 1994; Fernandez et al., 1997; Alonso et al., 1998; Alonso, Aguirre, & Marzo, 2000a). Extrusion cooking has advantages including versatility, high productivity, low operating costs, energy efficiency and shorter cooking times. Extrusion cooking application to legume processing has developed quickly during the last decade, and can now be considered a technology in its own right. Legume extrusion cooking would allow reduction of antinutritional factors and therefore improve the nutritional quality at a cost lower than other heating systems (baking, autoclaving, etc.) due to a more efficient use of energy and better process control with greater production capacities (Reimerdes, 1990; Alonso et al., 1998; Quintana, Canovas, Morales, Morales, & Ramos, 1998; Alonso, Grant, Dewey, & Marzo, 2000b).

The content of antinutritional factors varies among varieties and crops of plants. For instance, modern varieties of rapeseeds and cottonseeds are bred to contain low levels of glucosinolates and gossypol, respectively. The glucosinolate content of bred rapeseed varieties is usually below 200 µg/g, which should be tolerated by salmonid fish. Glandless cottonseeds contain essentially no gossypol, but the culture of such varieties is limited. Soybeans are bred to contain lower levels of, and less potent, proteinase inhibitors and agglutinating lectins. Noteworthy, soy cultivars already exist with low inherent levels of these antinutritional factors (Han et al., 1991; Douglas et al., 1999). Such criteria are important when selecting plant seeds for salmonid diets.

When practically feasible, the recommended method for removal of condensed tannins is dehulling to remove the tannin rich seed coat. This also reduced the level of cellulose and lignin, which are the main constituents of seed coats. Certain seeds such as rapeseeds are, however, too small for efficient dehulling. In this situation, the tannin level may be reduced

by thermal treatments such as autoclaving (Griffiths, 1991) or extrusion (Mukhopadhyay et al., 2007). Tannins may also to some extent be removed by alkali treatment (Griffiths, 1991) or lactic acid fermentation (Mukhopadhyay and Ray (1999a).

Moderate heating of soybeans reduces the soy TI and lectin activities to levels tolerated by salmonids. Thermal denaturation and inactivation of proteinase inhibitors and agglutinating lectins requires moist heat (McNaughton and Reece, 1980). Defatted soy flakes are typically toasted (steam-cooked) at 105 °C for 30 minutes to remove solvent residues after the oil extraction procedure. This reduces the TI activity down to levels tolerable by salmonids. It is also paralleled by denaturation and inactivation of the lectins (Maenz et al., 1999). Furthermore, soybean meals in modern salmonid diets are subjected to a second moist heating during high-pressure moist extrusion manufacturing of the diets. Thus, proteinase inhibitors and lectins are rarely problematic when using defatted soybean meal in salmonid feeds. They may, however, cause problems when producing feeds from insufficiently toasted full-fat soy and certain unheated beans.

Isoflavones, saponins,  $\alpha$ -galactoside oligosaccharides, soluble non-starch polysaccharides (NSP), and factor(s) inducing distal enteritis in salmonids are all soluble in alcohol. Thus, such antinutritional factors are removed by alcohol washing when manufacturing plant protein concentrates.

Fermentation of raw grains yields a significant reduction in concentration of both phytic acid (Marklinder et al., 1996; Mukhopadhyay and Ray, 1999b; Skrede et al., 2002) and  $\alpha$ -galactoside oligosaccharide (Skrede et al., 2001; Refstie et al., 2005).

In conclusion, processing of raw materials and of the complete fish diets may potentially alter nutritional properties of plant materials. Processing, particularly of raw materials, may remove or inactivate harmful substances. Extrusion processing has the potential to induce chemical changes which may result in the appearance of harmful substances. However, the negative effects appear to be modest under practical conditions.

## **4.8. PATHOLOGIES LINKED TO FEED**

Like other developed husbandry, modern finfish aquaculture faces problems such as bone and skeletal deformities, cataracts, heart disorders, unspecific ulceration (Vågsholm & Djupevik 1998; Wall 1998; Kvellestad et al. 2000; Farrel 2002; Waagbø et al. 2005; Bjerås et al 2006; Seierstad 2008; Waagbø 2008), various digestive disorders (A.M. Bakke, personal communication) including intestinal colic in Atlantic cod (National Veterinary Institute, 2008), gastric dilatation (bloat) in rainbow trout (Anderson, 2006; Baeverfjord et al., 2006), and intestinal tumours (low incidence) in Atlantic salmon broodstock (National Veterinary Institute, 2008). The mentioned problems have been related to malnutrition, feed, intensive growth and/or unfavorable environmental conditions. 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 increase the risk for such production-related disorders in intensive fish farming. Below is a description of the disorders that have been characterized to some extent – cataracts, skeletal deformities and heart disorders.

### **4.8.1. CATARACTS**

Significant occurrence of permanent (irreversible) bilateral cataracts (opacity of the lens or its capsule) have been observed in farmed Atlantic salmon during the last decade, lately more recurrently and with varying incidences (Wall 1998; Midlyng *et al.* 1999; Breck 2004; Bjerås *et al.* 2006). There is a risk of nutritional related cataract development in modern intensive aquaculture following major changes in both feed technology and composition, leading to variation in nutrient digestibility, nutrient content and requirements,

which finally may lead to suboptimal lens nutrition. Serious outbreaks of cataracts in farmed Atlantic salmon in Europe were associated with the omission of blood meal in salmon feeds onwards 1995 (Wall 1998). Blood meal is a rich source of the amino acid histidine. Leaving out blood meal as feed ingredient seems to uncover critical roles of histidine in the Atlantic salmon, especially in lens cell volume regulation (Breck et al 2003; Breck 2004; Bjerkås et al 2006). The histidine level required to prevent cataracts in Atlantic salmon smolt was found to be in excess of the established minimum requirement for growth (NRC 1993).

Several nutritional related cataracts have appeared as a consequence of introducing novel feed ingredients in fish feeds, such as “spleen and liver cataracts”, “white fish meal cataract”, or “rancid low quality feed cataracts”, reflecting nutrient deficiencies, reduced nutrient availability and oxidative challenges, respectively (Roberts 2002; Ketola 1979; Tacon 1992). Animal sources of many vitamins, such as riboflavin (Cooperman & Lopez 1991), are better absorbed and hence more available than plant sources. Waagbø *et al.* (2003) demonstrated that cataract development in Atlantic salmon could be influenced by dietary means, where high levels of nutrients of pro-oxidant nature (dietary lipid, iron, copper and manganese levels) increased, while excess of antioxidant nutrients (vitamin E, vitamin C and astaxanthin) alleviated the cataract incidence. This is consistent with the hypothesis of prevention of oxidative cataracts by antioxidant nutrition in humans (Bourgeois 2003). The natural concentrations of nutrients among feed ingredients differ largely, and for carnivorous fish, animal ingredient composition is often superior to plant ingredients. Thus, care should be taken to avoid risks for cataracts in searching for and using plant feed ingredients.

In a recent study it was demonstrated that dietary lipid sources affected cataract development in farmed Atlantic salmon adults in sea (Waagbø et al 2004). Fish fed a diet coated with a blend of plant oils (palm oil, linseed oil and rapeseed oil) grew equally from start-feeding and until 3 kg body weight, but developed more severe cataract than fish fed the same diet added marine oil. The dietary lipids altered lens fatty acid composition, which may have affected lens osmoregulation or oxidative status, and thereby cataractogenesis. However, potential impact of plant contaminants could not be excluded. Later studies have showed diverging results, supporting a multifactorial cause relationship for the cataract development, with temperature variation as a confounding factor.

#### **4.8.2. SKELETAL DEFORMITIES**

Bone deformities in juvenile and adult fish are periodically observed with high prevalence in intensive aquaculture, and are also regarded as disorders of multidisciplinary origin (Vågsholm and Djupevik 1998; Cahu et al. 2003; Waagbø et al. 2005; Waagbø 2008). Several nutrients in deficiency and excess cause bone disorders, of which vitamin C deficiency probably has caused the largest losses in the aquaculture history (Dabrowski 2001). From a nutritional point of view, both development and maintenance of the bone tissue can be affected, and may include impairments in bone cell differentiation and function, matrix composition and bone tissue mineralization (Huysseune 2000). Recent research shows that early developmental periods in fish seem to be most sensitive to nutritional related developmental bone disorders (like vitamin A and lipid in European sea bass and vitamin A in Atlantic salmon), mainly at the level of gene regulation of bone morphogenesis (Ørnsrud et al., 2004a; 2004 b; Villeneuve et al., 2005 a; 2005 b; 2006). For fish growers, however, imbalanced mineral nutrition is related to development of bone disorders, with dietary phosphorous (P) as the main element (Shearer and Hardy 1997). Recently, Fjellidal *et al* (accepted) showed that dietary P above established recommendations was needed to reduce incidences of vertebrae deformities in fast growing Atlantic salmon under-yearling smolt in sea, and that the deformities were related to reduced vertebrate mechanical strength and bone mineralization. In plant meals, P often occurs as phytate-P, with low bioavailability in fish.

The indigestible parts of plant meals may also bind and chelate dietary minerals and reduce their bioavailability, with increased risk for suboptimal P supply.

In relation to bone deformities the role of vitamin D and its active metabolites in calcium and P regulation of fishes have been discussed, especially potential toxic effects of excess dietary vitamin D from marine ingredients on bone health (Graff 2002). While plant ingredients contain minimal vitamin D levels, inherent vitamin D from a minor inclusion of marine ingredients will cover the vitamin D requirement in feeds based on plant ingredients.

#### **4.8.3. HEART DISORDERS**

It is well documented that fish develop various heart disorders including lesions and arteriosclerosis, both in the wild and in farms (Seierstad et al. 2008; Poppe et al. 2007; Toerud et al. 2006; Ferguson et al. 2005; Kongtorp et al. 2006; Kongtorp et al. 2004b; Kongtorp et al. 2004a; Farrell 2002). In some cases, these conditions are part of the disease progression, as with sleeping disease (Graham et al. 2006) and pancreas disease (Taksdal et al. 2007). In other cases, the aetiology is uncertain and although diseases are sometimes suspected, as with heart and skeletal muscle inflammation disease (Kongtorp et al. 2006), it is now being accepted that many of these conditions are “facts of life”. For example, the development of arteriosclerosis of the coronary artery seems to increase gradually with time (Seierstad et al. 2008; Poppe et al. 2007; Saunders et al. 1992).

The dietary correlation to the progression of heart disorders is less well documented in fish, and there is particularly little information linking these conditions to feeding high levels of plant oils. A possible connection was suggested by Bell et al. (Bell et al. 1991; Bell et al. 1993) when they found a marked increase in heart lesions (thinning of the ventricular wall and muscle necrosis) in Atlantic salmon fed high levels of sunflower oil. However, these conditions have not been reproduced in other feeding studies, some of which were as extreme and long-term as the original study (Grisdale-Helland et al. 2002; Saunders et al. 1992). The reason for these discrepancies is not known. The development of arteriosclerosis has also been suggested to relate to dietary lipid. This became particularly important as a short-term feeding study (5 months) gave indications of higher frequencies in Atlantic salmon fed rapeseed oil enriched diets (Seierstad et al. 2005). However, in a later study covering the entire lifespan of the fish, a plant oil blend diet (rapeseed, palm and linseed oil) did not affect the frequency of arteriosclerosis (Seierstad et al. 2008).

It does, however, appear likely that dietary lipids may affect heart function. Although not extensively studied, there are indications that metabolic rates, swimming performance and heart function may be altered by dietary lipid sources (McKenzie 2001; McKenzie et al. 1998b; Agnisola et al. 1996; Chatelier et al. 2006; McKenzie et al. 1998a; Wagner et al. 2004). However, results are inconclusive and only performed using a few mixtures of oils. But further work in this area is warranted.

## 5. RISK CHARACTERIZATION / CONCLUSIONS

*The Norwegian Food Safety Authority asked the Scientific Committee for Food Safety, Panel 6, to assess criteria to be used when evaluating plant ingredients to be used in fish feed, so that these fulfil the Feed regulative §7 to “not induce health damages to the animal”, and in this context aquacultured fish.*

*Further requested in particular was to identify plant ingredients which might induce long-term negative effects affecting fish health and should therefore be recommended limited. “Long-term” is meant to include substances that might affect the fish health beyond normal production time for consumption, e.g. when included in broodstock diets.*

Limited information exists on the interactive effects when exchanging large parts of the marine feed ingredients, both the fishmeal and the fish oil, in diets for Atlantic salmon, rainbow trout, cod and halibut. Very few, if any, results from long-term experiments exist. Most data report production and health results when single ingredients, immunostimulants, or single nutrients have been in focus, and are mostly based on studies of short duration, from weeks to a few months. Based on these data, however, which are (mostly) published in peer-referee journals, we have given the background for the answers to terms of reference in the present report. Ongoing at the moment are studies where several plant ingredients are utilized in the diet, to maximize plant ingredients without compromising nutrient needs. Preliminary data show that unexpected results occur on e.g. lipid metabolism due to simultaneous changes in both the lipid and protein part of the diet. The use of plant ingredients in aquafeeds will expose the fish to various antinutritional factors. With their effects on feed intake, digestive processes and possibly metabolism, this will change the recommended levels of various nutrients that need to be present in aquafeeds. More research in this area is needed. Furthermore, combinations of various plant ingredients may also give rise to combinations of antinutritional factors/plant components, which can have detrimental effects. Whether or not this affects fish health in a different manner than when single components are tested individually remains to be clarified. Some plant ingredients have however been used for several decades due to their starch contents (energy) and binding properties, especially grinded (and extruded) wheat and maize. So far, no detrimental effects from these additions have been reported. However, new data are needed when new ingredients or higher levels of plant proteins are added in addition, as these also add to the starch level in the diets. Studies on Atlantic salmon, rainbow trout, cod and halibut have all shown a limitation to utilize high dietary starch levels.

It is a prerequisite that the use of feed ingredients of plant origin complies with Norwegian and European feed legislation, which aims to ensure good fish health and consumer safety. The feed ingredients must comply with current maximum levels set for a range of undesirable substances. The list of undesirable substances is, in general, sufficient. However, as feed production is a rapidly developing area, with new feed ingredients being introduced frequently, the legislation must develop accordingly to ensure fish health and consumer safety. With the increased use of feed material of plant origin in feed for fish, the list of pesticides and mycotoxins included in the current legislation ought to be revised.

### ***In particular the risk assessment should address the following issues:***

*Assess if plant ingredients contain specific protein types or protein fractions that should be limited in fish diets, and identify these.*

When considering the nutritional quality of proteins in plant ingredients, the amino acid balance of the protein fraction is of importance, as is whether the amount of any amino acids may be limiting relative to the nutritional needs of the fish. Most proteins in plant ingredients are limited in certain essential amino acids, most commonly lysine and

methionine. Therefore, purified amino acids may need to be added to feeds containing high levels of proteins from plant. On the other hand, other amino acids may be overabundant in plant ingredients and may lead to imbalances in amino acid availability or even toxicities.

Furthermore, in mammals many plant ingredients contain proteins/peptides (protein fractions) that may elicit a hypersensitivity reaction in sensitized individuals. Generally, antigens are short peptides that may be inherent in the food/feed ingredient or they may be a product of protein digestion. For salmonids, components of soybeans appear to cause an inflammatory response in the distal intestine in all individuals that consume SBM-containing diets. Although T cells appear to be involved in the reaction, it is not clear whether the inflammation is caused by proteins/peptides, antinutrients, pathogens (bacteria, virus etc.), or combinations of these. Nor is it clear whether it can be classified as a hypersensitivity reaction since many characteristics of fishes' immune system and its responses are as yet unknown. For example, granules of mast cells in most fish species do not contain histamine and it is therefore questionable whether fish can react allergically to allergens.

*Identify anti-nutrients in plants that are already in use or are planned to be used, and assess to what extent the various plant ingredients can be tolerated by the fish.*

Antinutrients are defined as innate components of a food/feed ingredient that have a limiting effect on the food/feed intake, digestion, and/or nutrient absorption. This report lists and describes antinutrients present in plant ingredients that may be considered appropriate for use in formulated feeds for farmed fish. The authors consider saponins, proteinase inhibitors, and lectins to be the most potentially deleterious to fish health. Tolerance levels for the various known antinutrients and thus for inclusion levels for the plant ingredients have been identified in the report when sufficient data is available. Many studies indicate that inclusion levels of more than 20% of the total diet, of a specific plant ingredient, can have consequences to fish growth and nutrient digestibility, and therefore potentially to fish health. Thus as a general rule of thumb, an upper limit of about 20% inclusion level of total diet for most plant ingredients in feeds for carnivorous fish species may appear to be applicable. An exception is full-fat and extracted soybean meal for both Atlantic salmon and rainbow trout, which should be limited to approximately 10 and 5% inclusion levels, respectively, of total diet. However, cod and halibut appear to tolerate these soybean products well, but the exact tolerance level is impossible to state.

However, long-term effects of plant ingredients and/or their isolated antinutrients have generally not been conducted. Nor is there sufficient data on effects of combinations of antinutrients/plant ingredients. Further research is needed to identify long-term effects on both individual and combinations of antinutrients as well as to further characterize plant feedstuffs to identify other possible components that may be deleterious to fish health. Until more information is available, only highly processed plant ingredients should be used. Especially plant ingredients processed using heat in combination with enzyme/fermentation treatment and/or alcohol extraction appear to be well tolerated by carnivorous fish.

*Assess interactions between anti-nutrients, and how such interactions should be considered when plant ingredients are to be used in diets for various aquacultured fish species.*

Recent data indicate that interactions between antinutrients may be of more practical importance to fish/animal health than formerly appreciated. Of particular interest are apparently deleterious interactions between saponins and other antinutrients, as well as between lectins and proteinase inhibitors. Such interactions appear to dramatically lower the tolerance of even individual feed ingredients, as exemplified by soybean meal's effects in salmonids. Research is needed to study other possible interactions and the mechanisms involved in the potentiating effect such interactions may have. A valuable tool to boost

efficiency in procuring knowledge of both individual and combined effects would be for researchers and the industry to increase their efforts in identifying and quantifying levels of antinutrients in the plant ingredients as well as in the feeds. In this way it may also be possible to link practical fish health issues with antinutrient exposure over the longer term.

The use of *in vitro* methods may be helpful initially to screen for effects of such interactions on tissue/cell function and viability, especially for antinutrients and combinations of antinutrients that are difficult to isolate and therefore expensive. Ultimately, however, *in vivo* trials are needed to confirm the *in vitro* data as well as assess effects on the whole animal and its health, especially in the long term.

*Determine whether the use of plant ingredients with high fibre contents should be limited in fish diets.*

Fibres are polymers made up of monosaccharides. They vary in their solubility in water, size and molecular structure. Dietary fibres alter flow, impair interactions, affect intestinal receptors, restrict nutrient diffusion, change microbial diversity and activities, and change absorptive surfaces. The variability of the compounds belonging to the fibre complex and the varying degrees that they affect these parameters makes it difficult to conclude on a general basis regarding maximum inclusion level. Little is known on their specific effects in fish since few experiments have been conducted with isolated fibre in the diets. No data from long-term studies exist describing fibre effects on fish health, with the possible exception of diets high in krill meal (high chitin contents). As these are marine ingredients and not derived from alternative plant ingredients these are not included in the present report.

*Assess if plant lipids should be limited in fish diets.*

Addition of medium chain triglycerides (MCT) should be performed with caution. Maximum 1-3% of total diet in larval fish, but little data exist on cod, halibut and salmonid fish. In larger fish, both Atlantic salmon and rainbow trout, a cautionary limit of 10% of total diet seems safe as does 5% of total diet in cod. But this also depends on type of MCT used.

Fish may have an absolute requirement for phospholipids (mainly phosphatidylcholine) but exact figures are lacking. Dietary inclusion of 2-6% of total diet for larval fish and 1-3% of total diet for larger fish could be considered safe.

In marine fish addition of plant oils must not reduce the level of marine essential fatty acids below minimum requirement levels. The actual level of inclusion will thus depend on type of marine lipid used in the diets. Actual essential fatty acid levels for Atlantic cod and Atlantic halibut is not known but rough estimates exist. Broodstocks may have higher requirements for some marine PUFAs, including arachidonic acid (ARA).

In freshwater fish 100% replacement of fish oil with plant oil is possible as these fish produce their essential fatty acids (marine) from 18:2n-6/18:3n-3 with some question marks for DHA. Essential fatty acid requirements are assumed to be in the range 0.5-1.5% of total diet depending on type of fatty acid and size of fish. Broodstocks may have higher requirements for some marine HUFA, including ARA.

Plant oils may alter eicosanoid cascade, immunology and stress responses in salmonid fish, particularly with high alterations in the n-6/n-3 ratio from the typical “marine” ratio. At present, a higher inclusion level of than 50% plant oils is not recommended.

Using mixtures of different plant oils (e.g. soybean, linseed, palm oil) producing a “marine” ratio n-6/n-3 may lower the problems and may increase safe inclusion levels above the recommended 50% of the dietary lipid.

Oxidative stress is not a general problem, with the possible exception of linseed oil. An upper inclusion level of 25% of total dietary lipid is suggested, but there are few data available.

*Assess whether feed ingredients containing glucans, nucleotides or other potent molecules, added due to their immunostimulatory effects, should be limited in diets for aquacultured fish species.*

As limited knowledge is available about potentially negative effects of dietary  $\beta$ -glucans, alginate, nucleotides or other potent molecules, especially with respect to long-term effects on fish health and efficiency, more knowledge is needed on this topic before it is possible to recommend any inclusion level and/or recommend any limitations in fish feed.

More effort on challenge studies, both *in vivo* and *in vitro*, should be given high priority in the future studies.

As limited information is available about the effect of immunostimulants, prebiotic and nucleotides on gut morphology, these topics should be given high priority in future studies. This is of high relevance as the gastrointestinal tract of fish is one of the major infection routes.

As only some information is available about the effect of nucleotides on growth, gut function structure and whether toxification is possible, we are not able to conclude on any limitations or recommended inclusions. Studies on this topic have to be carried out in future.

Interactive effects of immunostimulants and anti-nutritional factors in plants may exist but there is a total lack of knowledge, and no recommendation is possible to make on e.g. plant-based diets and additions of immunostimulants. However, as mentioned in the background for this risk assessment, several of the anti-nutritional factors possess, to a certain degree, some immunostimulatory effects.

*Assess if processing methods, including the use of processing aids, could influence the ingredient to such an extent that the processing aid might be a risk factor for the aquacultured fish species.*

Extrusion processing is the predominant method used for shaping fish feeds into particles with suitable shape and handling properties. Due to the heat and moisture applied, this process has a potential to affect chemical components in the feed. These changes may be beneficial in terms of inactivating substances which would otherwise be harmful, or they may be detrimental in terms of forming new compounds with harmful effects. At temperatures above 55 °C, the tri-dimensional structure of proteins will be altered through breakage of non-covalent bonds. Heat may therefore affect the functional properties of the proteins. Hence, harmful proteins such as enzyme inhibitors may be eliminated through feed processing. Amino acid racemisation and protein cross-links may take place during processing. These heat-induced chemical changes may reduce protein digestibility, but will mainly be formed under conditions of high pH.

Fibre is a component strictly associated with plant material. The use of plant ingredients will therefore inevitably result in fibre being introduced into fish feeds. It has been shown that solubility of fibres may increase with processing. Thus, plant fibres may increase their antinutritive properties as a consequence of extrusion processing. Soluble fibres with antinutritive properties are mainly found in fibre-rich cereals such as rye, oats and barley, although wheat may also exhibit antinutritive properties caused by soluble fibres. Since small quantities of these cereals are used in fish feeds, soluble fibre does not appear to be a major problem.

Processing aids are components that are primarily added to the feed mix prior to processing to facilitate processing and/or enhance physical quality of the feed. Such aids are not very commonly used in extruded feeds for fish, but may include fibrous viscous components and enzymes. Fibrous components may have detrimental effects as described for

fibres above, while the enzymes added to aid flow are not likely to have any negative effects due to the fact that they do not hydrolyse feed components into harmful substances, e.g. amylase.

In conclusion, processing of diets for fish may potentially alter nutritional properties of plant materials. Processing, particularly of raw materials, may remove or inactivate harmful substances. Extrusion processing has the potential to induce chemical changes which may result in the appearance of harmful substances. However, the negative effects appear to be modest under practical conditions.

## 6. CHALLENGES (GAPS OF KNOWLEDGE)

As seen throughout the present assessment, most results have been obtained from short-term studies which have focused on single dietary ingredients or even single components specific for the ingredients. Very few studies have been performed to evaluate the long-term effects, as questioned in the present assessment. There is accordingly, an urgent need for studies investigating long-term effects and combined effects in fish when using feed plant ingredients (both plant proteins and plant lipids). The consequence not only for the fish itself, but for the final product quality, e.g. changes in nutrient composition, is also important.

### *In particular:*

For the plant proteins new data are needed when new ingredients or higher levels of plant proteins are added.

Studies of the long-term effects of plant ingredients and/or their isolated antinutrients have generally not been conducted. Nor is there sufficient data on the effects of combinations of antinutrients/plant ingredients. Further research is needed to identify long-term effects of both individual antinutrients and combinations thereof, as well as to further characterize plant feedstuffs to identify other possible components that may be deleterious to fish health.

The use of *in vitro* methods may be helpful to initially screen for effects of such interactions on tissue/cell functions and viability, especially for antinutrients and combinations of antinutrients that are difficult to isolate and therefore expensive. Ultimately, however, *in vivo* trials are needed to confirm the *in vitro* data as well as to assess the effects on the whole animal and its health, especially in the long-term studies.

Actual essential fatty acid requirements for Atlantic cod and Atlantic halibut are unknown and only rough estimates exist for practical diets for salmon and trout. There is only fragmentary knowledge on the long-term effects of the different plant lipids on fish health and welfare. There is also a general lack of knowledge on the effects of dietary interactions with other feed components, e.g. various oil sources, lipid-protein interactions, and lipid-vitamin interactions.

For the immunostimulants more efforts on challenge studies, both *in vivo* and *in vitro*, should be given high priority in future studies. The use of short-time studies is insufficient as a basis to conclude on any limitations or recommended inclusions. Studies on these topics have to be carried out in future.

Interactive effects of immunostimulants and anti-nutritional factors in plants may exist but knowledge is lacking, and no recommendation is possible to make on e.g. plant-based diets and additions of immunostimulants.

As feed production is a rapidly developing area, with new feed ingredients being introduced frequently, the legislation must develop accordingly to ensure fish health and consumer safety. Revisions of the current legislation must be based on risk assessments, which rely on scientific data. Today such data are scarce, and there is an urgent need for

studies on known and emerging undesirable substances in feed ingredients of plant origin. Such studies include a characterization of their occurrence, their effects in fish, and the possible transfer of the compounds from feed to fish fillet.

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## 8. APPENDIX (IN NORWEGIAN ONLY), FEED INGREDIENTS

### APPENDIX 8.1: OVERSIKT OVER IMPORTERTE VEGETABILSKE RÅVARER TIL BRUK I FISKEFÔR HVOR DET ER INNVILGET TOLLFRITAK (2005):

Hvete: 73 500 tonn  
 Soyamel: 60 000 tonn  
 Mel av andre bønner enn soyabønner: 31 000 tonn  
 Bønnevikker/hestebønner: 22 000 tonn  
 Hvetegluten: 13 500 tonn  
 Solsikkemel: 4700 tonn  
 Bukkerter: 2200 tonn  
 Rapsmel: 1900 tonn  
 Grasmel (eller lignende): 1500 tonn  
 Maisgluten: 1000 tonn  
 Rapsolje: 39 000 tonn  
 Palmeolje: 14 000 tonn  
 ”Andre” vegetabiliske oljer: 14 500 tonn

### APPENDIX 8.2: VEGETABILSKE FØRRÅVARER BENYTTET AV INDUSTRIEN PR MAI 2008 (ETTER FORESPØRSEL)

<i>Oljekilder</i>	Rapsolje
<i>Kilde for protein/bindere</i>	Hvetegluten Solsikkemel/protein Fababønner (hestebønner) Hvetegluten Hvete Maisgluten Soya protein <i>Ekstrahert soya</i> <i>Fullfett soya</i> <i>Soya konsentrat</i> Erter Tapioka
<i>Tilsetningsstoff/prosesshjelpemiddel</i>	Maisstivelse i formuleringsmatriks for pigment

### APPENDIX 8.3: TILLATTE GM-RÅVARER (MATTILSYNET).

Genmodifiserte fôrprodukter som er tillatt å omsette på det norske markedet inntil 15 september 2008, forutsatt at de er merket "genmodifisert".

For mer informasjon om produktene, se EUs oversikt: [http://ec.europa.eu/food/dyna/gm\\_register/index\\_en.cfm](http://ec.europa.eu/food/dyna/gm_register/index_en.cfm)

Produkt	Opprinnelses GMO	Opprinnelses GMOs "unike identifikasjon"	Status i EU
Maisgluten	Mais Bt 11	SYN-BT 011-1	Tillatt. Vurdering av fornyet søknad pågåår.
Maisgluten	Mais MON810	MON-00810-6	Tillatt. Vurdering av fornyet søknad pågåår.
Maisgluten	Mais MON863	MON-00863-5	Tillatt. Vurdering av fornyet søknad pågåår.
Maisgluten	Mais NK 603	MON-00603-6	Tillatt. Vurdering av fornyet søknad pågåår.
Maisgluten	Mais NK 603 X MON810	MON-00603-6 X MON-00810-6	Godkjent inntil 23/10-2017
Maisgluten	Mais DAS1507	DAS-01507-1	Tillatt. Vurdering av fornyet søknad pågåår.
Maisgluten	Mais T25	ACS-ZM003-2	Tillatt. Vurdering av fornyet søknad pågåår.
Maisgluten	Mais GA21	MON-00021-9	Tillatt. Vurdering av fornyet søknad pågåår.
Maisgluten	Mais MON863 X MON810	MON-00863-5 X MON-00810-6	Tillatt. Vurdering av fornyet søknad pågåår.
Maisgluten	Mais MON863 X MON603	MON-00863-5 X MON-00603-6	Tillatt. Vurdering av fornyet søknad pågåår.
Soya, ekstrahert Soya, konsentrat Soya, fullfett Soya, lecithin - tokoferoler - mono- og diglyserider - olje	Soya  RR soya (MON 40-3-2)	MON-04 032-6	Tillatt. Vurdering av fornyet søknad pågåår.
Mel (Protein)	Bomull	MON-01445-2	Tillatt.
	MON 1445		Vurdering av fornyet søknad pågåår.
Mel (Protein)	Bomull MON 531	MON-00531-6	Tillatt. Vurdering av fornyet søknad pågåår.
Mel (Protein)	Bomull MON 531 X MON 1445	MON-00531-6 x MON- 01445-2	Tillatt. Vurdering av fornyet søknad pågåår.
Mel (Protein)	Bomull MON15985	MON-15985-7	Tillatt. Vurdering av fornyet søknad pågåår.
Mel (Protein)	Bomull MON15985 X MON1445	MON-15985-7 x MON- 01445-2	Tillatt. Vurdering av fornyet søknad pågåår.
Olje, mel	Raps GT 73	MON-00073-7	Tillatt. Vurdering av fornyet søknad pågåår.
Olje, mel	Raps MS8,RF3,MS8 X RF3	ACS-BN005-8 ACS-BN003-6 ACS-BN005-8 x ACS- N003-6	Tillatt. Vurdering av fornyet søknad pågåår.
Olje, mel	Raps T45	ACS-BN008-2	Tillatt. Vurdering av fornyet søknad pågåår.