ISBN 978-82-575-1015-2
ISSN 1503-1667

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Philosophiae Doctor (PhD) Thesis 2011:52

Upgrading plant ingredients in feed for salmonids by thermo-mechanical treatment and acid salts

Oppgradering av planteråvarer i fôr til laksefisk ved bruk av termomekanisk behandling og syresalter

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Acknowledgments

The work presented in this thesis was carried out at the Aquaculture Protein Centre (APC) during 2008–2011. Financial support was provided by APC via grants from the Norwegian Research Council and our collaborative partners, BioMar AS and ADDCON Nordic AS. I was supported by a PhD grant from the Norwegian University of Life Sciences.

This work would not have been possible without the supervision, help and support I received from many of my colleagues, friends and family. First, I would like to thank my supervisors, Dr. Margareth Øverland, Dr. Mette Sørensen and Dr. Olav Fjeld Kraugerud for their support, inspiration and excellent supervision during these years. Especially, I would like to thank my main supervisor Margareth for her encouragement and for giving me the opportunity to do research abroad. Thanks to Mette and Olav for their great humor and willingness to help despite their busy days. The three of you have been the perfect team for supervising me during my time as a PhD student.

Thanks are due to my co-authors Trond Storebakken, Marie Hillestad, Rune Christiansen, Rick Barrows, Francisco J. Moyano, Lorenzo Márques and Liv Torunn Mydland for their valuable input to the different papers. I am also indebted to many of my colleagues, whose support and inspiring discussions have made an important contribution to my work. Especially, I would like to thank Anders Skrede and Øystein Ahlstrøm for their helpful advices on the mink experiments and Liv Torunn Mydland for help with the chemical analyses. Thanks to Jon Øvrum Hansen and Felipe Reveco for being great office mates. I would also like to thank all the other PhD students and colleagues at APC for all the good times and social events outside of work.

Finally, I am grateful to my parents who encouraged me for higher education and to my dearest Helgi for his support and patience during these years.

Ås, September 21, 2011

Thea Morken

Abstract

Morken, T., 2011. Upgrading plant ingredients in feed for salmonids by thermo-mechanical treatment and acid salts. Norwegian University of Life Sciences, Philosophiae Doctor Thesis, 2011:52; ISSN: 1503-1667; ISBN: 978-82-575-1015-2.

Inclusion of plant ingredients in diets for salmonids is restricted due to low energy contents, unbalanced amino acid (AA) composition and presence of antinutritional factors (ANF). The objective of this thesis was to investigate methods for improving the nutritional and physical quality of plant ingredients in diets for salmonids by use of thermo-mechanical treatment and supplemental organic acid salts.

Diets containing plant ingredients derived from soybeans or barley were exposed to thermo-mechanical treatment at different temperatures by (1) expander pretreatment, (2) extrusion and (3) autoclaving, with or without the supplementation of 12 g kg^{-1} potassium diformate (KDF) or 10.6 g kg^{-1} sodium diformate (NaDF). Nutritional quality was evaluated by the content of dietary AA, available lysine and trypsin inhibitor activity (TIA), as well as *in vivo* apparent digestibility in Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*) and mink (*Neovison vison*). In addition, a two-step *in vitro* gastrointestinal model (GIM) was used to assess protein solubility and bioavailability of AA by use of digestive enzymes from Atlantic salmon. Physical quality of extruded diets was evaluated by measuring hardness, expansion ratio, durability and water stability index (WSI).

Expander pretreatment of full-fat soybean meal (FFSBM) at 100 and 120 ºC improved ($P<0.05$) the digestibility of arginine, glutamine and tyrosine in Atlantic salmon. The higher digestibility of expander pretreated FFSBM was confirmed in mink. The improved digestibility of AA in Atlantic salmon and mink by expander pretreatment of FFSBM coincided with the reduction in dietary TIA. Extrusion of diets containing toasted soybean meal (SBM) at 150 ºC improved ($P<0.05$) the digestibility of crude protein (CP) and several individual AA in Atlantic salmon compared to extrusion at 110 ºC. Similar findings were observed for diets containing barley protein concentrate (BPC) when fed to rainbow trout. Extrusion at 141 ºC improved ($P<0.05$) the digestibility of starch, CP, arginine and

several dispensable AA, but reduced ($P<0.01$) the digestibility of phenylalanine compared to extrusion at 110 ºC. The improved digestibility of protein and AA in rainbow trout with increasing extrusion temperatures was associated with higher availability of lysine in diets extruded at 141 ºC. Heat treatment at 130 ºC by autoclaving reduced the dietary AA content ($P<0.05$), the *in vivo* digestibility of CP and all individual AA in mink, as well as the *in vitro* bioavailability of AA ($P<0.01$) compared to heat treatment at 100 ºC. The adverse effects of heat treatment at long treatment times on the nutritional quality of diets were also shown by reductions in dietary contents of reactive and available lysine ($P<0.001$), protein solubility ($P<0.01$) and increased browning ($P<0.001$).

Supplementation of KDF to FFSBM and SBM diets did generally not ($P>0.1$) affect nutrient digestibility in Atlantic salmon and mink, whereas supplementation of NaDF improved ($P<0.05$) the digestibility of most major nutrients and individual AA in rainbow trout. Addition of NaDF did not ($P>0.1$) affect the digestibility of CP or individual AA in mink.

Physical quality of the extruded diets was affected by both extrusion temperature and supplementation of organic acid salts. Increased pellet expansion was observed in diets with soybeans processed by expander pretreatment and increasing extrusion temperatures, while supplemental KDF reduced pellet expansion. In diets with BPC, increasing extrusion temperatures improved pellet durability. Addition of NaDF improved the expansion ratio, durability and WSI compared to diets without NaDF.

The improved digestibility of plant proteins in salmonids by thermo-mechanical treatment at increasing temperatures is explained by a reduction in heat-labile ANF concurrent with higher availability of AA, as a result of denaturation and structural unfolding of protein molecules. The negative effects of increasing temperatures in combination with long treatment times in the autoclave on digestibility of CP and AA in mink was explained by a reduction in AA availability. Supplementation of acid salts did not consistently improve the digestibility of plant ingredients in salmonids and did not protect the protein from heat-induced damage during prolonged heat treatment. The lack of consistency remains unclear and requires further investigation. Both increasing temperatures during extrusion and supplementation of acid salts affected the physical quality of the feeds. The improved physical quality by supplemental acid salts indicates increased binding of feed particles.

Sammendrag

Morken, T., 2011. Oppgradering av planteråvarer i fôr til laksefisk ved bruk av termomekanisk behandling og syresalter. Universitetet for miljø- og biovitenskap, doktorgradsavhandling, 2011:52; ISSN: 1503-1667; ISBN: 978-82-575-1015-2.

Inkludering av planteråvarer i fôr til laksefisk er begrenset på grunn av lavt energiinnhold, ubalansert aminosyresammensetning og innhold av antinæringsstoffer. Formålet med denne avhandlingen var å undersøke metoder for å forbedre ernæringsmessig- og fysisk kvalitet av planteråvarer i fôr til laksefisk ved bruk av termomekanisk behandling og tilsetning av organiske syresalter.

Fôr som inneholdt planteråvarer fra soyabønner eller bygg ble eksponert for termomekanisk behandling ved ulike temperaturer under (1) ekspanderforbehandling, (2) ekstrudering og (3) autoklavering, med eller uten tilsetning av 12 g kg^{-1} kalium diformiat (KDF) eller 10.6 g kg^{-1} natrium diformiat (NaDF). Ernæringsmessig kvalitet ble evaluert som aminosyreinnhold i fôret, tilgjengelig lysin og trypsininhibitor-aktivitet (TIA), samt *in vivo* apparent fordøyelighet i Atlantisk laks (*Salmo salar*), regnbueørret (*Oncorhynchus mykiss*) og mink (*Neovison vison*). En tostegs *in vitro* fordøyelighetsmodell (GIM) ble i tillegg brukt for å vurdere proteinløselighet og biotilgjengelighet av aminosyrer ved bruk av fordøyelsesenzymer fra Atlantisk laks. Fysisk kvalitet av de ekstruderte fôrene ble vurdert ved å måle hardhet, ekspansjon, durabilitet og vannstabilitetsindeks (WSI).

Ekspanderforbehandling av fullfett soyamel (FFSBM) ved 100 og 120 ºC ga økt ($P<0.05$) fordøyelighet av arginin, glutamin og tyrosin hos Atlantisk laks. Den høyere fordøyeligheten av ekspanderforbehandlet FFSBM ble bekreftet i mink. Den økte fordøyeligheten av aminosyrer i Atlantisk laks og mink ved ekspanderforbehandling av FFSBM var sammenfallende med reduksjon av TIA i fôret. Ekstrudering av dietter som inneholdt varmebehandlet og avfetta soyamel (SBM) ved 150 ºC ga forbedret ($P<0.05$) fordøyelighet av råprotein og flere individuelle aminosyrer sammenliknet med ekstrudering ved 110 ºC. Liknende resultater ble observert da dietter med byggproteinkonsentrat (BPC) ble fôret til regnbueørret. Ekstrudering ved 141 ºC ga en høyere ($P<0.05$) fordøyelighet av stivelse, råprotein, arginin og flere ikke-essensielle aminosyrer, men resulterte i redusert ($P<0.01$)

fordøyelighet av fenylalanin sammenliknet med ekstrudering ved 141 ºC. Varmebehandling ved autoklavering på 130 ºC ga redusert innhold av aminosyrer i fôret ($P<0.05$), redusert *in vivo* fordøyelighet av råprotein og alle essensielle aminosyrer i mink, samt redusert *in vitro* biotilgjengelighet av aminosyrer ($P<0.01$) sammenliknet med varmebehandling ved 100 ºC. De negative effektene av varmebehandling og lang behandlingstid på ernæringsmessig kvalitet på fôret ble også vist ved reduksjoner i fôrets innhold av reaktivt og tilgjengelig lysin ($P<0.001$), redusert proteinløselighet ($P<0.01$) og økt bruning ($P<0.001$).

Tilsetning av KDF til fôr med FFSBM og SBM ga generelt ingen ($P>0.1$) effekt på fordøyeligheten av næringsstoff hos Atlantisk laks og mink, mens tilsetning av NaDF ga en forbedret ($P<0.05$) fordøyelighet av de fleste hovednæringsstoffer og aminosyrer hos regnbueørret. Tilsetning av NaDF påvirket ikke ($P>0.1$) fordøyeligheten av råprotein eller individuelle aminosyrer hos mink.

Fysisk kvalitet av det ekstruderte fôret ble påvirket av både ekstruderingstemperatur og syresalter. Ekspanderforbehandling og økt ekstruderingstemperatur ga økt ekspansjon av fôr som inneholdt soya, mens tilsetning av KDF ga redusert ekspansjon. Økende ekstruderingstemperatur ga forbedret durabilitet av fôr med BPC, mens tilsetning av NaDF ga økt ekspansjon, durabilitet og WSI sammenliknet med fôr uten NaDF.

Forbedringen av fordøyeligheten av planteråvarer hos laksefisk ved bruk av termomekanisk behandling med økte temperaturer er forklart ved en reduksjon i varmelabile antinæringsstoff, samtidig med økt tilgjengelighet av aminosyrer som et resultat av denaturering og åpning av proteinstrukturer. Negative effekter av økende temperatur i kombinasjon med lang behandlingstid under autoklavering på fordøyeligheten av råprotein og aminosyrer er forklart ved redusert aminosyretilgjengelighet. Tilsetning av syresalter ga variable resultater på fordøyeligheten av planteråvarer hos laksefisk. Årsaken til dette er fortsatt uklart og krever videre undersøkelser. Både økende temperaturer under ekstrudering og tilsetning av syresalter påvirket fysisk kvalitet på fôret. Forbedringen i fysisk kvalitet ved tilsetning av syresalter indikerer en økt binding mellom fôrpartikler.

Abbreviations

AA	Amino acid
ANF	Antinutritional factor
ANOVA	Analysis of variance
BPC	Barley protein concentrate
BW	Body weight
CP	Crude protein
CTTAD	Coefficient of total tract apparent digestibility
DM	Dry matter
DORIS	Durability on a Realistic Test
EAA	Essential amino acid
EC	European Commission
FFSBM	Full-fat soybean meal
FM	Fish meal
GIM	Gastrointestinal model
GLM	General Linear Model
HDI	Holmen durability index
KDF	Potassium diformate
N	Newton
NaDF	Sodium diformate
NEAA	Non-essential amino acid
NSP	Non-starch polysaccharide
RPM	Revolutions per minute
SBM	Solvent-extracted and toasted soybean meal
SCF	Screw configuration
SD	Standard deviation
SEM	Standard error of the mean
SGR	Specific growth rate
SME	Specific mechanical energy
TI	Trypsin inhibitor
TIA	TI activity
WF	White flakes
WSI	Water stability index

List of publications

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:

I. **Thea Morken, Olav F. Kraugerud, Mette Sørensen, Trond Storebakken, Marie Hillestad, Rune Christiansen, Margareth Øverland, 2011.** Effects of feed processing conditions and acid salts on nutrient digestibility and physical quality of soy-based diets for Atlantic salmon (*Salmo salar*). **Aquaculture Nutrition (in press), DOI: 10.1111/j.1365-2095.2011.00872.x.**

II. **Thea Morken, Olav F. Kraugerud, Frederic T. Barrows, Mette Sørensen, Trond Storebakken, Margareth Øverland, 2011.** Sodium diformate and extrusion temperature affect nutrient digestibility and physical quality of diets with fish meal and barley protein concentrate for rainbow trout (*Oncorhynchus mykiss*). **Aquaculture 317, 138-145.**

III. **Thea Morken, Francisco J. Moyano, Lorenzo Márquez, Mette Sørensen, Liv T. Mydland, Margareth Øverland.** Effects of heat treatment and sodium diformate on amino acid composition, *in vivo* digestibility in mink and *in vitro* bioavailability using digestive enzymes from Atlantic salmon. **Animal Feed Science and Technology (submitted).**

Contents

1. General introduction

Aquaculture supplied nearly half of the total fish production for human consumption in 2006 and is expected to outpace the production from capture fisheries (FAO, 2009). In Norway, the production of farmed salmonid fish increased almost two-fold from 474,000 tons in 1999 to 937,000 tons in 2009 (Figure 1) (FAO, 2011a). Concurrently, the global fish meal (FM) use within the aquaculture feed industry doubled over the last decade (Tacon and Metian, 2008). This has resulted in high price and low availability of FM on the world market. To meet the future challenges with population growth and need for aquatic food, the aquaculture industry is dependent on alternatives for FM to support a rapid and sustainable development.

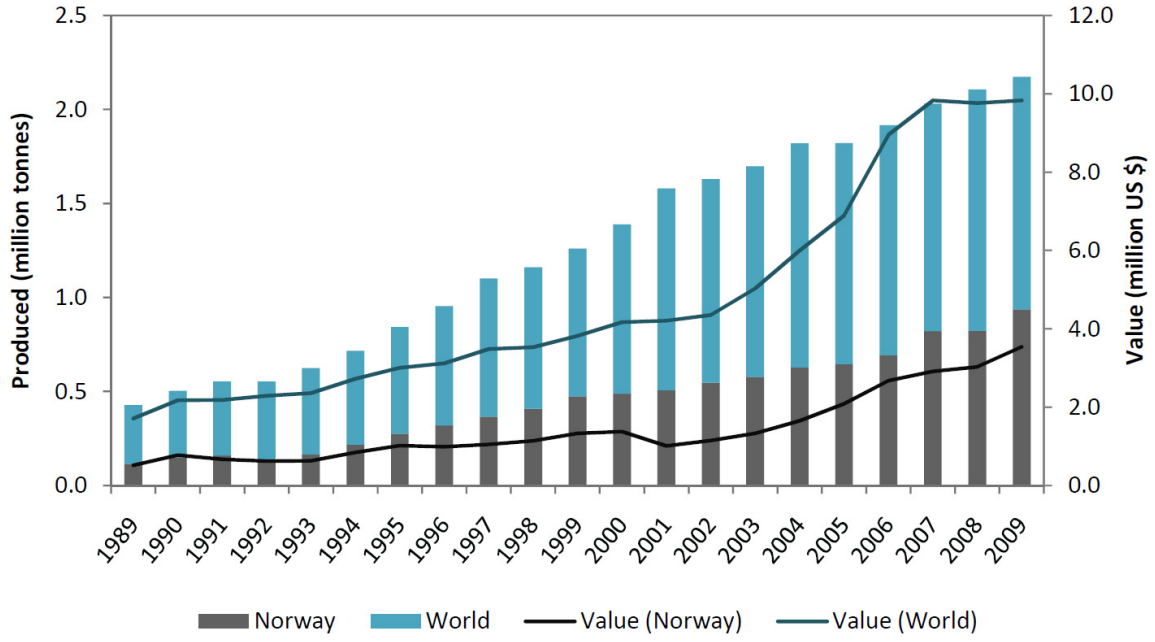

Figure 1. Norwegian share (grey bars) of the total world aquaculture production (blue bars) of Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) from 1989 to 2009 in relation to the production value (FAO, 2011a).

A variety of ingredients have been evaluated as alternatives for FM in diets for carnivorous fish, including plants (Gatlin et al., 2007), single-cell protein (Øverland et al., 2010), rendered by-products from fish and terrestrial animals (Naylor et al., 2009), as well as

organisms at lower trophic levels, such as krill (*Euphausia superba*) (Hansen, 2011). The use of animal by-products such as feather meal, poultry by-product meal and blood meal from ruminants are currently prohibited for use in fish feeds in the EU and EEA due to the hazard of propagating transmissible animal diseases to humans through the food chain (European Commission [EC] Regulation No 1774/2002). Although blood meal from porcine and poultry sources is permitted for use in fish feeds (EC Regulation No 1234/2003), there is low market acceptance for use of these products in Norway and the UK (EWOS, 2010). This has resulted in a more widespread use of plant ingredients in the European aquaculture industry.

Plant ingredients are considered promising alternatives to FM because of their wide availability, low cost and viable environmental profile. The use of plant ingredients such as legumes, oilseeds and cereal grains is, however, restricted due to low nutrient density, high content of fiber, unbalanced amino acid (AA) composition and a variety of antinutritional factors (ANF) (Francis et al., 2001). In order to overcome these challenges, processing methods or feed additives can be used to upgrade the nutritional characteristics of plant ingredients (Drew et al., 2007; Gatlin et al., 2007). Such modifications will not only affect their nutritional quality, but also their functional properties and ability to produce pellets of good physical quality (Sørensen et al., 2009). Physical quality is less commonly reported in ingredient-replacement studies, but should be considered when evaluating the potential of plant ingredients in diets for fish (Glencross et al., 2007).

Modern fish feeds have developed towards lower inclusion levels of marine resources, which results in reduced wild fisheries inputs to farmed fish outputs, and consequently, a net fish protein production (Naylor et al., 2009; Bendiksen et al., 2011). Estimates on the dietary FM and fish oil use in salmon feeds for 2020 suggests inclusion rates as low as 8 and 6%, respectively (Tacon and Metian, 2008). In order to accomplish this, there is a need to increase the inclusion rates of alternative ingredients such as plant proteins to replace FM. Furthermore, increased inclusion rates of plant proteins depend on development of methods to upgrade their nutritional quality in order to sustain high inclusion rates without compromising fish performance. The overall objective of this work was, therefore, to investigate methods for improving the nutritional and physical quality of plant ingredients in diets for salmonids by use of thermo-mechanical treatment and organic acid salts.

2. Background

2.1 Alternative plant ingredients in diets for fish

Expansion of the aquaculture industry has been accompanied by rapid growth in the production of fish feeds, leading to increased pressure on marine commodities such as FM and fish oil (Bostock et al., 2010). One factor contributing to this growth is the increased share of farmed fish produced by using compound feeds in Asia (Naylor et al., 2009). The high demand for marine commodities by the aquaculture sector given the static global supply has resulted in low availability and high prices of FM (Figure 2). High ingredient prices will have a negative impact on the profitability in Atlantic salmon production, where feed accounts for more than 50% of the production costs (Waagbø, 2006). This has resulted in an increased interest in identifying less costly alternatives to FM and fish oil in diets for carnivorous fish.

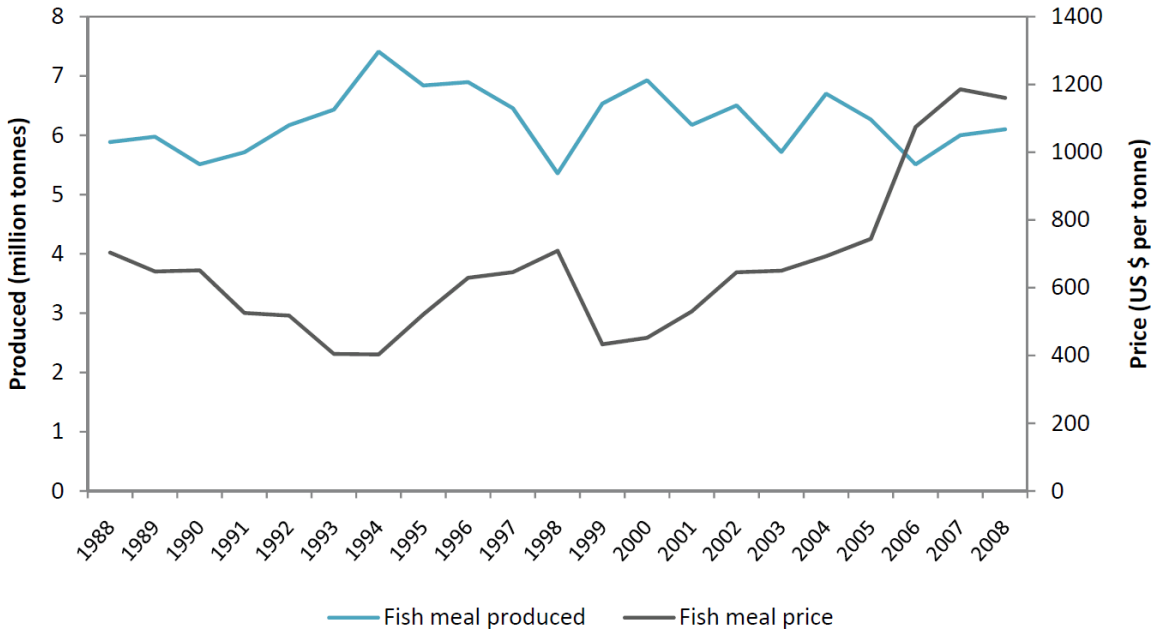

Figure 2. Annual production of FM (blue line) and price in US $ per ton FM (grey line) from 1988 to 2008 (IFFO, 2008; IMF, 2011).

Use of alternatives to FM has not only been driven by the possible economical issues of higher commodity prices. Increased awareness about the sustainability of the aquaculture industry has also evoked questions about the long-term viability of aquaculture as long as it remains a net consumer of marine resources (Bendiksen et al., 2011). Reducing the dependence on marine commodities through extended use of alternative ingredients will improve the future sustainability of the aquaculture industry.

A variety of protein sources have been explored as alternatives to FM, including plant proteins, by-products from fish and terrestrial animal productions, products from organisms at lower trophic levels and single-cell proteins (reviewed in Gatlin et al., 2007; Naylor et al., 2009; Øverland et al., 2010). Although many of these alternatives may offer considerable potential in diets for fish, plant proteins are considered to have the greatest possibilities because of their availability, low cost and sustainable environmental profile compared to FM. Currently, a wide range of plant ingredients are being used in commercial fish feed, including products of oilseeds (soybean, sunflower, rapeseed and cottonseed), legumes (peas and beans), tubers (potato), cereal grains (corn, barley and wheat), as well as co-products from the ethanol industry (Naylor et al., 2009). Among these, soybeans, canola/rapeseed, sunflower, peas and lupins are the most widely used plant ingredients in salmonid feeds.

The crude protein (CP) content of defatted oilseed meals ranges from 450 to 500 g kg^{-1} for soybean meal (SBM) (Storebakken et al., 2000), 350 to 390 g kg^{-1} for rapeseed meal and 250 and 500 g kg^{-1} for corticated and decorticated sunflower meal, respectively (Hertrampf and Piedad-Pascual, 2000). Among the legumes, peas has the lowest CP content (237 g kg^{-1}) and lupins the highest (345 g kg^{-1}) (Hertrampf and Piedad-Pascual, 2000). The essential amino acid (EAA) composition for soybeans, rapeseed, sunflower, peas and lupins in relation to FM is shown in Table 1. Soybean protein has a good AA composition compared to other oilseed meals, but is lower in methionine and higher in cysteine when compared to FM (Storebakken et al., 2000). Rapeseed and sunflower protein is especially limited in lysine and methionine. Methionine is also the main limiting AA in pea protein, which is otherwise characterized by high lysine content (Øverland et al., 2009). White lupins has an overall desirable AA composition, but is also limited in lysine and methionine relative to FM.

Table 1.

The essential amino acid composition of fish meal (FM) and commonly used plant protein sources in fish feeds.

g (16 g N)$^{-1}$	FM [a]	Soybean [b]	Rapeseed [c]	Sunflower [d]	Pea [e]	Lupin [f]
Arginine	5.4	6.7	2.1	3.6	8.2	11.2
Histidine	2.0	2.4	1.0	1.0	2.7	1.8
Isoleucine	3.6	4.0	1.4	2.1	4.5	3.9
Leucine	6.3	6.7	2.6	3.0	7.5	7.7
Lysine	6.6	5.1	2.1	0.7	7.4	4.9
Methionine	2.5	1.1	0.7	0.8	0.9	0.5
Phenylalanine	3.5	4.6	1.4	2.2	4.9	3.8
Threonine	3.9	3.7	1.6	1.7	3.7	4.0
Tryptophan	1.0	1.5	0.4	0.6	0.9	0.7
Valine	4.1	4.1	1.8	2.3	4.8	3.5

[a] Low-temperature dried FM (Romarheim et al., 2005).

[b] Hexane-extracted and toasted SBM with hulls (Romarheim et al., 2005).

[c] Defatted rapeseed meal (Hertrampf and Piedad-Pascual, 2000).

[d] Defatted and dehulled sunflower meal (Hertrampf and Piedad-Pascual, 2000).

[e] Pea protein concentrate, 350 g kg^{-1} CP (Øverland et al., 2009).

[f] White lupin (Hertrampf and Piedad-Pascual, 2000).

The inclusion level of plant ingredients in diets for fish is limited by their nutritional characteristics, including low nutrient density, high content of indigestible organic matter, unbalanced AA composition and presence of ANF or antigens (Francis et al., 2001; Gatlin et al., 2007). Additionally, plant proteins are less palatable for fish that have been adapted to FM-based diets (Gomes et al., 1995; Refstie et al., 2000). Use of diets where a substantial proportion of the FM is replaced with plant ingredients may, therefore, result in reduced feed intake and growth rate, as well as increased risk of life-style related disorders and susceptibility to infectious diseases (Waagbø, 2006). These impediments limit the use of high inclusion levels of some plant ingredients in diets for salmonids. Consequently, the majority of formulated fish feeds are based upon a partial replacement of FM with a variety of plant ingredients. Partial replacement of FM has often proven successful

(Carter and Hauler, 2000; Romarheim et al., 2008; Øverland et al., 2009), whereas complete replacement of FM with plant ingredients in diets for salmonids have sometimes resulted in reduced growth (Gaylord et al., 2006; Torstensen et al., 2008; Barrows et al., 2009). Nevertheless, studies have reported that trout fed FM-free diets showed growth comparable to trout fed FM-based diets (Kaushik et al., 1995; Barrows et al., 2007a). Thus, the development towards higher inclusion levels of plant ingredients in diets for salmonid fish depend on methods for upgrading their nutritional quality, such as feed processing technologies or feed additives.

2.2 Processing to improve nutritional quality of feed

Nutritional quality of a feed is determined by the content and availability of nutrients compared to the requirements of the target animal (Phillips, 1989). It is usually defined in terms of chemical composition and the ability of the animal to utilize the nutrients for growth. Several processing methods can be applied to improve the nutritional quality of plant ingredients in diets for carnivorous fish. The beneficial effect of processing on nutritional quality of feeds is a result of the physical and chemical modifications which occur when the feed material is subjected to heat, moisture and shear over a certain time. Plant ingredients for use in fish feeds are mainly processed in order to alter nutrient concentrations (reviewed in Drew et al., 2007) and to reduce antinutrient levels (reviewed in Francis et al., 2001).

There are two main strategies for enhancing nutritional quality of plant ingredients by processing: (1) pre-processing of individual ingredients and (2) processing of complete feed mixtures (Gatlin et al., 2007). The overall goal for ingredient pre-processing is to alter the inherent nutritional characteristics of plant products prior to dietary inclusion, whereas processing of complete diets aims to improve nutrient availability through physicochemical and chemical modification of the nutrients present in the feed mixture. Consequently, final nutritional quality of the diet depends on nutrient composition and pre-processing history of the ingredients used, in addition to the choice of equipment, processing and system variables applied during processing of the complete feed mixture.

2.2.1 Nutritional effects of ingredient pre-processing

Plant ingredients can be pre-processed by methods such as heat treatment, mechanical fractionation, fermentation processes, or combinations of these, depending on the nutritional characteristics of the plant material. These methods may be used separately or incorporated directly to the feed manufacturing process. Thermal processing inactivates antinutritional compounds by denaturing the biologically active protein structures of the heat-labile antinutrients such as soybean protease inhibitors and lectins (Liener, 1994; Francis et al., 2001; Romarheim, 2007). Oilseeds are normally pre-processed with heat treatment to facilitate oil extraction and removal of solvent residues (Storebakken et al., 2000), but also to inactivate heat-labile antinutrients. Studies on trypsin inhibitor activity (TIA) in raw and routine pre-processed soybeans have shown that toasting reduced the mean TIA from 30.2 to 8.5 mg g^{-1} (Rackis et al., 1985). Further reduction of the TIA of soybeans occurs when the complete diet is extruded (Clarke and Wiseman, 1999; 2007; Romarheim et al., 2005); resulting in dietary TIA levels within the tolerance level of salmonids (Olli et al., 1994; Romarheim et al., 2005; **Paper I**). Thus, heat-labile antinutrients in soy generally do not pose a problem in commercial feeds, as the soy products is heat treated both during pre-processing and diet processing (Storebakken et al., 2000; Romarheim et al., 2005; 2006). A greater concern is the possible adverse effects of heating on nutritional quality of plant ingredients (further discussed in chapter 2.2.2.2).

Fiber and heat-stabile ANF in plant ingredients are not reduced by heat treatment. These components have to be removed mechanically by fractionation technologies, biologically by fermentation, or by chemical extraction processes. For example, aqueous or methanol extraction can be used to purify the CP of SBM (400 g CP kg^{-1}) into soya protein concentrate (700 g CP kg^{-1}) or soya protein isolate (900 g CP kg^{-1}), products with lower levels of fiber and antinutrients (Drew et al., 2007). The use of fractionation processes, such as de-hulling, sieving, air-classification and aqueous or solvent extraction to purify proteins results in plant products with nutrient concentrations relative to the fractions removed.

Modification of ingredients using fermentation takes advantage of microorganisms and their enzymes (Nout and Motarjemi, 1997). Besides reducing the level of antinutrients, microorganisms consume and convert fermentable carbohydrates into cell mass that adds

essential nutrients to the final product (Gatlin et al., 2007). Distiller's by-products is an example of plant-products enriched with vitamins and nucleotides as a result of synthesis by yeast (Hardy and Barrows, 2002; Thiessen et al., 2003). Previous studies have shown that fermentation with microorganisms such as yeast (Servi et al., 2008; Gao et al., 2010), bacteria (Mukhopadhyay and Ray, 1999; Skrede et al., 2002; Refstie et al., 2005) and fungi (Kiers et al., 2000) reduces starch levels, content of antinutrients and concentrates the protein of plant ingredients. Additionally, combining fractionation technologies with biological processing will further improve their nutritional characteristics. For instance, plant products from soybean, barley and corn co-products of ethanol and biodiesel production are characterized by a low level of starch, but relatively high levels of fiber (Chevanan et al., 2009). Removing the hulls from these crops prior to ethanol fermentation increase their protein content and thus, nutritional value for fish (Gatlin et al., 2007).

2.2.2 Nutritional effects of feed processing

Commercial fish feeds are commonly produced by extrusion, a process which was introduced by the Norwegian fish feed industry in the early 1980's. The extrusion process and its effects on nutritional quality of feed will thus be the main component of the following discussion.

2.2.2.1 The extrusion process

Smith (1976) defined extrusion as "the process by which moistened, expansile, starchy and/or proteinaceous materials are plasticized and cooked in a tube by a combination of moisture, pressure, temperature and mechanical shear" (according to Huber, 2000). Extrusion is a versatile process, which allows the use of a wide range of raw materials to produce feed with specific nutritional and physical properties. The extruded pellets assert textural attributes which promotes the absorption and retention of up to 400 g oil kg^{-1} feed (Sørensen et al., 2011), which is an important property of high-energy salmon feeds. The extrusion process involves a series of unit operations such as particle size reduction, mixing, preconditioning, extrusion, drying, cooling and vacuum coating.

Preconditioning is generally the first step in the extrusion process after particle size reduction and ingredient mixing. The objective of this process is to heat and moisten the

feed material prior to the final cooking step in the extruder. During preconditioning, the feed material is mixed with a total of 25 to 30% water and steam at retention times ranging from 1 to 5 minutes, resulting in temperatures from 80 to 95 ºC (Strahm, 2000; Beyer, 2007). Preconditioning at temperatures higher than 100 ºC can be accomplished by using pressurized conditioners such as expanders (Lucht, 2007). Expanders are commonly used in the production of ruminant feeds to increase the percentage of rumen undegraded dietary protein (Prestløkken, 1999), but they may also be used as a standalone machine for special applications (Lucht, 2007). In **Paper I** of this thesis, expander technology was used to precondition full-fat soybean meal (FFSBM) at temperatures up to 120 ºC.

The heart of the process is the extruder barrel, which consists of 5–7 barrel sections and one or two screw shafts, depending on the application. Twin-screw extruders are commonly used for fish feed production as they are designed to operate at higher moisture and fat levels than single-screw extruders (Rokey, 1994). These have usually co-rotating and intermeshing screws. Both screw shafts are threaded with screw elements and the configuration of these can easily be modified to accommodate different raw materials and extruder functions (Sämann, 2008). The screw configuration (SCF) is generally built up from three major zones, including the feeding zone, the kneading zone and the final cooking zone (Huber, 2000). An example on the temperature profile in the different extruder zones is shown in Figure 3.

Following preconditioning, feed material is continuously fed into the first barrel section of the extruder and transported towards the die by the rotation of the screw (Huber, 2000). Fish feeds are typically extruded at temperatures below 150 ºC with moisture contents around 25 to 30% (Sørensen, 2003). Retention time inside the extruder barrel may vary from 20 to 40 seconds (Edwards et al., 1999), depending on the throughput and screw speed of the extruder. The die contains one or several orifices which restrict the material to flow, resulting in increased back-pressure and energy input. When the pressure is large enough, the feed material is extruded through the die orifice and shaped by rotating knifes. The pressure differential between the extruder and the atmosphere results in a steam flash-off as the material leaves the die and expansion of the extrudate (Chinnaswamy, 1993). The extruded pellets are subsequently dried, vacuum coated and cooled prior to packaging.

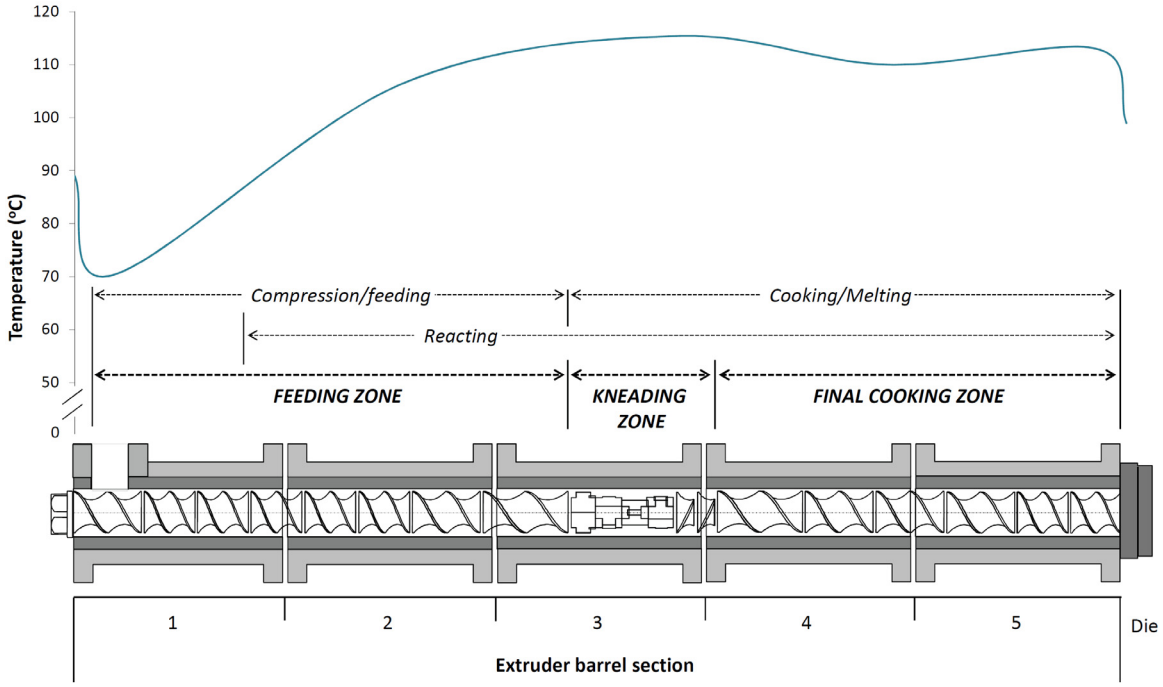

Figure 3. Example illustrating the actions occurring in the different processing zones of the extruder barrel, from inlet (left) to outlet (adapted from Huber, 2000). The temperature graph demonstrates the temperature development during extrusion of a FFSBM diet (**Paper I**), using a standardized SCF and a five-section twin-screw extruder (modified from Kraugerud, 2008).

2.2.2.2 Nutritional effects of extrusion

Several reviews of chemical and nutritional changes during extrusion cooking have been published in food research (Björck and Asp, 1983; Cheftel, 1986; Phillips, 1989; Arêas, 1992; Camire, 1991; 1998; 2000; 2001; Singh et al., 2007). The nutritional changes during extrusion can be regarded as an effect of the nutritional characteristics of the ingredients used (chemical composition, pre-processing history and ingredient combination) and the conditions applied during processing (water content, feeding rate, screw speed, SCF, barrel temperature, steam injection and die configuration) (Camire, 2001). The reactions occurring in the feed material during extrusion can be classified as physicochemical or chemical changes (Table 2).

Physicochemical changes are usually initiated at an early stage of the extrusion process, while chemical changes occur in the portion of the extruder barrel in front of the die (Camire, 2000), where the feed is plasticized under high temperature and pressure (Riaz,

2001). The change in the native structure of starch and proteins can be regarded as a precursor of the chemical changes occurring at a later stage of the extrusion process (Sørensen et al., 2005). These reactions may either improve or damage nutritional quality of the feed, depending on the temperature, shear, moisture and time applied during processing (Papadopoulos, 1989). Moist heat in combination with short retention time generally improves nutritional quality of plant proteins fed to fish due to denaturation and inactivation of biologically active compounds, as well as making the starch and proteins more susceptible to digestive enzymes (Håkansson et al., 1987; Phillips, 1989; Camire, 1991, 2001; Svihus et al., 2005; Barrows et al., 2007a). Extrusion result in a high degree of starch gelatinization (Lundblad et al., 2011), which improves the utilization of starch in carnivorous fish species (Storebakken, 2002; Krogdahl et al., 2005). In general, the short retention time and high moisture content of the extrusion process allows use of high temperatures without compromising nutritional quality (Sørensen et al., 2002). Conversely, feed materials are more sensitive to nutritional damage when the moisture content is low. This is further aggravated by increased processing time (Papadopoulos, 1989).

Proteins are especially exposed to nutritional damage during processing (Opstvedt et al., 1984; Moughan, 2003). Apart from denaturation, proteins may react with other proteins (disulphide or isopeptide crosslinks), or the protein side-chains may undergo racemization, oxidative degeneration and reactions with reducing substances (Phillips, 1989). These reactions lead to disruption and reformation of strong covalent bonds which are less susceptible to digestive proteases, and hence, less digestible. The Maillard condensation between the free ε-amino group of AA and the carbonyl group of reducing sugars

Table 2.

Physicochemical and chemical reactions during extrusion (modified from Camire, 1998).

Physicochemical changes	Chemical changes
Binding of smaller molecules	Thermal degradation of sugars and amino acids
Volatilization	Depolymerization of starch, dietary fiber and protein
Change in native structure - Starch gelatinization - Protein denaturation	Recombination of fragments from starch and dietary fiber

may give a loss of AA with reactive side-chains such as arginine, cysteine, lysine, histidine and tryptophan (Cheftel, 1986; Iwe et al., 2001; Singh et al., 2007). Among these, lysine is considered to be the most reactive (Belitz et al., 2009). Available lysine, or the amount of reactive lysine in percentage of total dietary lysine, can therefore be used as a measure on protein damage during processing (Moughan and Rutherfurd, 1996; Rutherfurd et al., 2007; Singh et al., 2007). Extrusion processing may also reduce heat sensitive nutrients, such as crystalline vitamins (Marchetti et al., 1999).

Several studies have assessed the effect of heat treatment of diets for salmonid fish (Opstvedt et al., 1984; Arndt et al., 1999; Sørensen et al., 2002; 2005; Barrows et al., 2007a) and monogastric carnivores such as mink (*Neovison vison*[1]) (Skrede and Krogdahl, 1985; Ljøkjel et al., 2000; 2004; Opstvedt et al., 2003; Romarheim et al., 2005; Aslaksen et al., 2006), but the results are inconsistent. Extrusion temperatures up to 150 ºC have no negative impact on nutrient digestibility or growth in salmonids (Sørensen et al., 2002), whereas mink is more sensitive to high extrusion temperatures (Ljøkjel et al., 2004). Reductions in CP and AA digestibility in mink are particularly observed when applying heat treatment at long retention times, such as by autoclaving (Skrede and Krogdahl, 1985; Ljøkjel et al., 2000). Nevertheless, the difficulty in defining optimal extrusion temperatures is related to the complex interrelationship among processing parameters, extrusion equipment and diet formulation (Björck and Asp, 1983). In addition, acceptable processing conditions also depend on the target animal (Romarheim, 2007).

2.3 Effects of processing and ingredients on physical quality of feed

The use of processing to improve nutritional quality of plant ingredients will also have implications for the physical quality of the feed. Physical quality is the ability of feed pellets to withstand repeated handling without creating excessive amounts of fines (Lundblad, 2009). Plant ingredients have shown to assert specific processing responses and physical properties when included in fish feed (Sørensen et al., 2009; 2011; Øverland

[1] Errata: The American mink is commonly included in the sub-genus *Vison* of the genus *Mustela*. However, cytogenetic and biochemical differences between the American mink and other *Mustela* species supports placement of the American mink in the mustelid genus *Neovison* (Kurose et al., 2008). The authors of **Papers I** and **II** were not aware of this and mink was, therefore, indicated by the genus *Mustela*.

et al., 2009; Kraugerud et al., 2011). Thus, it is important to optimize feed processing to improve both nutritional and physical quality of plant-based diets for fish.

Physical properties of feed pellets must accommodate the feeding behavior and the culture environment of the target species (Thomas and van der Poel, 1996; Glencross et al., 2010). Physical quality of extruded salmonid feed is usually defined by properties such as durability, hardness, expansion ratio, density, oil absorption capacity, oil leakage, water stability index (WSI) and sinking velocity. Physicochemical methods such as diet viscosity and starch gelatinization also play an integral role in determining both physical and nutritional characteristics of the feed (Evans, 1999; Hansen et al., 2010). These methods have previously been used to assess ingredient functional properties in relation to physical quality of feeds (Glencross et al., 2010; Hansen et al., 2010; Kraugerud et al., 2011; Sørensen et al., 2011). Different methods and procedures are used to measure pellet properties. For instance, pellet durability can be measured using the Holmen durability tester, tumbling box procedure, Lignotest and DORIS (durability on a realistic test) tester (Sørensen et al., 2010; 2011; Aas et al., 2011a). The majority of these methods was developed for steam-pelleted feeds, and may not give adequate information of pellet degradation when high-energy extruded fish feed is conveyed by feeding systems similar to the ones used in the Norwegian aquaculture industry. For that reason, the DORIS tester was developed to simulate forces extruded high-energy salmon feed is exposed to when conveyed by pneumatic feeding systems (Sørensen et al., 2011; Aas et al., 2011a).

In intensive aquaculture production, feeds are exposed to degradation through various handling processes during manufacture, transportation, storage and feeding. The feed is normally conveyed from a holding bin into the sea pens by pneumatic feeding systems with air velocities from 30 to 70 ms^{-1} (Aarseth, 2004). The feeding system has pipelines ranging from a few hundred up to 1,500 m (Sørensen et al., 2011). During feeding, pellets collide with other pellets and the pipe wall, leading to attrition, abrasion or chipping of the feed (Aarseth, 2004; 2006a; Aas et al., 2011a). Loss of feed particles does not only represent a direct economical loss for the farmer, but can also lead to microbial growth inside the pipe walls or cause downtime of the feeding system due to accumulation of feed particles in the pipe system (Aas et al., 2011a; b). In addition, feed particles may increase emission of nutrients to aquatic areas. Consequently, physical quality is of great importance to sustain cost-efficiency in intensive aquaculture. It is also important that the

physical quality does not compromise fish performance by interfering with feed intake or nutrient utilization, as suggested in some publications (Hilton et al., 1981; Baeverfjord et al., 2006; Hansen and Storebakken, 2007; Venou et al., 2009; Aas et al., 2011b).

Physical quality is affected by several factors, including ingredients (Refstie et al., 2006; Aarseth et al., 2006b; Hansen and Storebakken, 2007; Sørensen et al., 2009; Øverland et al., 2009; Glencross et al., 2010; Hansen et al., 2010; Draganovic et al., 2011; Aas et al., 2011b), extruder configuration (Sørensen et al., 2009; 2010) and processing parameters (Aarseth et al., 2006b; Øverland et al., 2007; Sørensen et al., 2009; 2010; 2011). Despite increased focus on alternative ingredients as replacements for FM, relatively few studies have reported the effects of ingredients and processing on physical quality of extruded fish feed. Plant ingredients are known to yield unique processing responses (Sørensen et al., 2009; 2010; 2011; Draganovic et al., 2011; Kraugerud et al., 2011), which highlights the importance of understanding the relationship between ingredient functional properties and processing parameters in relation to physical quality.

The effect of dietary ingredient composition on physical quality of feed can be attributed to physicochemical changes which occur when the feed material is subjected to moisture, heat and shear forces during processing (Thomas et al., 1998). Ingredients with viscous properties, e.g. starchy materials, will enhance the binding between feed particles (Thomas and van der Poel, 1996; Svihus et al., 2005). Different starch sources have different gelatinization optimums, and hence, binding properties depending on their granular microstructure and amylose to amylopectin ratio (Svihus et al., 2005). For instance, Sørensen et al. (2011) showed that pea and wheat starch had different viscosity profiles, resulting in different binding properties when included in extruded feeds. Furthermore, Kraugerud et al. (2011) reported that diets with starch-rich legumes produced less durable feed pellets than diets containing protein-rich oilseeds such as rapeseed and sunflower meal. Globular proteins in plant ingredients may also have structuring capabilities (Arêas, 1992; Li and Lee, 1996). Sørensen et al. (2009) suggested that the improved physical quality of diets containing toasted or untoasted SBM could be an effect of the stabilizing forces of disulphide linkages.

The choice of extruder configuration and processing parameters used during feed processing will also affect physical quality of extruded feed (Thomas et al., 1997). The configuration of the extruder and the process parameters employed can be used to mani-

pulate the flexibility to the extrusion process according to the material that is to be extruded. Processing can therefore be considered a tool for utilizing the physicochemical properties of ingredients to optimize physical quality of the feed. Sørensen et al. (2009) observed that diets containing FM, toasted SBM or white flakes extruded with a standardized SCF resulted in different specific mechanical energy (SME) among the feeds. Changing the SCF to a less shearing profile in combination with lower screw speed resulted in SME levels similar to the diets containing FM or toasted SBM, but the feed had a significantly higher breaking force compared to the feed extruded with the standardized screw. In line with this, Sørensen et al. (2010) reported that SCF accounted for about 40% of the variation in expansion and durability of feeds containing different starch sources.

Extrusion processing parameters can be divided into independent (adjustable) and dependent (observed) processing variables (Table 3). The most critical parameters for the response in physical quality are moisture content, retention time, mechanical and thermal energy input, which can be manipulated by adjusting the independent processing variables (Plattner, 2007). The physical quality is affected by the critical parameters alone or in combination with the ingredients used. In line with this, Kraugerud et al. (2011) showed that diets containing either protein-rich or starch-rich plant ingredients gave different physical qualities in response to different extrusion processing parameters.

The interaction between physical and nutritional quality is not well explored, but is currently receiving increased attention in both industry and aquaculture research. One of the first studies suggesting that there are such interactions present was conducted 30 years ago by Hilton et al. (1981). This study reported that extruded feeds gave prolonged gastric retention time and depressed feed intake in rainbow trout compared to steam-pelleted feeds. In line with this, Venou et al. (2009) showed that extrusion processing decreased feed intake and doubled gastric evacuation time in gilthead sea bream (*Sparus aurata*). The reason for the prolonged gastric retention time and reduced feed intake was explained by the high water stability of extruded feed compared to the steam-pelleted feed. Literature concerning the effect of water stability on feed intake is, however, incomplete. Aas et al. (2011b) observed a lower feed intake for feeds with high water stability compared to feeds with low water stability. Studies have also shown that low water stability affects the separation and accumulation of free oils in the stomach, a condition which may lead to oil-belching (Baeverfjord et al., 2006; Aas et al., 2011b). Information on the interactive

effects between physical quality and nutrition is not well understood and should be given more attention in future studies. One way to approach this is to report physical quality in addition to common measures such as feed intake.

Table 3.
Independent and dependent extrusion processing variables (Plattner, 2007).

Independent variables	Dependent variables
Preconditioning	
Feeding rate	Throughput
Moisture level	Feed moisture
Shaft speed	Retention time
Configuration	Temperature
Extrusion	
Moisture level	Feed moisture
Screw speed	Retention time
Barrel heating elements	Temperature
Screw configuration	Mechanical energy input
Die configuration	Pressure

2.4 Organic acids to improve nutritional quality of feed

Organic acids occur naturally as constituents of plants or animal tissue or as products from microbial metabolism (Partanen and Mroz, 1999). Organic acids and their salts have received much attention as feed additives for terrestrial monogastric species, especially pigs (reviewed in Partanen and Mroz, 1999; Mroz et al., 2006; Metzler and Mosenthin, 2007). Improvements in health and disease resistance, nutrient utilization, growth performance and carcass quality following dietary supplementation of organic acids have frequently been reported (Mroz et al., 2006). The beneficial effects of organic acids have mainly been explained by their strong antimicrobial activity (Freitag, 2007). It is considered that organic acids lower gastric pH, resulting in an increased proteolytic activity and hence, protein digestibility and growth (Partanen and Mroz, 1999). They may also

selectively inhibit the growth of harmful bacteria such as *Escherichia coli* and serve as energy substrates in the intermediary metabolism (Metzler and Mosenthin, 2007). To date, limited information exists on the use of organic acids and their salts in diets for fish. Based on the successful reports from livestock research, it could be interesting to investigate if organic acid salts can improve the nutritional quality of plant ingredients in diets for carnivorous fish.

Organic acids are weaker compared to the strong mineral acids, meaning that they will only partially dissociate in water. Organic acids used for food and feed purposes are usually short-chained (C1–C7) and have acid dissociation constants (pK_a) in the range from 3 to 5 (Table 4). Formic acid is a commonly used preservative (Partanen and Mroz, 1999), but its use is associated with problems related to handling, odor and corrosiveness to skin and equipment (Øverland et al., 2000; Canibe et al., 2001). Acid salts are therefore more favorable compared to liquid acids. Formic acid may combine with minerals (e.g. K, Na and Ca) to form acid salts. Salts of formic acid are commonly referred to as formates or diformates, depending on their number of formate groups. Formates consist of a monovalent mineral ion combined with the conjugate base of formic acid through an ionic bond, such as potassium formate (HCOO-K), while diformates consist of a divalent mineral ion bound to two formate groups, such as calcium diformate ($Ca(HCO_2)_2$). Other compounds are also referred to as diformates, although not being 'true' diformates in that they are conjugated salts of formic acid and formates. Such examples are potassium diformate (HCOOH··HCOO-K; KDF) and sodium diformate (HCOOH··HCOO-Na; NaDF). Formates and diformates has different chemical properties in that when dissolved in aqueous media, the formates will dissociate into the conjugate base of the organic acid and the mineral ion, while diformates such as KDF and NaDF will dissociate into a mineral ion, a formate and a formic acid molecule. Depending on the pH of the solution, the majority of formic acid may dissociate (if $pH>pK_a$) or remain undissociated (if $pH<pK_a$). Salts of acids such as potassium formate will not affect the pH of the solution but act as a buffer.

Although several biological mechanisms of organic acids have been suggested (reviewed in Mroz et al., 2006), their exact mode of action is complicated and has not yet been fully elucidated (Chaveerach et al., 2002). A common approach is the uncoupling theory which suggests that the antimicrobial activity of organic acids results from their bactericidal and

Table 4.

Chemical characteristics of some organic acids used for food and feed purposes (modified from Foegeding and Busta, 1991; Aylward and Findlay, 1994).

Organic acid	Formula	Form	Dissociation constant [1]		
Monocarboxylic			**pK_a**		
Formic	HCOOH	liquid	3.74		
Acetic	CH_3COOH	liquid	4.76		
Propionic	CH_3CH_2COOH	liquid	4.87		
Lactic	$CH_3CH(OH)COOH$	liquid	3.86		
Butyric	$CH_3(CH_2)_2COOH$	liquid	4.82		
Sorbic	$CH_3CH=CHCH=CHCOOH$	solid	4.76		
Dicarboxylic			**pK_{a1}**	**pK_{a2}**	
Malic	$COOHCH(OH)CH_2COOH$	solid	3.40	5.10	
Tricarboxylic			**pK_{a1}**	**pK_{a2}**	**pK_{a3}**
Citric	$COOHCH_2C(OH)(COOH)CH_2COOH$	solid	3.13	4.76	6.40

[1] The acid dissociation constant (K_a) is expressed by its negative logarithm ($pK_a = -\log_{10}K_a$), the pH at which 50% of the acid molecules are dissociated ($HA \rightleftharpoons A^- + H^+$) (Mroz et al., 2006).

bacteriostatic properties (Russell, 1992). The latter author suggested that the undissociated form of a short-chained organic acid can permeabilize and/or passively diffuse through bacterial membranes when the intraluminal environment has a pH lower than the pK_a value of the acid (Canibe et al., 2001; Mroz et al., 2006). Once inside the cellular cytoplasm, where the pH is maintained around 7, the organic acid (R-COOH) dissociates into protons (H^+) and anions ($RCOO^-$). Acidification of the cytoplasm inhibits cellular enzyme functions and suppresses microbial growth (Russell, 1992; Partanen and Mroz, 1999). The suggested antimicrobial mode of action is shown in Figure 4. However, the antimicrobial effects of organic acids on bacteria could also be caused by an accumulation of polar anions within the bacteria cell rather than acidification of the cellular cytoplasm (Russell, 1992).

Organic acids may also be hydrolyzed in the stomach, causing liberation of protons and a lowering of gastric pH, which may increase the overall proteolytic activity and inhibit bacterial growth. This may be beneficial in animals with suboptimal hydrochloric acid production, such as piglets (Partanen and Mroz, 1999; Mroz et al., 2006). Furthermore, intestinal hydrolyzation with subsequent liberation of protons is also expected to lower the intraluminal pH, serving as a pH barrier against pathogen colonization (Mroz et al., 2006). Organic acids may, therefore, selectively stimulate the growth of beneficial bacteria while reducing the growth of pathogenic bacteria which are not able to resist internal pH change. This has shown to be effective in altering the microbial populations in the gastrointestinal tract of weanlings (Canibe et al., 2001), which potentially may reduce the risk of postweaning diarrhea (Partanen and Mroz, 1999). Organic acid salts have also shown to affect the gut microbiota of hybrid tilapia (Zhou et al., 2009). The antimicrobial efficacy of organic acids *in vivo* is a result of several factors, including carbon-chain length, dietary inclusion level, dissociation characteristics, the intraluminal digesta acidity and acid-binding capacity, retention time and exposure in the different segments of the gastrointestinal tract, specific potency of pathogens for colonization and enterotoxin production and the intestinal status of the animal (Mroz et al., 2006).

Early studies on the application of free organic acids in diets for fish have mainly focused on acid-preserved fish silage. One of the first studies was published by Rungruangsak and Utne (1981). They tested diets with 0, 40, 60 and 100% formic acid-preserved fish silage. The results from this study showed that increasing dietary concentrations of formic acid reduced the growth, proteolytic activity and feed utilization of rainbow trout (*Salmo gairdneri* Richardson). Organic acids and their salts have gained increased interest in recent years, especially following the ban of antibiotic growth promoters in the EU (Ng and

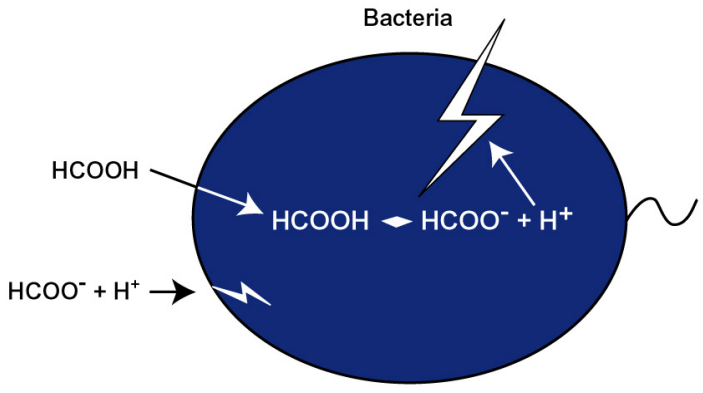

Figure 4. The suggested antimicrobial mode of action of formic acid (reprinted with permission from Christiansen and Lückstädt, 2008).

Koh, 2011). Recent studies have focused on the effects of organic acids and their salts on growth performance, nutrient utilization and disease resistance in several fish species (reviewed in Lückstädt, 2007, 2008a; Ng and Koh, 2011). In this chapter, special emphasis is given to potassium and sodium salts of formic acid when added to diets for salmonid fish species.

Among the formic acid salts, KDF is the most extensively studied. KDF was the first substance approved as a preservative for use in feeds for all species or categories of animals (EC Regulation No. 492/2006) and is frequently referred to as a possible non-antibiotic growth promoter (Zhou et al., 2009). Substantial effort has been made in evaluating the effects of KDF on nutrient digestibility, growth performance and disease resistance in pigs (Roth et al., 1998a, b; Øverland et al., 2000; Canibe et al., 2001; Mroz et al., 2002) and poultry (Mikkelsen et al., 2009). In recent years, some studies have also tested its potential in diets for fish, including Atlantic salmon (Lückstädt, 2008b; Lückstädt and Schulz, 2008; Lückstädt and Kühlmann, 2009; Storebakken et al., 2010), Nile tilapia (Ramli et al., 2005; Cuvin-Aralar et al., 2010; Liebert et al., 2010; Lim et al., 2010) and different hybrid species of tilapia (Ng et al., 2009; Zhou et al., 2009).

Lückstädt (2008b) reported an increase ($P<0.05$) in the apparent digestibility of CP, dry matter (DM) and gross energy of Atlantic salmon (650 g body weight [BW]) following dietary supplementation of 13.5 g KDF kg^{-1} added either prior to FM production or prior to feed extrusion. A significant increase in specific growth rate (SGR) was also observed for Atlantic salmon fed the diets where KDF was added to the raw fish prior to FM production. Similarly, Lückstädt and Kühlmann (2009) reported a significant increase in CP digestibility of Atlantic salmon fingerlings (30 g BW) fed diets supplemented with 13 g KDF kg^{-1} added prior to diet processing. However, feed intake and growth was reduced for Atlantic salmon fed the diet supplemented with KDF. Lückstädt and Schulz (2008), on the other hand, observed no effects of adding 10 g KDF kg^{-1} in different stages of the production process on the digestibility of CP, starch, DM and energy in Atlantic salmon (350 g BW). Although salmon fed diets with KDF has a significantly improved fat digestibility, KDF had no significant effects on SGR. Furthermore, Storebakken et al. (2010) observed that addition of 12 g KDF kg^{-1} to expander pre-treated (100, 110, 116 and 122 ºC) FFSBM and wheat mixtures prior to extrusion increased the digestibility of several AA in Atlantic salmon compared to non-supplemented diets. However, the

apparent digestibilities of fat, starch and ash were not affected. The inconsistency among the previously mentioned studies indicates that the effect of KDF on nutrient digestibility and growth in salmonids is variable and may depend on factors such as diet composition, feed processing conditions and fish size.

Recently, Liebert et al. (2010) evaluated the effects of adding 3 g KDF kg^{-1} or 3 and 5 g NaDF kg^{-1} to a plant-based diet for tilapia fingerlings. The results showed a slight, but not significant, increase in growth of tilapia fed diets containing KDF and NaDF. Fish fed the diet with 3 g NaDF kg^{-1} showed a significantly improved feed efficiency and protein retention efficiency compared to the other dietary treatments. To our knowledge, no previous studies have evaluated the effects of NaDF in diets for salmonid fish species.

Currently, few reports are available on parameters such as pH in the diet and digesta of fish fed dietary organic acid salts. Lückstädt (2008b) observed that dietary KDF at 13.5 g kg^{-1} did not influence the intestinal pH of Atlantic salmon, whereas Ng et al. (2009) observed a small numerical ($P>0.05$) reduction in diet and stomach pH of red hybrid tilapia fed diets with 2 g KDF kg^{-1}. These observations indicate that the biological effects of acid salts may be species-dependent. More research is needed to clarify the effects of both KDF and NaDF on nutrient utilization and growth of salmonids. Studies should also focus on identifying biological mechanisms of acid salts in fish. In this context, it could be interesting to investigate if the efficacy of organic acid salts is an effect of pH, the salt, or combined effects resulting from interactions among the different components of the acid salt.

3. Objectives

The overall objective for this work was to increase the knowledge on how to improve the nutritional and physical quality of plant ingredients in salmonids by use of thermo-mechanical treatment and organic acid salts. This research was designed to test three main hypotheses: (1) that thermo-mechanical treatment at high temperatures increases the apparent digestibility of plant ingredients in salmonids, (2) that supplementation of organic acid salts increases the digestibility of plant ingredients in salmonids, and (3) that high temperatures in combination with organic acid salts reduces heat-induced structural changes to proteins, and hence, improves the digestibility of proteins in salmonids. The following sub-objectives were studied:

- To investigate the effect of expander pretreatment of FFSBM prior to extrusion on nutrient digestibility, TIA and physical quality of diets for Atlantic salmon (**Paper I**).
- To study the effect of extrusion temperatures on digestibility and availability of nutrients and physical quality of diets based on FM or a partial replacement with plant proteins (SBM or BPC) for Atlantic salmon (**Paper I**) and rainbow trout (**Paper II**).
- To examine the effect of heat treatment at long treatment times on dietary AA composition, *in vivo* digestibility in mink and *in vitro* bioavailability using digestive enzymes from Atlantic salmon (**Paper III**).
- To investigate the effect of supplemental organic acid salts on nutrient digestibility (**Papers I-III**) and physical quality (**Papers I** and **II**) of diets for Atlantic salmon, rainbow trout and mink.
- To investigate the interactive effects between heat treatment and organic acid salts on chemical composition, nutritional and physical quality of diets (**Papers I-III**).

4. Discussion of methods

The majority of the chemical methods used in this thesis are standard analytical procedures, which are described in **Papers I-III**. Those methods will not be discussed here. The purpose of this section is to discuss strengths and weaknesses of the various methods used in the different experiments. The main focus will be on ingredient selection, diets and processing, measurement of digestibility and bioavailability of nutrients, as well as allotment, experimental design and statistical treatment.

4.1 Ingredient selection

The plant ingredients used in **Papers I-III** originated from soybean (*Glycine max* L.) or barley (*Hordeum vulgare* L.). Soybean is the world's leading oilseed crop with a production of 223 mmt in 2009, whereas barley is ranked as the world's fourth largest grain crop with a production of 152 mmt in 2009 (FAO, 2011b). The majority of research on replacement of FM with plant ingredients has focused on soybeans (reviewed in Storebakken et al., 2000), while barley appears to be less commonly used (reviewed in Gatlin et al., 2007). The main reason for this is the low protein and energy content due to the high level of fiber in barley (Gatlin et al., 2007). Soybeans also contain a considerable amount of indigestible carbohydrates and a wide range of ANF (Francis et al., 2001). Removal of indigestible carbohydrates and ANF by ingredient pre-processing allows a higher inclusion level of plant ingredients in fish feeds, but also increases their costs relative to FM (Naylor et al., 2009). Thus, we aimed to study alternative cost-effective methods for improving the nutritional quality of low-cost soybean products such as FFSBM and SBM (**Paper I**). The heat treatment methods chosen for the research were expander treatment and extrusion.

In contrast to the soybean ingredients used in **Paper I**, the BPC used in **Papers II** and **III** was derived from a more advanced processing method. The protein content of the BPC was upgraded by an enzymatic method during ethanol fermentation. The BPC contained approximately 570 CP kg^{-1} DM, which is higher than the typical protein content of distiller's dried grains with solubles (DDGS) such as corn DDGS (Kannadhason et al.,

2010). The BPC is, therefore, a potential fish feed ingredient that can be produced in northern America as well as in northern Europe such as Norway.

4.2 Diets and processing

The majority of diets used in this research were formulated to partially replace FM by substituting 260–380 g kg^{-1} of the total dietary CP content with products from either soy or barley (**Papers I-III**). Diets were also formulated based on constant CP to crude lipid ratios (**Papers II** and **III**). In **Paper II**, both FM and BPC-based diets were used to assess treatment effects of extrusion temperature and NaDF. Details regarding diet formulations and processing of the experimental feeds are described in **Papers I-III**.

In brief, the diets used in **Papers I-III** were produced at three different research facilities. Diets used in **Paper I** were produced at the Centre for Feed Technology (FôrTek) at UMB (Ås, Norway), using a five-section Bühler co-rotating twin-screw extruder with a throughput capacity ranging from 200–1,400 kg h^{-1} (Bühler AG, BCTG 62/20 D), whereas the diets in **Paper II** were produced at Bozeman Fish Technology Center at the U.S. Fish and Wildlife Service (Bozeman, MT, USA), using a six-section Bühler co-rotating twin-screw extruder with a throughput capacity ranging from 20–80 kg h^{-1} (Bühler AG, DNDL-44). The diets used in **Paper III** were autoclaved in order to enable thermal processing at treatment times exceeding the possibilities of extrusion. More details on diet processing are described in **Papers I-III**.

The different extrusion temperatures were obtained by modifying the SCF (**Paper I, exp. I**), increasing the screw revolutions per minute (**Paper I, exp. II**) or the set-temperature of the water-driven heating jackets (**Paper II**). In **Paper I**, the SCF was adjusted for the expander-treated FFSBM diets in order to reach the targeted extrusion temperature of 110 ºC (Figure 5) due to the high lipid content in the FFSBM (226 g kg^{-1} DM).

The autoclave treatment in **Paper III** was carried out in a fully automated process, with the independent variables temperature and treatment time at the defined set-temperature (**Paper III**). The temperature was monitored by a temperature sensor fitted in a glass bottle with water, where the volume resembles the volume of the liquid to be autoclaved.

This volume determines the total treatment time in the autoclave because steam and pressure must heat the content of the glass bottle to the defined set-temperature. The volume and initial water temperature of the sensor was therefore kept constant for all diets during autoclaving. Variations in the total treatment time of the autoclave cycle was, however, not possible to manipulate due to the positive relationship between steam pressure and temperature (Ljøkjel, 2002). Thus, at higher set-temperatures, the total treatment time was slightly longer due to the time needed to generate higher steam pressure and temperature, in addition to the time needed to cool the autoclave.

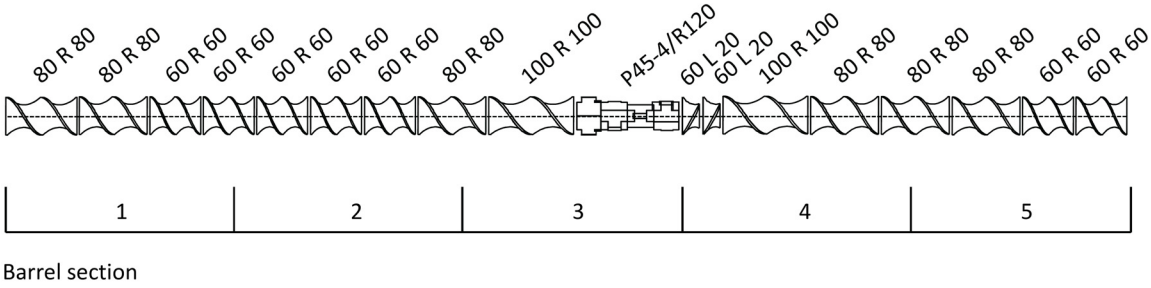

Figure 5. Illustration of the standardized screw configuration (SCF1) used during extrusion in **Paper I**, from inlet (left) to outlet. The notation of the individual screw elements are described in **Paper I**. The kneading section consisted of a polygon element ('P45-4/R120'), followed by two backward conveying elements ('60L20') that had a 90º twist-off angle. The expander-treated FFSBM diets were extruded using a similar screw configuration (SCF2), except that the two '60L20' elements were continuous to each other.

4.3 Measurement of digestibility and bioavailability of nutrients

In this thesis, digestibility is defined as the proportion of an ingested nutrient which is not excreted in the feces and which is, therefore, assumed to be absorbed by the animal (McDonald et al., 2002). Bioaccessibility is defined as the proportion of an ingested nutrient that is released from the food matrix and is available for intestinal absorption (Hedrén et al., 2002), whereas bioavailability is defined as the proportion of an ingested nutrient which is available for physiological utilization and storage (Parada and Aguilera, 2007). Nutrient digestibility was measured *in-vivo* as apparent total tract digestibility and does not account for endogenous losses from the animal (**Papers I-III**). Bioaccessibility in each diet was determined as solubilized protein by reproducing the pH profile and total

reaction time during the acid and alkaline phases of the hydrolysis in the *in vitro* assay. *In vitro* bioavailability of AA was estimated using a two-step GIM (Hamdan et al., 2009) with crude enzyme extracts from Atlantic salmon (**Paper III**).

Fecal samples were obtained by different methods for determination of digestibility in salmonids and mink. In the fish experiments (**Papers I** and **II**), samples were obtained by fecal stripping according to Austreng (1978). In the mink experiments (**Papers I** and **III**), feces were quantitatively collected from each animal at four consecutive days as described by Skrede et al. (1980). Apparent digestibility was measured indirectly, using the indicator method (**Papers I** and **II**), or directly with total collection of feed intake and fecal output (**Paper III**). The indicator method is commonly used for digestibility estimation in fish, due to the difficulty in obtaining quantitative measures of feed intake and total fecal output (Storebakken et al., 1998). Yttrium oxide (Y_2O_3) was used as inert marker in **Paper I**, whereas a combination of yttrium and chromic oxide (Cr_2O_3) were used in **Paper II** in order to ensure that at least one of the two markers could be analyzed following the experiment. The chromic oxide was included at 1 g kg^{-1} to the diets, which is lower than commonly referred levels of 5 to 10 g kg^{-1} diet (Austreng et al., 2000). The latter authors reported that high dietary levels (10 to 20 g kg^{-1}) of chromic oxide may disturb the absorption and metabolism of nutrients in fish. Thus, chromic oxide was included at the lowest possible level that could be analyzed in order to not bias the digestibility estimates (**Paper II**). Following the experiment, yttrium oxide was used to determine apparent digestibility.

The indicator method was also used for determination of digestibility in mink (**Paper I**). Earlier investigations have shown similar digestibility estimates when the indicator method using yttrium oxide was compared to total collection of feces (Vhile et al., 2007). However, the accuracy of the indirect method is dependent on high fecal recovery of the inert marker, as low recovery may underestimate digestibility estimates (Austreng et al., 2000). At a dietary inclusion level of 0.1 g kg^{-1}, Vhile et al. (2007) reported an average fecal recovery of yttrium oxide of 94.4% in mammalian carnivores (dogs, blue foxes and mink). The fecal recovery of the inert marker was lower (88%) in **Paper III** than reported in Vhile et al. (2007). The digestibility of CP and AA were, therefore, slightly underestimated. Consequently, the digestibility coefficients in **Paper III** were calculated quantitatively, based on total collection of feed intake and fecal output.

The *in vitro* assay in **Paper III** was carried out in order to predict nutritional quality of experimental diets by use of enzymes from Atlantic salmon in relation to *in vivo* digestibility in mink. Although *in vitro* techniques simplifies the mechanisms occurring *in vivo*, they are rapid and inexpensive tools for estimating nutrient availability of both feed ingredients (Morales and Moyano, 2010; Sáenz de Rodrigáñez et al., 2011) and experimental diets (Rungruangsak-Torrissen et al., 2002). In the present work, the *in vitro* method enabled evaluation of potential bioavailability of protein in unpelleted feed, which is an advantage in small-scale experiments. Differences in the nutritional quality of feeds, as measured by *in vitro* techniques, have shown to correspond with *in vivo* digestibility estimates (Bassompierre et al., 1997; 1998; Chong et al., 2002; Rungruangsak-Torrissen et al., 2002). However, the main issue with *in vitro* techniques is related to their sensitivity, and thus, ability to separate between different experimental treatments and how accurately they predict *in vivo* digestibility estimates (Carter et al., 1999). Sensitivity of the assays can be improved when using enzymes obtained directly from the targeted fish species (Alarcón et al., 2002; Rungruangsak-Torrissen et al., 2002). In addition, a better simulation of the *in vivo* conditions taking place in carnivorous fish is obtained when an acid pre-digestion is followed by alkaline hydrolysis (Morales and Moyano, 2010). The *in vitro* assay employed in **Paper III** considered the two aforementioned features and was based on a two-step GIM using a semipermeable membrane reactor as described by Hamdan et al. (2009), which allowed continuous removal of digestion products during hydrolysis of substrates. It should be emphasized that *in vitro* techniques only estimate the amount of hydrolyzed nutrients during gastric and/or intestinal digestion and cannot mimic the absorption of nutrients. Consequently, the results obtained *in vitro* are only partially consistent with digestion values obtained *in vivo*. The results from the *in vitro* assays in **Paper III** are, therefore, expressed as an estimate on the amount of bioavailable AA resulting from hydrolysis of dietary protein by salmon proteases.

4.4 Allotment, experimental design and statistical treatment

The experiments in **Papers I-III** were planned as factorial designs with 3 or 4 replicates per treatment, depending on the experimental animal. The feeding experiment with Atlantic salmon in **Paper I** was carried out with 12 diets, each fed to fish in 12 sea cages over three succeeding feeding periods to obtain three replicates per treatment, where cage

within period was the experimental unit. Each feeding period was terminated by stripping and marking the fish that were stripped. A new feeding period was then started by randomly re-allotting the 12 diets to the 12 sea cages, so that each diet was assigned to a new sea cage. This regime ensured that no fish were fed the same diet, or stripped twice. Triplicate cages of fish per treatment would have been preferred; however, increasing the number of sea cages was not economically feasible.

During the course of the feeding experiment, it was evident that the environmental conditions were suboptimal and affected the feed intake of salmon. This may have contributed to the high within-treatment variability observed on the digestibility estimates among the different feeding periods. Consequently, a follow-up experiment was conducted with the FFSBM diets, using mink as experimental animal (**Paper I**). Although the use of mink only provide indirect information on apparent digestibility in salmon (Romarheim et al., 2005), there is a general consensus that apparent total tract digestibility of AA correlates between these species (Skrede et al., 1980; 1998; Øverland et al., 2006). Feeding experiments with mink are also advantageous for practical reasons, because it allows better control of each individual and the experimental conditions at which the animals are kept. The mink experiments were carried out with 4 replicates per treatment, where each individual represented an experimental unit (**Papers I** and **III**).

The feeding experiment with rainbow trout in **Paper II** was carried out with three replicate tanks per treatment, with each tank representing an experimental unit. The fish were stripped for feces three times during the experimental period in order to collect enough material for the chemical analyses. The number of recovery days between each of the three strippings was 8, 6 and 10 days, respectively. Following the experiment, one tank was randomly selected and fecal material from each stripping was analyzed for CP and yttrium oxide in order to elucidate potential variation caused by the different number of recovery days. The apparent CP digestibility showed low variation among the three different sampling points, and thus, the fecal samples from each sampling were pooled for each tank.

The statistical analyses of data in **Papers I-III** were performed using the General Linear Model (GLM) procedure of SAS 9.2 (1990). As discussed previously, the high within-treatment variability of the digestibility experiment with salmon was a concern in **Paper I**, because the independent variables seemed to have little influence on the dependent va-

riables. The statistical analyses unveiled that the proportion of variation explained by the model (R^2) was low (Table 5), indicating that an additional source of variation influenced the response (Shearer, 2000). The greater error variance may be due to the presence of confounding factors arising from variations in (1) the number of days in each feeding period, (2) feeding schedules (one vs. two daily feedings), (3) environmental factors, including low water temperature, short photoperiod and poor weather conditions, and/or (4) low feed intake. Although confounding factors decrease the sensitivity of a statistical test and could hide the presence of real differences (Motulsky, 1995; Norman and Streiner, 2008), they cannot be accounted for in the statistical model unless they are known (Townend, 2002). This was the main rationale for conducting the follow-up experiment with mink. The results from this experiment showed a substantially higher R^2 for the digestibility estimates of CP and AA (Table 5). This suggests that the experimental design and statistical model were adequate, and that the high error variance of the salmon experiment most likely was influenced by a combination of the aforementioned variations in experimental procedures, environmental factors, and feed intake.

Table 5.
The proportion of variation explained by the model (R^2) for data derived from the feeding experiments with Atlantic salmon and mink (Paper I).

	Atlantic salmon (n = 18)	Mink (n = 24)
Lipid	0.40	0.42
Ash	0.45	0.80
Starch	0.29	0.25
CP	0.33	0.78
Total AA	0.45	0.91

5. Discussion of main results

The present study aimed to increase the knowledge on how to improve the nutritional and physical quality of plant ingredients in diets for salmonid fish by use of thermo-mechanical treatment and acid salts. This chapter combines and discusses the main results from **Papers I-III** with respect to the effects of plant ingredients, processing temperatures and acid salts on nutritional and physical quality of feeds.

5.1 Inclusion of plant ingredients in diets for fish

FM is a limited resource (Naylor et al., 2009); it is therefore increasing focus on alternatives, such as plant proteins, plant by-products from breweries and single-cell protein (Gatlin et al., 2007; Burr et al., 2011). SBM is one of the most widely studied plant ingredient and is extensively used in diets for carnivorous fish. The results in **Paper I** showed that inclusion of soybeans in diets for Atlantic salmon gave similar apparent digestibility of macronutrients as previously reported by Aslaksen et al. (2007), but lower than described by Refstie et al. (1999; 2000; 2001). This could be an effect of low feed intake due to the presence of heat-stable ANF in soy (Refstie et al., 1998), as the TIA level of the diet was below the tolerance limit for Atlantic salmon (Olli et al., 1994; **Paper I**). Another reason may be that feed intake was reduced due to the low water temperatures (4.4 to 6.8 ºC) during the feeding experiment. Low water temperatures reduce the activity of digestive enzymes (Torrissen, 1984), and hence, the digestibility of macronutrients in salmonids (Watanabe et al., 1996; Olsen and Ringø, 1998). In addition, low feed intake may result in a greater proportion of endogenous protein in the fecal waste (Bureau et al., 2002). This may lead to a decrease in the apparent, but not the 'true' digestibility of the diet (Azevedo et al., 1998). The low digestibility of macronutrients and especially protein in the soybean diets may, therefore, be explained as an effect of low feed intake due to heat-stable ANF in soybeans or low environmental temperatures.

The use of FFSBM compared to SBM in fish feeds is advantageous for several reasons. The FFSBM contains high levels of protein (~370 g kg^{-1}) and lipid (~190 g kg^{-1}), while

defatted SBM contains high levels of protein (~460 g kg^{-1}) but low levels of lipid (≤10 g kg^{-1}) (Hertrampf and Piedad-Pascual, 2000). Further, FFSBM has not been subjected to additional processing, which may add costs to the final product and/or result in a reduced protein quality. Thus, the use of FFSBM in fish feeds could be cost-efficient while at the same time providing both native protein and lipids in the diet. There is, however, a need to inactivate the heat-labile trypsin inhibitors (TI) in raw FFSBM in order to ensure a high protein digestibility and growth of salmonid fish (Romarheim et al., 2006). The results in **Paper I** showed that the digestibility of several AA was significantly lower in diets with FFSBM compared to SBM when both diets had undergone extrusion at 110 ºC. This was probably caused by ANF other than TIA in soybeans, as previously suggested in studies with fish and terrestrial animals (Olli et al., 1994; Clarke and Wiseman, 2005).

In contrast to soybean products, BPC is a relatively new feed ingredient and only one report exists on the use of this product in diets for fish. Protein digestibility of the BPC diet in trout was 84% on average, and the digestibility of all EAA was significantly lower than the FM-based diets (**Paper II**). This might be due to the lower concentration of EAA in BPC compared to FM, as discussed in **Paper II**. In comparison, Burr et al. (2011) reported protein digestibility values of 96% for Atlantic salmon fed BPC from the same manufacturer as used in the present study. The lower digestibility of protein in **Paper II** might be due to differences in nutrient availability among different batches of BPC. The low digestibility of protein in trout fed BPC may also be due to a negative effect of microbial membrane and cell wall components of the yeast residues in BPC (Rumsey et al., 1991). Similar effects have also been observed on protein digestibility in salmonids fed diets containing bacterial meal (reviewed in Øverland et al., 2010).

The findings presented in **Papers I** and **II** highlights the importance of using apparent digestibility as a tool for evaluating the nutritional quality of plant ingredients, although growth data would have provided more information on nutrient utilization. Although the soybean products contained low levels of TI, there seemed to be other compounds present that negatively affected nutrient digestibility in Atlantic salmon. In contrast, the BPC contains lower levels of ANF but may contain a considerable amount of soluble fiber. The BPC could potentially replace a considerable proportion of FM in diets for salmonids if other essential nutrients are provided as supplements. The research indicated that this product is a promising ingredient for future production in northern America and Europe.

5.2 Feed processing temperatures

5.2.1 Heat treatment and effects on nutritional quality

Expander pretreatment of FFSBM at two different temperatures prior to extrusion enhanced the apparent digestibility of total AA and some individual AA in Atlantic salmon and improved the digestibility of most major nutrients and all individual AA in mink (**Paper I**). The improvement was more consistent in the latter species, which may in part reflect the overall lower variation among experimental animals when using mink as a model animal for fish (see section 5.4). The improved digestibility of AA in both Atlantic salmon and mink was most likely caused by the reduction in dietary TIA following heat treatment, which is in agreement with previous research (Romarheim et al., 2005; 2006). Expander pretreatment reduced the dietary TIA content by more than 40% compared to extrusion without pretreatment, but the overall TIA levels were within the tolerance level of Atlantic salmon irrespective of the heat treatment applied (Olli et al., 1994). These findings suggest that extrusion is sufficiently extensive to reduce TIA to safe levels for Atlantic salmon (Romarheim et al., 2005), but that the reduction in TIA and improvement in digestibility of AA is greater when applying expander pretreatment at moderate temperatures prior to extrusion. Furthermore, there were no differences in dietary TIA between diets that were expander pretreated at 100 and 120 ºC. This could be explained by the longer exposure time of the FFSBM to moist heat, as increased treatment times reduce the activity of TI in soybeans (Kaankuka et al., 1996). Also, the improved digestibility of AA by expander pretreatment may be explained by increased protein solubility by additional heat treatment (Clarke and Wiseman, 2007).

The apparent digestibility of most major nutrients and individual AA were improved when increasing the extrusion temperature from 110 to 150 ºC (**Paper I**) and from 110 to 141 ºC (**Paper II**). The improved digestibility by increasing extrusion temperatures can be explained by increased protein solubility due to denaturation and structural unfolding of the protein molecules (Cheftel, 1986). Such structural changes render the protein more accessible to digestive proteases (Phillips, 1989). This is in line with the observed increase in the content of available lysine with increasing temperatures (**Paper II**). Reduction in nutritional quality due to heat treatment is likely to affect some AA more than others, such as arginine, cysteine and lysine (Ljøkjel et al., 2000). No reductions were

observed on the digestibility of these AA in the present work (**Papers I** and **II**), which supports the conclusion that extrusion temperatures in the range between 100 and 150 ºC is lenient regarding nutritional quality of feeds for salmonids (Sørensen et al., 2002; 2005). However, the results from **Papers I** and **II** did not differentiate between improvements in digestibility caused by inactivation of TIA or increased protein solubility due to protein denaturation.

In contrast to the improved apparent digestibility of CP and AA in salmonids with increasing extrusion temperatures (**Papers I** and **II**), the results in **Paper III** indicated that the digestibility of macronutrients and individual AA in mink decreased linearly in response to increasing autoclave temperatures. The reduction in CP and AA digestibility was evident when increasing the temperature from 110 to 120 ºC. Increasing the autoclave temperature from 120 to 130 ºC gave a further reduction in the digestibility of methionine and cysteine in mink (**Paper III**). The reduced digestibility of CP and individual AA due to heat treatment is in agreement with previous results where mink was fed diets autoclaved at temperatures from 130 to 135 ºC (Skrede and Krogdahl, 1985; Ljøkjel et al., 2000). In comparison, extrusion at temperatures between 100 to 150 ºC did not give the same reduction in CP and AA digestibility in mink (Ljøkjel et al., 2004; **Paper I**). This indicates that the longer treatment time in the autoclave aggravated heat-induced structural changes to the proteins, resulting in reduced nutritional quality of the diets (Papadopoulos, 1989). The decrease in nutritional quality was evidenced by a reduction of dietary AA and available lysine concurrent with an increased browning. This could be explained by the formation of Maillard browning products between reducing sugars and the amino group of lysine, resulting in reduced dietary content of chemically reactive lysine (Moughan, 2003). Structural changes to proteins due to heat treatment is typically shown by a reduction in protein solubility (Phillips, 1989), especially at increasing treatment times (Arndt et al., 1999). The results from the *in vitro* model in **Paper III** showed that protein solubility was significantly reduced for diets autoclaved at 130 ºC. This coincided with the observed decrease in the amount of AA released during *in vitro* hydrolysis by digestive enzymes from Atlantic salmon. It can, therefore, be concluded that the heat treatment applied in **Paper III** was more excessive than the extrusion in **Papers I** and **II**, mainly due to the adverse combination of high temperatures and long retention times.

5.2.2 Effects of extrusion on physical quality of the feed

Physical quality of the extruded feed was affected by extrusion temperature (**Papers I** and **II**). Extrusion at 150 ºC increased the pellet diameter and expansion ratio compared to pellets extruded at 110 ºC, but resulted in decreased hardness, durability and WSI (**Paper I**). These results can be explained by the higher SME levels of diets extruded at 150 ºC, leading to increased starch gelatinization and pellet expansion (Chinnaswamy, 1993; Garber et al., 1997). Increased expansion is commonly associated with lower hardness and durability due to a more porous pellet structure (Aarseth et al., 2006b; Hansen and Storebakken, 2007; Sørensen et al., 2009). It is reasonable to assume that porous pellets will disintegrate faster in water than compact pellets with a low expansion, and this could partly explain the reduced WSI at increasing extrusion temperatures.

In contrast to the findings in **Paper I**, the results from **Paper II** showed that extrusion at 141 ºC yielded pellets that were more durable than pellets extruded at 110 ºC, whilst no effects of extrusion temperature were observed on pellet hardness, expansion ratio and WSI. This may reflect the overall low expansion ratio of pellets in this study. Compared to **Paper I**, the low expansion and response in physical quality to different extrusion temperatures could be attributed variations in extruder equipment, processing conditions and/or diet formulation. The experimental diets in **Paper II** contained higher amounts of starch and could explain the overall high physical quality of feeds compared to the results obtained in **Paper I**. Also, the extruder used in **Paper II** had a lower throughput capacity and required the use of heating jackets to increase the extrusion temperature up to 141 ºC. The SME was not quantified in that study, but it may be reasonable to assume that this extruder generated a lower mechanical energy input and resulted in low expansion of the pellets and, thus small differences in physical quality parameters.

These findings imply that extrusion temperatures affect the physical quality of pellets, as shown by the different response in **Papers I** and **II**. This is most likely due to variations in the extrusion equipment (**Papers I** and **II**) and the functional properties of the individual ingredients present in the diet formulation. In order to optimize physical quality of feeds, factors such as processing equipment and conditions should be considered in relation to functional properties of the ingredients. The relationship between ingredients and processing conditions on physical quality of feeds requires further investigation.

5.3 Supplementation of acid salts

5.3.1 Effects of organic acid salts on nutrient digestibility in salmonids

Apparent digestibility of main nutrients and individual AA were generally not affected by dietary KDF in Atlantic salmon (**Paper I**), but were improved by dietary NaDF in rainbow trout (**Paper II**). The observed differences in digestibility of CP and AA in salmon and trout by dietary acid salts are summarized in Table 6. The differences in apparent digestibility of CP and AA with supplemental acid salts ranged from −1.5 to 0.8 in **Paper I** and from −0.6 to 2.9 in **Paper II**. Previous research on the use of KDF in diets for Atlantic salmon is variable and does not show consistent improvements in nutrient digestibility (reviewed in section 2.4). This could be explained by the specific taste preferences of fish (Kasumyan and Doving, 2003), and the fact that salmon is generally known to be more sensitive to off-flavors and variations in diet composition than trout (Refstie et al., 2000). It is reasonable to assume that the 0.4 U reductions in pH of diets with KDF affected the acceptability in salmon, which in turn, reduced the intake and efficiency of the acid salt.

The ability of organic acids and their salts in reducing dietary pH appears to be an important property of acidifiers in improving the performance of pigs (Blank et al., 1999; Partanen and Mroz, 1999). The acidifying properties of KDF and NaDF were demonstrated by a 0.4 to 0.5 U reduction in diet pH (**Papers I-III**). Atlantic salmon and rainbow trout has a higher gastric pH compared to mammals due to their low innate acid secretory capacity (Sugiura et al., 2006). The two acid salts should, therefore, have the potential to increase gastric acidity and complement the endogenous acid production in salmonid fish. The lack of effect of dietary KDF in Atlantic salmon may indicate that the pH-lowering effect of the acid salt was affected by external factors, such as different rearing environments in the two fish experiments. The main difference between the fish experiments in **Papers I** and **II** was that the salmon was kept in saltwater while the trout was kept in freshwater. Fish in saltwater drinks water in order to maintain osmoregulation (Lall, 2002); hence, there is a possibility that the greater water consumption diluted the gastric acidity of the salmon stomach. In addition, the minerals present in saltwater could have weakened the pH-lowering effect of KDF due to the high buffering capacity of minerals

Table 6.

Influence of dietary acid salts on the apparent digestibility of crude protein (CP), essential amino acids (EAA) and non-essential amino acids (NEAA) in salmonids, using digestibility values from the feeding experiments with Atlantic salmon (Paper I) and rainbow trout (Paper II).

Paper	I				II	
Diet/Acid salt	FFSBM/KDF (12)		SBM/KDF (12)		NaDF (10.6)	
	D (%)	ΔD	D (%)	ΔD	D (%)	ΔD
CP	79.6	+ 0.9	82.4	+ 0.2	82.8	+ 1.0**
EAA						
Arginine	91.3	+ 0.2	93.0	+ 0.8*	89.9	+ 1.2***
Histidine	85.2	+ 0.2	87.7	+ 0.5	87.1	+ 0.9**
Isoleucine	87.8	+ 0.1	90.4	+ 0.3	91.7	+ 0.6*
Leucine	88.6	+ 0.3	91.2	+ 0.4	91.7	+ 0.6*
Lysine	90.4	+ 0.5	92.4	+ 0.3	89.5	+ 0.7**
Methionine	89.6	− 0.1	91.3	+ 0.5	88.6	+ 1.1**
Phenylalanine	85.6	− 0.1	89.0	+ 0.6	84.9	− 0.6
Threonine	82.2	+ 0.2	85.5	+ 0.5	86.4	+ 1.0**
Valine	87.0	+ 0.2	89.7	+ 0.3	90.3	+ 0.6*
NEAA						
Alanine	89.3	+ 0.2	91.2	+ 0.4	86.5	+ 1.6***
Aspargine	83.3	+ 0.2	85.3	+ 0.3	78.5	+ 1.3*
Cysteine	71.3	+ 0.6	73.4	+ 0.3	76.7	+ 1.5*
Glutamine	90.3	+ 0.3	92.0	+ 0.4	91.7	+ 0.6**
Glycine	85.6	+ 0.2	87.0	+ 0.6	76.3	+ 2.9***
Proline	85.9	+ 0.4	88.8	+ 0.4	85.4	+ 1.9***
Serine	84.3	+ 0.1	86.9	+ 0.6	86.4	+ 1.1**
Tyrosine	96.9	− 1.5	96.0	+ 0.8	94.2	+ 1.3***

Diet: FFSBM, full-fat soybean meal diet; SBM, hexane-extracted and toasted soybean meal diet. Acid salt (g kg^{-1}): KDF, potassium diformate; NaDF, sodium diformate. D, digestibility main effect of diets without acid salt; ΔD, difference in digestibility main effects when adding acid salt to the diets. Significant differences are indicated by asterisks (*P<0.05; **P<0.01; ***P<0.001). Table style adapted from Partanen and Mroz (1999).

(Jasaitis et al., 1987). These factors could have lowered or masked the beneficial effect of the acid salt on digestibility in salmon and may, in part, explain the different response towards dietary acid salts between salmon and trout in **Papers I** and **II**. The form of the organic acid used is crucial for the effectiveness in diets for salmonids. In the present study, we used potassium or sodium diformate, which are acid salts in that they contain both a salt and an acid. On the other hand, Gao et al. (2011) used salts of organic acids such as sodium butyrate and sodium formate and found no effects on dietary pH or nutrient digestibility in rainbow trout.

5.3.2 Nutrient digestibility in salmonids versus mink and *in vitro*

The present work demonstrated that inclusion of KDF did not affect apparent digestibility in mink and Atlantic salmon (**Paper I**). However, supplementation of NaDF improved the digestibility of most major nutrients and AA in rainbow trout (**Paper II**), whereas no significant effects of dietary NaDF were observed in mink fed similar diets (**Paper III**). This led to the assumption that the effectiveness of organic acid salts is species-dependent (**Paper III**). The main physiological difference between mink and salmonids is stomach acidity. At pH conditions lower than the dissociation constant of KDF and NaDF (pK_a 3.75), such as in mink (pH ~2), the majority of the acid moiety would remain undissociated. Undissociated short-chain organic acids are rapidly absorbed through the stomach wall and metabolized, or they proceed to the intestine at which they may assert antimicrobial properties. The latter aspect has been evidenced in species with similar gastric pH as mink, in this case red hybrid tilapia, where inclusion of 0.2 g KDF kg^{-1} resulted in a significant reduction in fecal bacteria counts without significantly affecting stomach pH and nutrient digestibility (Ng et al., 2009). Compared to tilapia, mink has a low degree of post-ileal fermentation (Skrede, 1979; Zhou et al., 2009). Consequently, the potential antimicrobial effect of organic acid salts is not expected to have a substantial impact on nutrient digestibility in mink. Salmonid fish such as salmon and trout, on the other hand, has a gastric pH in the range of ~4.0–4.5 (Austreng et al., 2000; Sugiura and Ferraris, 2004). Furthermore, the proteolytic activities in the digestive tracts of salmon and trout has an optimum pH of about 2 for the peptide-like activity from the stomach extracts and about 9 for the tryptic-like activities from the pyloric ceca and intestinal extracts (Rungruangsak and Utne, 1981; Torrissen, 1984). Thus, a reduction in gastric pH due to

supplemental exogenous acids could serve the endogenous acid production in these species and potentially improve the digestibility of CP and AA due to increased proteolytic activity. Similar hypotheses have been proposed to explain the improved CP and AA digestibility in early-weaned pigs fed diets with organic acids (Blank et al., 1999). This implies that organic acid salts have a greater potential to improve the performance of non-ruminant animals that has an inadequate HCl secretion and/or considerable post-ileal fermentation. As mink seem to not benefit from the pH-lowering and antimicrobial effect of organic acids, it may be questionable to use mink as a model animal for salmonids when assessing the effectiveness of organic acid salts in diets for fish. The potential species-dependent response to dietary organic acid salts were hypothesized based on the observations obtained in the present work. More research is needed to fully elucidate the mechanisms of acid salts on nutrient digestibility in monogastric species.

In vitro assays are rapid and inexpensive tools for measuring bioavailability of nutrients in fish. From an ethical standpoint, *in vitro* methods have a greater social acceptance due to the controversy of using experimental animals in scientific research. The two-step *in vitro* GIM applied in **Paper III** was used to estimate the bioavailability of AA in diets by use of crude enzyme extracts from Atlantic salmon. The results indicated an increased release of AA during hydrolysis in the presence of NaDF, in particular when the diet was treated at the highest temperature (130 ºC). In contrast, the *in vivo* digestibility of total AA in mink showed a numerical ($P>0.05$) decrease with NaDF (**Paper III**). Although the *in vitro* and *in vivo* response to NaDF is not directly comparable, the *in vitro* assay may provide important information on the bioavailability of AA in salmon. In future research, this type of information should be correlated with the *in vivo* response in the targeted animal species in order to validate the predictions made by the *in vitro* model.

5.3.3 Effects of organic acid salts on physical quality of feeds

Physical quality of feed pellets was generally improved by inclusion of KDF (**Paper I**) and NaDF (**Paper II**), as summarized in Table 7. The improvement in pellet durability was greater in diets with KDF compared to NaDF, which may reflect the initial differences in durability in diets containing no acid salt. The pellet expansion decreased by adding KDF to both FFSBM and SBM diets (**Paper I**). Pellet expansion is a measure on

the ratio between pellet diameter and die diameter. Thus, the negative expansion ratio obtained in diets with KDF could be explained by shrinkage of the pellets after expansion due to loss of moisture and collapse of the pellet structure (Arhaliass et al., 2003). As discussed in **Paper I**, the reduced expansion could also be explained by the loss of hydrophilic properties of the starch molecules in the presence of formic acid due to a lower degree of starch gelatinization. The pellet expansion was generally low in **Paper II**, but increased slightly when adding NaDF. Reasons for the small differences in physical quality of the feeds in response to NaDF in **Paper II** could be explained by the extrusion equipment applied in that experiment, as previously discussed (see section 5.2.2). The WSI of the extruded pellets were improved by adding acid salts (**Papers I** and **II**), which may indicate that the binding properties were improved in the presence of acid salts.

Table 7.

Influence of dietary acid salts on physical quality parameters, using values from Papers I and II.

Paper	I				II	
Diet/Acid salt	FFSBM/KDF (12)		SBM/KDF (12)		NaDF (10.6)	
	Mean	Δ	Mean	Δ	Mean	Δ
DORIS durability (%)	24.4	+ 16.1***	70.4	+ 15.5***	91.7	+ 3.2***
Hardness (N)	12.5	+ 5.2***	29.9	+ 14.2***	25.0	+ 1.2
Expansion ratio (%)	9.0	− 8.5***	13.3	− 3.6**	1.3	+ 1.4***
Water stability (%)	74.5	+ 5.5**	80.2	+ 4.7***	85.9	+ 5.2***

Diet: FFSBM, full-fat soybean meal diet; SBM, hexane-extracted and toasted soybean meal diet. Acid salt (g kg^{-1}): KDF, potassium diformate; NaDF, sodium diformate. Mean, physical quality main effect of diets without acid salt; Δ, difference in physical quality main effects when adding acid salt to the diets. Significant differences are indicated by asterisks (*P<0.05; **P<0.01; ***P<0.001). Table style adapted from Partanen and Mroz (1999).

6. Conclusions

- Thermo-mechanical treatment at high temperatures improved the digestibility of plant ingredients in salmonids. This was a result of decreased content of heat-labile ANF and higher availability of AA due to denaturation and structural unfolding of protein molecules.

- Heat treatment at long treatment times by autoclaving had a negative effect on the nutritional quality of feeds, as shown by the reduced content of dietary AA, *in vivo* digestibility and *in vitro* bioavailability. These negative effects were mainly explained by decreased AA availability due to heat-induced structural changes in the protein.

- Supplementation of acid salts did not give consistent improvements in digestibility of plant ingredients in salmonids. The mode of action of organic acid salts in salmonids remains unclear and further investigation is needed to verify this hypothesis.

- There were no interactive effects of heat treatment and acid salts on the digestibility of protein in salmonids. Also, no such effects were observed during prolonged heat treatment, indicating that acid salts do not protect proteins from heat-induced structural changes.

- Both increasing temperatures during extrusion and supplementation of acid salts affected the physical quality of feeds. The improvement in physical quality by acid salts indicates increased binding of feed particles.

7. Implications and future directions

- Although expander pretreatment of FFSBM had a positive effect on the digestibility of some AA, the improvement was moderate and indicated that use of FFSBM in diets for Atlantic salmon is limited due to heat-stable ANF or other compounds that may interfere with digestive processes (Olli et al., 1994; Refstie et al., 1998).

- The work presented in this thesis did not elucidate the mode of action of organic acid salts when added to diets for Atlantic salmon and rainbow trout. Considering the inconsistent results reported on the use of organic acid salts in salmon, there is a need to investigate the underlying biological mechanisms of acid salts in fish. Future research with organic acid salts in diets for salmonids should include measures on feed intake to eliminate potential interference with the results. Determination of gastrointestinal pH and microflora, as well as recovery of the organic acid in the various segments of the digestive tract could increase the understanding of the mechanism of acid salts in fish. Also, little information exists on the metabolic effects of organic acids in salmonids such as their effects on the acid-base balance.

- The *in vitro* GIM applied in the present work is a promising tool for assessing effects of heat treatment and feed additives on the bioaccessibility and bioavailability of diets by use of digestive enzymes from the target species. This method is rapid, inexpensive and reduces the number of research animals needed. Nevertheless, there is a need to improve the method to obtain a better correlation between results obtained in *in vitro* and *in vivo* assays.

- Extrusion can be used to obtain fish feeds with specific nutritional and physical properties. In order to improve the understanding of the complex relationship between raw materials, functional properties and processing conditions on nutritional and physical quality of feeds, more details regarding processing conditions need to be reported in future research. Also, when including measures of physical quality the understanding of the relationship between physical quality and biological response would increase.

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Paper I

Aquaculture Nutrition

Aquaculture Nutrition 2011 doi: 10.1111/j.1365-2095.2011.00872.x

Effects of feed processing conditions and acid salts on nutrient digestibility and physical quality of soy-based diets for Atlantic salmon (*Salmo salar*)

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Abstract

Two experiments were conducted to investigate effects of feed processing conditions and potassium diformate (KDF) supplementation on apparent digestibility of nutrients in Atlantic salmon (*Salmo salar*) and physical quality of extruded feed. In Exp. 1, diets with raw or expander pretreated full-fat soybean meal (FFSBM) at 100 or 120 °C were extruded at 110 °C. Expander pretreatment significantly ($P < 0.05$) improved the digestibility of arginine, glutamine and tyrosine in Atlantic salmon. The higher digestibility of expander pretreated FFSBM was confirmed in mink (*Mustela vison*). In Exp. 2, diets with defatted soybean meal (SBM) were extruded at 110, 130 or 150 °C. The results showed that increasing extrusion temperatures significantly ($P < 0.05$) improved the digestibility of most major nutrients and amino acids in Atlantic salmon. In general, KDF supplementation to FFSBM and SBM diets did not affect digestibility of nutrients in Atlantic salmon or mink. Expander pretreatment and increasing extrusion temperatures increased pellet expansion, while KDF supplementation reduced pellet expansion.

KEY WORDS: digestibility, expander, extruder, physical quality, potassium diformate, soybeans

Received 16 November 2010, accepted 29 March 2011

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Introduction

Soybeans are used as protein source in salmonid diets because of its high crude protein content, reasonably balanced amino acid profile, abundance and low price per protein unit (Hertrampf & Piedad-Pascual 2000; Storebakken *et al.* 2000; Gatlin *et al.* 2007). The potential of soybean meal (SBM) for salmonids is, however, limited by the presence of several antinutrients (Storebakken *et al.* 2000; Francis *et al.* 2001), of which one of the most important is the heat-labile trypsin inhibitors (TI) (Liener 1994). Full-fat soybean meal (FFSBM) has a higher content of TI than conventional defatted SBM, which has been toasted following oil extraction (Hardy & Barrows 2002). In addition to the temperature itself, the duration of heating and moisture content are also important factors for reducing the TI activity (TIA) and thereby improving the nutritional value of soybeans (Zarkadas & Wiseman 2005). Leeson & Atteh (1996) reported that the heat generated during extrusion was sufficient to reduce TIA activity in FFSBM, and Romarheim *et al.* (2006) showed that extrusion of feed was sufficient to reduce TIA in flaked, defatted, untoasted soybeans (white-flakes, WF) to less than 5 g kg^{-1} feed when dietary WF inclusion was 250 g kg^{-1}. Previous results have shown that a dietary TIA level at 5 g kg^{-1} may be considered acceptable in diets for Atlantic salmon (Olli *et al.* 1994). Heating aimed at completely inactivate TI in the feed is not an option, as overheating may reduce protein solubility and nutritional value without further lowering the TI content of the feed (Arndt *et al.* 1999; Iwe *et al.* 2001).

The FFSBM contains approximately 370 g kg^{-1} protein and 190 g kg^{-1} fat, whereas defatted SBM contains on average 460 g kg^{-1} protein and low levels of fat (≤10 g kg^{-1}) (Hertrampf & Piedad-Pascual 2000). The fat content of FFSBM may cause challenges during feed processing when the total lipid level of the diet exceeds 70 g kg^{-1} (Rokey 2007). Moreover, high fat contents are known to negatively influence physical quality of extruded feed (Sørensen *et al.*

2009). During extrusion processing, raw materials are cooked by applying moisture, pressure, temperature and shear forces. Cooking initiates a series of physiochemical reactions, including starch gelatinization (Colonna *et al.* 1989) and protein unfolding (Phillips 1989), which affects the binding between feed particles and hence physical quality (Thomas & van der Poel 1996). Fat interferes with the binding between particles, first by reducing the degree of cooking by lubricating the interface between the dough and the extruder elements and secondly by acting as an insulating agent and thus preventing water from being absorbed by the particles (Lin *et al.* 1997). In intensive aquaculture production, it is important that feed pellets are of high physical quality to avoid feed losses and water emissions (Aarseth 2004; Aarseth *et al.* 2006a; Sørensen *et al.* 2011). Additionally, physical quality may also affect feed intake and nutrient utilization of fish (Hilton *et al.* 1981; Baeverfjord *et al.* 2006; Venou *et al.* 2009) and should therefore be taken into consideration when formulating fish feeds.

Organic acids salts have been reported to promote growth and health performance in warm-blooded monogastric animals and fish because of their antimicrobial effects (Øverland *et al.* 2000; Mikkelsen *et al.* 2009; Zhou *et al.* 2009). A recent study with Atlantic salmon has shown that the addition of potassium diformate (KDF) to a plant-based diet improved protein digestibility (Storebakken *et al.* 2010). Furthermore, interactive effects between KDF supplementation and heat treatment showed that the digestibility of individual amino acids in diets with KDF increased with increasing temperatures, while the digestibility decreased in diets without KDF. Research investigating the effect of acid salts on digestibility of plant-based diets for salmonids is, however, limited. Therefore, the objective of this study was to investigate the effects of ingredients and processing conditions on apparent digestibility in Atlantic salmon and physical quality of feed when (i) dietary fish meal was partially replaced with FFSBM or SBM, (ii) without or with KDF supplemented to the diets and (iii) pretreating FFSBM with an expander prior to extrusion or extrusion of SBM at increasing temperatures.

Materials and methods

Experimental design

Two experiments were carried out using either FFSBM (Exp. 1) or defatted, solvent-extracted SBM (Exp. 2) as a partial replacement for fish meal in the diets (Table 1). Each of the two test ingredients made up approximately 260 g kg^{-1} of the total crude protein content of the diet. FFSBM was either added directly to the diets prior to extrusion or pretreated with an expander. In Exp. 1, the dietary treatments were organized according to a 3×2 factorial design. Three different feed processing conditions were used [(i) extrusion at 110 °C without pretreatment; (ii) expander pretreatment at 100 °C prior to extrusion at 110 °C; (iii) expander pretreatment at 122 °C prior to extrusion at 110 °C], and two dietary treatments (− or + KDF) were applied. The effect of feed processing conditions of FFSBM diets will be referred to as the effect of pretreatment hereafter. In Exp. 2, the treatments were organized according to a 3×2 factorial design with three extrusion temperatures (110; 130; 150 °C) and two dietary treatments (− or + KDF).

Ingredients and diet preparation

The FFSBM and SBM used in this study were derived from the same batch of soybeans (Deno-Soy®; Denofa AS, Fredrikstad, Norway). From this batch, a subsample from whole full-fat soybeans with hulls was taken out before the soybeans were processed into hexane-extracted and toasted SBM with hulls. The dietary macroingredients (fish meal, wheat, FFSBM and SBM) were thoroughly ground using a hammer mill (Münch, HM 21.115, Wuppertal, Germany) to pass a 3-mm screen. Diet formulations, ingredients and analysed chemical composition of the diets are presented in Table 1.

In Exp. 1, the FFSBM was mixed (Dinnissen Pegasus Menger 400 l, Sevenum, The Netherlands) with wheat in a 2 : 1 ratio, preconditioned with steam to 91 °C and pretreated using a 75-kWh OE15.2 Kahl expander (Amandus Kahl GmbH & Co. KG, Reinbek, Germany) with cone pressures of 5 and 11 bar (bar; 1 bar = 10^5 Pa) to raise the temperature to 100 and 122 °C, respectively. The expandate was pelleted through 3-mm dies (Münch MDZAD8, MD 350 RPM, Wuppertal, Germany) and cooled in a counter flow drier (Miltenz VD010 gas; Millbank Technology Ltd, Auckland, New Zealand) prior to grinding through a 3-mm screen.

For both the experiments, dietary macroingredients (fish meal, wheat and either FFSBM [untreated; pretreated at 100 °C; pretreated at 122 °C] or SBM) were thoroughly mixed as 200 kg batches and ground using a hammer mill with a 1-mm screen. The microingredients (vitamin and mineral premix, yttrium oxide, and KDF) were manually added to the mixture during a second mixing cycle. Following mixing, the diets were preconditioned for 62 s and extruded through a 6-mm die (Sørensen *et al.* 2011). Two different screw configurations were used to reach the targeted extrusion temperatures. Screw configuration 1 (SCF1) was built up from the following elements, from inlet to outlet:

Table 1 Diet formulation and analyzed contents of main nutrients, amino acids and TIA in the experimental diets

	Experiment 1 – FFSBM-based diets						Experiment 2 – SBM-based diets					
	Extruded 110 °C		Expanded 100 °C		Expanded 120 °C		Extruded 110 °C		Extruded 130 °C		Extruded 150 °C	
Treatment[1]	–	+	–	+	–	+	–	+	–	+	–	+
Feed formulation (g kg^{-1})												
Fish meal[2]	383	377	379	374	379	381	388	385	388	385	388	385
Fish oil[3]	202	202	210	209	210	194	252	252	252	252	252	252
Wheat starch[4]	126	124	124	123	124	125	130	126	130	126	130	126
FFSBM[5]	286	281	283	279	283	284	0	0	0	0	0	0
SBM[6]	0	0	0	0	0	0	226	221	226	221	226	221
Mineral and vitamin premix[7]	3.8	3.8	3.8	3.8	3.8	3.8	3.9	3.8	3.9	3.8	3.9	3.8
Astaxanthin[8]	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Yttrium oxide[9]	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
KDF[10]	0	12	0	12	0	12	0	12	0	12	0	12
Dry matter (DM) (g kg^{-1})	952	943	963	952	954	949	944	943	948	952	966	952
Chemical composition (g kg^{-1} DM)												
Lipid	304	298	301	314	317	290	283	280	299	280	293	296
Ash	71	75	69	73	69	75	72	76	69	77	70	78
Starch	80	88	94	90	90	86	102	78	88	80	91	82
Crude protein	425	411	405	414	409	424	423	420	417	411	406	417
Total amino acids	408	391	424	423	428	419	433	448	428	436	436	426
Essential amino acids												
Arginine	26.3	25.3	28.5	28.9	29.5	28.2	28.1	30.6	27.9	29.8	28.5	29.5
Histidine	9.9	9.5	10.2	10.0	10.0	10.0	10.5	10.8	10.4	10.4	10.5	10.2
Isoleucine	20.2	18.9	20.5	20.6	20.7	20.3	21.1	22.1	21.1	21.2	21.5	20.6
Leucine	33.1	31.6	34.1	34.1	34.5	33.8	35.0	36.2	34.6	35.2	35.3	34.4
Lysine	33.0	31.3	33.5	33.9	34.0	33.7	34.5	35.4	34.1	34.5	34.7	33.9
Methionine	11.0	10.4	11.2	11.4	11.4	11.2	11.5	11.8	11.4	11.6	11.6	11.2
Phenylalanine	19.1	18.3	19.9	19.8	20.1	19.6	20.3	21.0	20.1	20.5	20.6	19.9
Threonine	18.7	17.9	19.3	19.2	19.5	19.1	19.8	20.3	19.5	19.9	19.8	19.4
Valine	22.0	20.7	22.1	22.2	22.4	21.9	23.0	24.1	23.0	23.1	23.4	22.4
Non-essential amino acids												
Alanine	23.0	22.0	23.8	23.9	24.1	23.6	24.3	24.9	24.1	24.4	24.5	23.8
Aspargine	43.8	42.0	45.1	44.8	45.4	44.6	46.4	48.0	45.9	46.7	46.7	45.6
Cysteine[11]	4.7	4.6	5.0	5.2	5.2	4.9	5.0	5.2	5.0	4.9	5.0	4.9
Glutamine	69.7	67.1	73.3	72.6	73.4	71.9	74.4	76.4	73.4	74.4	74.8	72.9
Glycine	21.3	20.4	21.9	22.0	22.3	21.7	22.5	23.1	22.3	22.6	22.7	22.1
Proline	19.0	18.4	19.6	19.5	19.6	19.7	20.5	21.2	20.5	20.6	20.7	20.2
Serine	20.1	19.6	21.3	20.8	21.2	20.8	21.7	22.0	21.2	21.7	21.6	21.3
Tyrosine	13.5	12.9	14.5	14.4	14.6	14.2	14.3	14.8	14.1	14.8	14.5	14.0
TIA[12] (mg g^{-1} diet)	3.0	3.0	1.6	1.9	1.6	1.9	Na[13]	Na	Na	Na	Na	Na

FFSBM, full-fat soybean meal; KDF, potassium diformate; TIA, trypsin inhibitor activity.

[1] Treatments without KDF are denoted '–' and treatments with KDF are denoted '+'.

[2] NorsECO-LT, Egersund Sildoljefabrikk AS, Egersund, Norway.

[3] NorSalmOil, Egersund Sildoljefabrikk AS, Egersund, Norway.

[4] Whole wheat, Felleskjøpet Agri, Ski, Norway.

[5] Deno-Soy®, whole full-fat soybeans with hulls, Denofa AS, Fredrikstad, Norway.

[6] Deno-Soy®, hexane extracted and toasted soybean meal with hulls, Denofa AS, Fredrikstad, Norway.

[7] Mineral and vitamin premix for fish, Normin AS, Hønefoss, Norway.

[8] Carophyll® Pink, DSM, JH Heerlen, The Netherlands.

[9] Yttrium Oxide (Y_2O_3), Metal Rare Earth Limited, Shenzhen, China.

[10] Formi®, Potassium diformate, Addcon Nordic AS, Porsgrunn, Norway.

[11] Cystine and cysteine.

[12] Inhibited bovine trypsin.

[13] Not analyzed.

80R80-80R80-60R60-60R60-60R60-60R60-60R60-60R60-80R80-100R100-P120-60L20-(90° twist off)-60L20-100R100-80R80-80R80-80R80-60R60-60R60. The first number gives the flight length, 'R' denotes a forward conveying element, 'L' indicate a backward conveying element and the last number gives the screw element length (mm). The element 'P120' is a polygon block (kneading element). Screw configuration 2 (SCF2) was similar to SCF1, except that the two '60L20' elements were continuous to each other. In Exp. 1, the untreated FFSBM was extruded using SCF1, whereas the pretreated FFSBM were extruded using SCF2. In Exp. 2, SBM-based diets were extruded using SCF1, and the extruder temperature was increased from 110 to 130 and 150 °C by increasing the screw revolutions per minute (rpm). Samples of feed were collected at steady-state conditions in the extruder and dried in a fluidized bed dryer (Bühler OTW-50, Uzwil, Switzerland). According to the method described by Sørensen *et al*. (2011), the final water content in the feeds was adjusted in small dryers to approximately 930 g kg^{-1} dry matter (DM). Extruder parameters were continuously recorded during the feed production (Table 2). Fish oil was added to the dried pellets in amounts of 200–260 g kg^{-1} using a vacuum coater (Dinnissen, Sevenum, The Netherlands). The feed was stored at 4 °C until analyses of physical feed quality, and the digestibility trials were conducted.

Digestibility trial with Atlantic salmon

The salmon experiment was carried out at Helgeland Forsøksstasjon (Dønna, Norway) during the period December 2008–February 2009. Apparent digestibility was measured in the six FFSBM-based diets from Exp. 1 and the six SBM-based diets from Exp. 2. All experimental diets contained 0.1 g kg^{-1} yttrium oxide as an indigestible marker (Austreng *et al*. 2000). A total of 1644 Atlantic salmon with mean initial weight of 1500 ± 200 g were distributed to twelve sea pens, 5.5 × 5.5 m wide and 7 m deep. The sea temperatures ranged from 4.4 to 6.8 °C during the experimental period. The fish had been fed a commercial diet (BioMar AS, Trondheim, Norway) prior to the experiment.

Table 2 Processing parameters during extrusion of the experimental diets

	Experiment 1 – FFSBM-based diets						Experiment 2 – SBM-based diets					
	Extruded 110 °C		Expanded 100 °C		Expanded 120 °C		Extruded 110 °C		Extruded 130 °C		Extruded 150 °C	
Treatment[1]	–	+	–	+	–	+	–	+	–	+	–	+
Preconditioning												
Temperature (°C)	88	85	87	85	84	86	87	88	100	100	100	100
Moisture addition (kg h^{-1})												
Water	26	27	29	29	28	28	42	41	35	41	38	36
Steam	13	12	11	11	11	10	15	15	12	12	12	13
Extrusion												
Throughput[2] (kg h^{-1})	226	227	223	230	228	228	242	241	235	291	244	296
Temperature[3] (°C)												
Section 1	70	64	68	71	59	73	69	71	98	99	99	99
Section 3	115	115	114	115	115	115	114	116	132	126	140	145
Section 5	111	113	114	116	110	110	117	115	125	124	133	134
Die	101	98	100	105	94	106	101	97	132	136	148	150
Screw speed (rpm)	274	328	275	287	340	336	166	202	589	666	877	818
Screw configuration[4]	SCF1	SCF1	SCF2	SCF2	SCF2	SCF2	SCF1	SCF1	SCF1	SCF1	SCF1	SCF1
Die pressure (bar)	21	17	19	17	18	18	30	26	40	40	42	47
Torque (Nm)	204	192	216	194	218	203	346	341	304	313	301	326
Cutter speed (rpm)	1801	1801	1802	1803	1801	1801	2004	2004	2004	2204	2004	2205
SME[5] (Wh kg^{-1})	26	29	35	25	34	31	25	29	80	75	114	95
Vacuum coating												
Oil addition[6] (g kg^{-1})	210	210	220	220	220	200	260	260	260	260	260	260

FFSBM, full-fat soybean meal; KDF, potassium diformate.

[1] Treatments without KDF are denoted '–' and treatments with KDF are denoted '+'.

[2] Dry feed mash and water added in the preconditioner.

[3] Temperature is measured by sensors mounted on the extruder barrel.

[4] Screw configuration (SCF1 = screw configuration 1; SCF2 = screw configuration 2).

[5] Specific mechanical energy.

[6] Diets were coated with different levels of fish oil in order to obtain sinking pellets.

Each of the 12 diets was fed to one group of fish over the course of three feeding periods to obtain three replicates. Each feeding period was initiated by randomly switching the 12 diets to the 12 sea cages, ensuring that no fish were fed the same diet twice throughout the experimental period. The fish were manually fed to satiation once per day during the first feeding period because of limited number of daylight hours and twice per day during the second and third feeding periods. Uneaten feed was collected by a lift-up system (LiftUP Akva AS, Fusa, Norway), and feed intake was recorded each day during the experiment. The three feeding periods were targeted to last for 10 days each; however, harsh weather conditions at the research station affected the number of feeding days for each period. To obtain 10 feeding days in each of the three feeding periods, each period lasted for 28, 19 and 11 days, respectively.

At termination of a feeding period, fish were collected using a net and anesthetized with 60 mg L^{-1} tricane methanesulfonate (MS-222) dissolved in salt water. Approximately 40 fish from each cage were manually stripped for faeces by gently applying pressure to the lower abdominal region to express faecal material in a tray and carefully ensuring that urine was excluded from the sample (Austreng 1978). No fish was stripped more than once in the course of the experimental period. Stripped fish were marked by either removing the adipose fin (1st stripping) or making a mark in the gill cover (2nd stripping) before releasing the fish back into the sea cage. Faecal samples were immediately frozen after the stripping, freeze-dried and ground prior to analyses.

Digestibility trial with mink

Because the digestibility trial with salmon had high within-treatment variability, it was decided to verify the digestibility results with mink, using the six FFSBM-based diets from Exp. 1. Mink was chosen as a test species because of the close relationships in digestibility capacities between mink and salmonids (Skrede *et al.* 1980, 1998; Romero *et al.* 1994; Øverland *et al.* 2006). The mink experiment was carried out at the Department of Animal and Aquacultural Sciences (UMB, Ås, Norway). A total of 24 adult male mink of the genotype Standard dark with a weight ranging from 2022 to 2755 g ($\bar{x}$ = 2391, σ = 210) were used in the experiment. The feeding trial lasted for 9 days and consisted of a 5-day preliminary period and a 4-day faecal collection period. The experiment was otherwise conducted as described by Aslaksen *et al.* (2006).

Physical quality of extruded diets

All physical quality measurements were undertaken at room temperature and performed in triplicate on uncoated pellet samples unless otherwise stated. Hardness, diameter, expansion ratio and Holmen durability index (HDI) were measured according to Sørensen *et al.* (2011) and reported as the average of 3 × 30 pellets, except for HDI. Doris durability was analysed on coated pellet samples, using a Doris tester (AKVAsmart, Bryne, Norway). Sifted pellets (350 g) were loaded into the Doris tester, conveyed through the apparatus, loaded onto a set of 5.6-, 3.55-, 2.36- and 0-mm screens and sieved for 60 s at amplitude 0.5 in a sieving machine (Retsch AS 200 Control, Haan, Germany). Doris durability was calculated as the percentage of whole pellets remaining on the 5.6-mm screen. Water stability index (WSI) was measured on coated pellets incubated for 120-min intervals in a shaking water bath at 23 °C, according to Baeverfjord *et al.* (2006).

Chemical analysis

Diets and freeze-dried faecal samples were analysed for DM (Commission dir. 71/393/EEC), ash (Commission dir. 71/250/EEC), crude lipid by HCl hydrolysis followed by diethylether extraction (Commission dir. 98/64/EC) and starch (AOAC enzymatic method 996.11). The diets were defatted by acetone prior to the starch analysis. The nitrogen content in diets was determined by the Kjeldahl method (Commission dir. 93/28/EEC). As a result of low amount of faecal material, the nitrogen content in faeces was determined by the Dumas method as described by Denstadli *et al.* (2006). Crude protein was calculated from the nitrogen content in the material ($N \times 6.25$). Amino acids in diets and faeces (Commission dir. 98/64/EC) were analysed on a Biochrom 30 amino acid analyzer (Biochrom Ltd., Cambridge, UK). Samples for analysis of yttrium oxide were prepared as described by Denstadli *et al.* (2006) and analysed with an ICP-AES Perkin Elmer Optima 5300 DV (Perkin Elmer Inc., Shelton, CT, USA). The FFSBM diets from Exp. 1 were analysed for TIA in accordance with the method of Hamerstrand *et al.* (1981). The pH value of the diets was measured as described by Pandey & Satoh (2008).

Calculations and statistical analysis

Apparent digestibility of main nutrients and individual amino acids in diets fed to Atlantic salmon and mink was calculated as: $100 - (100\ M_D\ (M_F)^{-1}\ N_F\ (N_D)^{-1})$, where M

and N are marker and nutrient concentrations, and the subscripts $_D$ and $_F$ represent diet and faeces, respectively.

Analysis of variance was conducted using the general linear model procedure of the SAS 9.1 computer software (SAS Institute Inc., Cary, NC, USA). The effect of partially replacing fish meal with FFSBM or SBM on the digestibility of nutrients and physical quality of extruded feeds was determined using a one-way analysis of variance, with ingredient as the class variable. In Exp. 1, effects of pretreatment (extrusion at 110 °C; FFSBM expanded at 100 °C prior to extrusion at 110 °C; 3. FFSBM expanded at 120 °C prior to extrusion at 110 °C) and KDF supplementation (– or + KDF) were tested on data for digestibility in Atlantic salmon ($n = 3$), digestibility in mink ($n = 4$) and physical quality ($n = 3$). The data were analysed according to the following model:

$$Y_{ijk} = \mu + \tau_i + \beta_j + (\tau\beta)_{ij} + \varepsilon_{ijk},$$

where Y, the observed response; μ, the overall mean; τ_i, the effect of pretreatment ($i = 1, 2, 3$); β_j, the effect of KDF ($j = 1, 2$); $(\tau\beta)_{ij}$, the effect of interaction between pretreatment and KDF; and ϵ_{ijk}, the random error. In Exp. 2, the effects of extrusion temperature (110, 130 or 150 °C) and KDF supplementation (– or + KDF) were tested on data for digestibility in Atlantic salmon ($n = 3$) and physical quality ($n = 3$). The data were analysed according to the same model as for Exp. 1 except for the effect of pretreatment, which was exchanged for the effect of extrusion temperature. Orthogonal polynomials were used to test linear responses of increased extruder temperatures on digestibility in Atlantic salmon and physical quality of SBM-based diets.

Results from both experiments are presented as means for each treatment, and variance is expressed as the pooled standard error of means (SEM). The level of significance was set at $P < 0.05$, while tendencies were set at $0.10 > P \geq 0.05$. Significant differences among means were ranked by the Ryan-Einot-Gabriel-Welsch multiple range test. Correlation coefficients among the physical quality characteristics were carried out using the Pearson correlation coefficient procedure.

Results

Diets and feed processing

Chemical analyses of the diets revealed small variations in chemical composition (Table 1). The FFSBM diets had a slightly higher fat content (290-317 g kg^{-1} DM) compared with SBM diets (280–299 g kg^{-1} DM). Extrusion processing of FFSBM without pretreatment gave TIA levels of 3 mg g^{-1} diet, whereas expander pretreatment reduced TIA levels to 1.6 and 1.9 mg g^{-1} diet in treatments without and with KDF, respectively (Table 1).

Digestibility in Atlantic salmon

The effects of KDF supplementation and expander pretreatment on digestibility of FFSBM diets (Exp. 1) in Atlantic salmon are shown in Table 3. There was no significant effect of KDF supplementation on the digestibility of any major nutrients or amino acids. Also, there were no significant interactions between KDF supplementation and pretreatment for digestibility; therefore, only the main effects are presented. The use of expander pretreatment significantly increased the digestibility of the essential amino acid (EAA) arginine and the non-EAA glutamine and tyrosine and tended to increase the digestibility of lipid, total amino acids, several EAA and non-EAA compared with extrusion without pretreatment.

The effects of KDF supplementation and extrusion temperature on digestibility of SBM diets (Exp. 2) in Atlantic salmon are presented in Table 4. There were no significant interactions between KDF supplementation and extrusion temperature for the digestibility of major nutrients or amino acids; therefore, only the main effects are presented. The addition of KDF to diets resulted in a significantly higher digestibility of starch and arginine. There were significant effects of extrusion temperature on digestibility of ash, starch, crude protein and total amino acids, the EAA arginine, histidine, isoleucine, leucine, methionine, and the non-EAA cysteine and glutamine. Moreover, extrusion temperature tended to increase the digestibility of lipid and several essential and non-EAA. The digestibility of starch was significantly higher in the diet extruded at 150 °C compared with extrusion at 130 °C, but was not significantly different from extrusion at 110 °C. Extrusion processing with increasing temperatures resulted in significant linear increase in digestibility of crude protein ($P = 0.008$), total amino acids ($P = 0.015$) and the amino acids arginine ($P = 0.002$), histidine ($P = 0.016$), isoleucine ($P = 0.014$), leucine ($P = 0.015$), methionine ($P = 0.005$), valine ($P = 0.019$), cysteine ($P = 0.016$), and glutamine ($P = 0.01$) and a significant linear reduction in digestibility of ash ($P = 0.017$). A one-way ANOVA between FFSBM and SBM diets extruded at 110 °C showed a significantly ($P < 0.05$) higher digestibility of the EAA arginine, histidine, isoleucine, leucine, lysine and phenylalanine in SBM compared with FFSBM diets.

Table 3 Main effects of KDF supplementation and expander pretreatment on apparent digestibility (%) in Atlantic salmon fed FFSBM diets (Exp. 1)

	KDF			Pretreatment				*P*-values[4]	
	–	+	SEM[2]	Extruded 110 °C	Expanded 100 °C	Expanded 120 °C	SEM[3]	KDF	Pretreatment
Lipid	84.6	84.7	1.381	81.3	85.7	87.0	1.691	0.951	0.085
Ash	10.3	10.4	0.397	11.3	10.1	9.6	0.486	0.810	0.074
Starch	64.2	65.3	1.513	63.2	66.3	64.7	1.853	0.620	0.526
Crude protein	79.6	80.5	1.166	78.4	79.9	81.7	1.428	0.618	0.293
Total amino acids	87.7	87.8	0.717	85.9[b]	88.2[ab]	89.2[a]	0.878	0.853	0.052
Essential amino acids									
Arginine	91.3	91.5	0.408	89.7[b]	91.9[a]	92.6[a]	0.500	0.761	0.004
Histidine	85.2	85.4	0.915	83.4	85.7	86.9	1.120	0.848	0.117
Isoleucine	87.8	87.9	0.819	85.9	88.3	89.4	1.003	0.894	0.083
Leucine	88.6	88.9	0.766	86.8	89.2	90.2	0.938	0.813	0.068
Lysine	90.4	90.9	0.711	89.2	90.9	91.8	0.870	0.644	0.145
Methionine	89.6	89.5	0.639	88.0	89.9	90.8	0.782	0.910	0.075
Phenylalanine	85.6	85.5	1.150	82.9	86.0	87.7	1.408	0.973	0.086
Threonine	82.2	82.4	1.370	79.7	82.7	84.5	1.678	0.904	0.159
Valine	87.0	87.2	0.880	85.2	87.4	88.5	1.078	0.872	0.130
Non-essential amino acids									
Alanine	89.3	89.5	0.642	87.8	89.8	90.6	0.786	0.829	0.073
Aspargine	83.3	83.5	0.758	81.4[b]	83.9[ab]	84.9[a]	0.928	0.833	0.052
Cysteine[5]	71.3	71.9	1.549	67.6	72.7	74.5	1.897	0.796	0.060
Glutamine	90.3	90.6	0.517	89.0[b]	90.9[ab]	91.5[a]	0.634	0.640	0.048
Glycine	85.6	85.8	0.631	84.2	86.0	86.9	0.773	0.748	0.075
Proline	85.9	86.3	0.933	84.2	86.5	87.8	1.142	0.749	0.117
Serine	84.3	84.4	0.927	82.0	84.9	86.1	1.135	0.910	0.063
Tyrosine	96.9	95.4	0.640	93.3[b]	97.5[a]	97.6[a]	0.783	0.120	0.003

FFSBM, full-fat soybean meal; KDF, potassium diformate.
[1] Treatments without KDF are denoted '–' and treatments with KDF are denoted '+'.
[2] Standard error of the mean for the main effect KDF supplementation.
[3] Standard error of the mean for the main effect pretreatment.
[4] Significant ($P < 0.05$) differences among means within pretreatment are indicated by superscripts [a,b,c].
[5] Cystine and cysteine.

Digestibility in mink

The effects of KDF supplementation and expander pretreatment on the digestibility of FFSBM diets (Exp. 1) in mink are shown in Table 5. There were no significant interactions between KDF supplementation and pretreatment for the digestibility of major nutrients or amino acids except for cysteine; therefore, only the main effects are presented. The addition of KDF to diets significantly increased the digestibility of ash, but reduced the digestibility of aspargine. Moreover, KDF tended to increase the digestibility of alanine. Significant effects of expander pretreatment on digestibility were observed for all major nutrients and amino acids except for ash and starch. Expander pretreatment at 120 °C resulted in a significantly higher digestibility of lipid compared with extrusion without pretreatment. In general, expander pretreatment significantly increased the digestibility of crude protein and all amino acids compared with extrusion without preprocessing. A significant interaction between KDF supplementation and pretreatment was observed for digestibility of cysteine. In diets with KDF, expander pretreatment significantly increased the digestibility of cysteine compared with extrusion without pretreatment.

Physical quality

The effects of KDF supplementation, expander pretreatment and the interactions between KDF and pretreatment on physical quality of FFSBM diets are presented in Table 6. Supplementing diets with KDF gave a significantly lower pH for all diets and increased the pellet hardness compared with non-supplemented diets. Significant interactions were found between KDF supplementation and pretreatment for pellet diameter, expansion ratio, HDI, Doris durability and WSI. In diets containing no KDF, expander pretreatment at 100 or 120 °C significantly increased diametrical expansion com-

Table 4 Main effects of KDF supplementation and extrusion temperature on apparent digestibility (%) in Atlantic salmon fed SBM diets (Exp. 2)

	KDF			Extrusion temperature				*P*-values[4]	
	–	+	SEM[2]	110 °C	130 °C	150 °C	SEM[3]	KDF	Temperature
Lipid	81.6	84.6	1.406	79.9	83.6	85.8	1.722	0.165	0.092
Ash	10.6	10.7	0.220	11.1^{a}	10.7^{ab}	10.1^{b}	0.269	0.756	0.049
Starch	58.4^{B}	62.2^{A}	1.071	61.0^{ab}	57.2^{b}	62.6^{a}	1.312	0.028	0.036
Crude protein	82.4	82.6	0.478	81.2^{b}	82.5^{ab}	83.9^{a}	0.585	0.752	0.026
Total amino acids	89.8	90.2	0.348	89.1^{b}	90.1^{ab}	90.8^{a}	0.426	0.364	0.045
Essential amino acids									
Arginine	93.0^{B}	93.8^{A}	0.233	92.5^{b}	93.5^{a}	94.1^{a}	0.285	0.035	0.006
Histidine	87.7	88.2	0.408	87.0^{b}	88.1^{ab}	88.9^{a}	0.500	0.391	0.048
Isoleucine	90.4	90.7	0.376	89.5^{b}	90.8^{ab}	91.4^{a}	0.461	0.548	0.040
Leucine	91.2	91.6	0.351	90.4^{b}	91.6^{ab}	92.2^{a}	0.430	0.500	0.041
Lysine	92.4	92.7	0.261	92.0	92.6	93.1	0.320	0.513	0.087
Methionine	91.3	91.8	0.334	90.5^{b}	91.6^{ab}	92.5^{a}	0.410	0.256	0.018
Phenylalanine	89.0	89.6	0.508	88.0	89.7	90.1	0.622	0.400	0.072
Threonine	85.5	86.0	0.558	84.6	86.0	86.7	0.684	0.511	0.114
Valine	89.7	90.0	0.388	88.9^{b}	90.0^{ab}	90.7^{a}	0.476	0.542	0.053
Non-essential amino acids									
Alanine	91.2	91.6	0.331	90.6	91.6	92.1	0.406	0.413	0.063
Aspargine	85.3	85.6	0.393	84.8	85.2	86.4	0.482	0.550	0.087
Cysteine[5]	73.4	74.7	0.744	72.5^{b}	73.7^{ab}	76.1^{a}	0.911	0.247	0.046
Glutamine	92.0	92.4	0.280	91.4^{b}	92.3^{ab}	92.9^{a}	0.344	0.374	0.031
Glycine	87.0	87.6	0.384	86.5	87.2	88.2	0.470	0.292	0.066
Proline	88.8	89.2	0.418	88.0	89.1	89.9	0.512	0.517	0.067
Serine	86.9	87.5	0.466	86.1	87.3	88.2	0.571	0.455	0.066
Tyrosine	96.0	96.8	0.709	95.5	96.6	97.1	0.869	0.454	0.432

KDF, potassium diformate.
[1] Treatments without KDF are denoted '–' and treatments with KDF are denoted '+'.
[2] Standard error of the mean for the main effect KDF supplementation.
[3] Standard error of the mean for the main effect extrusion temperature.
[4] Significant ($P < 0.05$) differences among means within KDF supplementation are indicated by superscripts ^{A,B} and significant differences among means within extrusion temperature are indicated by superscripts $^{a,b,c.}$
[5] Cystine and cysteine.

pared with extrusion without pretreatment. Moreover, expander pretreatment at 120 °C significantly improved Doris durability compared with extrusion without pretreatment. In KDF supplemented diets, expander pretreatment at 120 °C significantly decreased HDI, Doris durability and WSI of pellets compared with extrusion without pretreatment. Furthermore, expander pretreatment at 100 °C significantly increased the diametrical expansion of pellets compared with expander pretreatment at 120 °C or extrusion without pretreatment. Among the diets with KDF, pellet expansion was negatively correlated to Doris durability ($r = -0.89$, $P = 0.001$) and positively correlated to pH of the diets ($r = 0.79$, $P = 0.011$). Moreover, pH of the feeds was negatively correlated to HDI ($r = -0.73$, $P = 0.025$), Doris durability ($r = -0.75$, $P = 0.019$) and WSI ($r = -0.74$, $P = 0.022$) in the same diets.

The effects of KDF supplementation, extrusion temperature and the interactions between KDF supplementation and extrusion temperature on physical quality of SBM diets are presented in Table 7. Supplementing diets with KDF gave a significantly lower pH for all diets. There were significant interactions between KDF supplementation and extrusion temperature for the parameters hardness, diameter, expansion ratio, HDI, Doris durability and WSI. In diets without KDF, there was no difference in physical quality parameters among the different extrusion temperatures except for WSI, which decreased at increasing extrusion temperatures (linear $P < 0.001$). The expansion of pellets without KDF was negatively correlated to WSI ($r = -0.68$, $P = 0.044$) and hardness ($r = -0.79$, $P = 0.012$) of pellets, and hardness was positively correlated with HDI ($r = -0.86$, $P = 0.003$). In diets supplemented with KDF, extrusion processing with increasing temperatures resulted in significant linear increase in diametrical expansion ($P < 0.001$) of pellets, but resulted in a significant linear decrease in hardness ($P < 0.001$), HDI ($P < 0.01$), Doris durability ($P < 0.001$) and WSI

Table 5 Main effects of KDF supplementation and expander pretreatment on apparent digestibility (%) in mink fed FFSBM diets (Exp. 1)

	KDF			Pretreatment				*P*-values[4]	
	–	+	SEM[2]	Extruded 110 °C	Expanded 100 °C	Expanded 120 °C	SEM[3]	KDF	Pretreatment
Lipid	96.3	96.6	0.194	95.9^{b}	96.5^{ab}	97.0^{a}	0.238	0.374	0.012
Ash	24.3^{B}	31.2^{A}	0.631	26.8	27.5	28.9	0.773	<0.001	0.167
Starch	99.0	98.8	0.256	98.7	99.1	98.8	0.314	0.665	0.645
Crude protein	81.7	82.0	0.276	79.7^{b}	82.9^{a}	82.9^{a}	0.338	0.394	<0.001
Total amino acids	88.2	88.2	0.167	85.9^{b}	89.2^{a}	89.5^{a}	0.205	0.945	<0.001
Essential amino acids									
Arginine	92.3	92.5	0.124	90.0^{b}	93.4^{a}	93.8^{a}	0.152	0.448	<0.001
Histidine	86.3	86.4	0.208	83.9^{b}	87.5^{a}	87.8^{a}	0.255	0.810	<0.001
Isoleucine	90.0	90.2	0.154	87.9^{b}	91.0^{a}	91.5^{a}	0.189	0.430	<0.001
Leucine	90.6	90.9	0.136	88.6^{b}	91.6^{a}	92.0^{a}	0.167	0.228	<0.001
Lysine	91.3	91.3	0.123	89.5^{b}	92.0^{a}	92.4^{a}	0.150	0.905	<0.001
Methionine	91.1	91.3	0.143	89.8^{b}	91.7^{a}	92.2^{a}	0.176	0.323	<0.001
Phenylalanine	87.6	88.1	0.251	85.2^{b}	88.9^{a}	89.5^{a}	0.307	0.181	<0.001
Threonine	84.4	84.0	0.278	81.2^{b}	85.5^{a}	85.9^{a}	0.340	0.393	<0.001
Valine	87.7	87.8	0.195	85.4^{b}	88.7^{a}	89.1^{a}	0.239	0.719	<0.001
Non-essential amino acids									
Alanine	88.8	89.3	0.164	87.1^{b}	89.9^{a}	90.1^{a}	0.200	0.050	<0.001
Aspargine	83.4^{A}	82.8^{B}	0.213	80.7^{b}	84.0^{a}	84.5^{a}	0.261	0.046	<0.001
Cysteine[5]	67.5	65.4	0.756	61.7^{b}	68.4^{a}	69.2^{a}	0.925	0.061	<0.001
Glutamine	90.5	90.7	0.154	88.5^{b}	91.5^{a}	91.8^{a}	0.189	0.447	<0.001
Glycine	85.2	85.3	0.203	83.3^{b}	86.1^{a}	86.2^{a}	0.249	0.760	<0.001
Proline	87.2	87.3	0.229	84.6^{b}	88.3^{a}	88.8^{a}	0.281	0.598	<0.001
Serine	85.6	85.6	0.218	82.7^{b}	86.9^{a}	87.3^{a}	0.267	0.934	<0.001
Tyrosine	89.3	89.0	0.220	86.2^{b}	90.4^{a}	90.8^{a}	0.269	0.242	<0.001

FFSBM, full-fat soybean meal; KDF, potassium diformate.

[1] Treatments without KDF are denoted '–' and treatments with KDF are denoted '+'.

[2] Standard error of the mean for the main effect KDF supplementation.

[3] Standard error of the mean for the main effect pretreatment.

[4] Significant ($P < 0.05$) differences among means within KDF supplementation are indicated by superscripts ^{A,B} and significant differences among means within pretreatment are indicated by superscripts ^{a,b,c}.

[5] Cystine and cysteine.

($P < 0.05$). Consequently, the expansion of pellets with KDF was negatively correlated to hardness ($r = -0.88$, $P = 0.002$), HDI ($r = -0.94$, $P < 0.001$), Doris durability ($r = -0.93$, $P < 0.001$) and WSI ($r = -0.70$, $P = 0.035$). Hardness was highly correlated to HDI ($r = 0.92$, $P < 0.001$) and Doris durability ($r = 0.97$, $P < 0.001$), and Doris durability was highly correlated ($r = 0.91$, $P < 0.001$) to HDI. No correlations were found between pH of the extruded diets and physical quality parameters. A one-way ANOVA between FFSBM and SBM diets extruded at 110 °C showed a significantly ($P < 0.05$) higher hardness, pellet diameter, expansion ratio, HDI, Doris durability and WSI in SBM compared with FFSBM diets.

Discussion

Soybean products are commonly used ingredients in diets for salmonids (Storebakken *et al.* 2000; Refstie *et al.* 2001; Mundheim *et al.* 2004; Aslaksen *et al.* 2007). The use of FFSBM is, however, limited in diets for carnivore fish because of ANF such as TI (Morris *et al.* 2005). In the current study, FFSBM was pretreated using an annular-gap expander at two different temperatures before the diets were extruded. The annular-gap expander resembles a single-screw extruder except that the material is discharged over an annular-gap outlet instead of being forced through a perforated die (Lucht 2007) and is primarily used for pretreatment of feed for terrestrial farm animals prior to pelleting. The increased digestibility of total amino acids and several EAA and non-EAA in Atlantic salmon is most likely explained by the reduction in TIA in FFSBM by the expander pretreatment. All diets were below the maximum acceptable TIA level of 5 mg g^{-1} diet for Atlantic salmon (Olli *et al.* 1994). Earlier studies have also shown that extrusion is an efficient process to reduce TI in soy to safe levels in feeds for fish. Barrows *et al.* (2007) observed that extrusion at 93 °C

Table 6 The effect of the interaction between KDF supplementation and expander pretreatment on physical quality and pH of processed FFSBM diets (Exp. 1)

	Extruded 110 °C		Expanded 100 °C		Expanded 120 °C			*P*-values[3]		
Treatment[1]	–	+	–	+	–	+	SEM[2]	KDF	Pretreatment	KDF × Pretreatment
Hardness (N)	12.4^{b}	18.6^{a}	11.9^{b}	16.6^{a}	13.1^{b}	17.8^{a}	0.884	<0.001	0.320	0.645
Pellet diameter (mm)	6.1^{b}	5.1^{c}	6.8^{a}	6.7^{a}	6.7^{a}	6.3^{b}	0.087	<0.001	<0.001	0.001
Expansion ratio (%)	1.3^{b}	−15.1^{c}	13.4^{a}	11.2^{a}	12.3^{a}	5.4^{b}	1.457	<0.001	<0.001	0.001
Holmen durability index (%)	0.3^{b}	11.0^{a}	0.4^{b}	0.8^{b}	0.4^{b}	2.4^{b}	1.843	0.013	0.038	0.035
Doris durability (%)	21.8^{e}	46.1^{a}	22.2^{e}	35.0^{c}	29.2^{d}	40.4^{b}	1.215	<0.001	<0.001	<0.001
Water stability index (%)	74.4^{bc}	85.8^{a}	74.3^{bc}	81.8^{ab}	74.8^{bc}	72.3^{c}	1.844	0.004	0.012	0.007
pH	6.5^{a}	5.8^{c}	6.4^{a}	6.0^{b}	6.5^{a}	6.0^{b}	0.025	<0.001	0.047	0.026

FFSBM, full-fat soybean meal; KDF, potassium diformate.
[1] Treatments without KDF are denoted '–' and treatments with KDF are denoted '+'.
[2] Standard error of the mean.
[3] Significant ($P < 0.05$) differences among means are indicated by different superscripts ^{a,b,c,d,e}.

Table 7 The effect of the interaction between KDF supplementation and extrusion temperature on physical quality and pH of processed SBM diets (Exp. 2)

	110 °C		130 °C		150 °C			*P*-values[3]		
Treatment[1]	–	+	–	+	–	+	SEM[2]	KDF	Temperature	KDF × Temperature
Hardness (N)	31.3^{b}	50.6^{a}	29.4^{b}	46.6^{a}	29.1^{b}	35.2^{b}	1.558	<0.001	<0.001	0.003
Pellet diameter (mm)	6.6^{b}	6.1^{c}	6.9^{ab}	6.7^{ab}	6.9^{ab}	7.0^{a}	0.079	0.006	<0.001	0.005
Expansion ratio (%)	10.2^{b}	0.8^{c}	14.3^{ab}	11.7^{ab}	15.5^{ab}	16.7^{a}	1.311	0.004	<0.001	0.004
Holmen durability index (%)	38.2^{b}	79.0^{a}	38.6^{b}	56.2^{ab}	35.9^{b}	38.5^{b}	6.191	0.002	0.016	0.029
Doris durability (%)	68.5^{d}	91.3^{a}	70.2^{d}	86.4^{b}	72.4^{d}	79.9^{c}	1.144	<0.001	0.022	<0.001
Water stability index (%)	87.5^{ab}	88.4^{a}	80.4^{c}	83.4^{abc}	72.7^{d}	82.8^{bc}	1.220	<0.001	<0.001	0.007
pH	6.5^{a}	5.8^{b}	6.5^{a}	5.9^{b}	6.4^{a}	5.8^{b}	0.015	<0.001	0.031	0.248

KDF, potassium diformate.
[1] Treatments without KDF are denoted '–' and treatments with KDF are denoted '+'.
[2] Standard error of the mean.
[3] Significant ($P < 0.05$) differences among means are indicated by different superscripts ^{a,b,c,d}.

decreased TI levels in soybeans to below detectable limits. Romarheim *et al.* (2005) reported that extrusion at 116–122 °C decreased TIA in SBM and white flake-based diets from 2.7 and 8.3 to 0.6 and 2.1 mg g^{-1} diet, respectively. Similar to that observed for salmon, the digestibility of major nutrients and amino acids in mink was significantly improved in response to pretreatment of FFSBM. This is in line with previous studies reporting close relationships in digestibility capacities between mink and Atlantic salmon (Skrede *et al.* 1980, 1998; Romero *et al.* 1994; Øverland *et al.* 2006). The present results, thus, suggest that TIA levels of FFSBM can be reduced to safe levels below 5 mg g^{-1} diet by expander pretreatment at moderate temperatures followed by low-temperature extrusion at 110 °C.

The digestibility of most individual amino acids was low in the FFSBM diets extruded at 110 °C without expander pretreatment compared with SBM diets extruded at 110 °C. These results are in line with Cheng & Hardy (2003), who observed reduced amino acid digestibilities with inclusion of raw FFSBM in diets for rainbow trout (*Oncorhynchus mykiss*). Although raw FFSBM contains more TI than processed SBM (Clarke & Wiseman 2005), the TIA content of FFSBM extruded at 110 °C was within the acceptable level in the present study. Therefore, the lower nutrient digestibility of FFSBM might be because of other ANF than TIA, as suggested by Olli *et al.* (1994) for fish and by Clarke & Wiseman (2005) for terrestrial animals.

The higher digestibility of protein and amino acids of SBM diets extruded at 150 °C compared with those extruded at 110 °C is not in line with other studies carried out with rainbow trout and mink. Sørensen *et al.* (2002) reported that extrusion temperatures ranging from 100 to 150 °C had no effect on digestibility of proteins or amino acids in rainbow trout fed a fish meal-based diet. Moreover, Barrows *et al.* (2007) did not observe any effects of extrusion at 93 or

127 °C on digestibility of protein in rainbow trout fed a plant-based diet. Ljøkjel *et al*. (2004), however, observed a reduction in protein and total amino acid digestibility in mink fed a fish meal-based diet, when extruder temperatures were increased from 100 to 150 °C. Other studies have shown that excessive heat treatment may lead to a reduction in digestibility of protein and amino acids, especially cysteine, because of the formation of disulphide bonds (Opstvedt *et al*. 1984; Ljøkjel *et al*. 2000, 2004). In the present study, increasing the extruder temperature from 110 to 150 °C increased the digestibility of cysteine. The higher digestibility of protein and amino acids of diets extruded at 150 °C may, therefore, indicate that elevated extrusion temperatures facilitated a higher protein digestion in Atlantic salmon by denaturing the protein without the formation of secondary disulphide cross-linkages (Opstvedt *et al*. 1984).

In contradiction to other studies, addition of KDF to FFSBM or SBM did generally not affect the digestibility of nutrients in Atlantic salmon or mink. Storebakken *et al*. (2010) showed improved digestibility of amino acids in Atlantic salmon when KDF was supplemented to FFSBM-based diets prior to extrusion. Improved digestibility of protein, DM and energy was also reported for Atlantic salmon fed diets where KDF was added prior to extrusion (Lückstädt 2008). Moreover, Johnsen *et al*. (2000) reported that Atlantic salmon fed diets with fish meal treated with KDF had a significantly higher fat and protein digestibility compared with fish fed diets without KDF. The same authors also reported a significant increase in protein digestibility of Atlantic salmon fingerlings fed diets where formic acid was added prior to feed production. In pigs, KDF was found to increase the apparent total tract digestibility of crude protein (Roth *et al*. 1998a), ash and several minerals (Roth *et al*. 1998b; Mroz *et al*. 2002). As suggested by Mroz *et al*. (2002), the inconsistent conclusions reported in the present study compared with previous reports could be attributed differences in the inclusion level of KDF, dietary composition, animal species or environmental factors, such as water temperature.

Increasing temperatures during expander pretreatment and extrusion gave greater diametrical expansion of pellets, and the higher expansion was associated with a reduction in hardness and durability, which is in line with previous studies (Aarseth *et al*. 2006b; Hansen & Storebakken 2007; Sørensen *et al*. 2009). This may be explained by a higher degree of starch gelatinization at increasing processing temperatures (Lin *et al*. 1997; Rolfe *et al*. 2000). The highest extrusion temperatures were obtained by increasing the screw rpm, resulting in higher SME. The reduction in hardness and durability for the diets extruded at 130 and 150 °C was, however, most likely associated with temperature and not mechanical energy input. Aarseth *et al*. (2006b) also reported lower hardness and durability for diets produced at higher temperatures without changing the rpm during extrusion. Other processing parameters, such as screw configuration, could also have contributed to the differences in physical quality for the FFSBM. The two diets pretreated with an expander was extruded with SCF2, whereas all other diets were extruded using SCF1. Sørensen *et al*. (2010) reported that screw configuration accounted for more than 40% of the variation in diametrical expansion and durability of extruded diets, which indicates that a systematic effect of screw configuration could have contributed to the high expansion in the two pretreated FFSBM diets. Expansion is associated with oil absorption capacity (Sørensen *et al*. 2011) and fat leakage (Øverland *et al*. 2007) and is an important parameter when producing high-energy feeds. Thus, expander pretreatment may be an important tool to obtain high expansion when FFSBM is included in extruded diets.

The more brittle and less durable appearance of FFSBM pellets could be associated with the higher fat content in FFSBM compared with SBM. Sørensen *et al*. (2009) suggested that even a small increase in fat content may negatively affect physical quality of extruded diets. The lubricating action of fat reduces mechanical energy inputs and processing temperatures, especially when fat is added to the feed mixture prior to extrusion (Lin *et al*. 1997). Low processing temperature combined with the hydrophobic properties of fat may reduce starch gelatinization, which in turn results in poor physical quality. In the current study, no additional fat was added to the feed mixture prior to extrusion. Thus, the effect of fat enclosed in the matrix of cell walls may have greater effect on physical quality than earlier suggested by Thomas *et al*. (1998).

The addition of 12 g kg^{-1} KDF to diets corresponded to a formic acid inclusion of 4.2 g kg^{-1} (Canibe *et al*. 2001; Mroz *et al*. 2002) and resulted in a pH drop equivalent of approximately 0.6 units in the processed feed. The use of acids is known to alter physiochemical properties of starch, such as gelatinization time and viscosity (Thomas & Atwell 1999; Wurzburg 2006). Formic acid has been reported to destructure native starch granules to starch formate (Divers *et al*. 2004; Bossard *et al*. 2008), and the resulting long-chain esters exhibit lower viscosity (Wolff *et al*. 1957) and hydrophobic properties (Aburto *et al*. 1999). The reduction in expansion and subsequent increase in hardness of FFSBM pellets with KDF might, therefore, be explained by reduced hydrophilic properties of the starch molecules in the presence

of formic acid, leading to a lower degree of starch gelatinization. The loss of hydrophilic character is also reflected by the inverse relationship between pH and WSI of pellets. Moreover, a decreased viscosity could also have affected expansion in diets supplemented with KDF because of lower pressure differentials between the extruder and the atmosphere (Arhaliass *et al*. 2003). In general, the modification of starch in the SBM diets was less prone to negatively affect physical quality because this diet had more inherent binders from other constituents than starch compared with FFSBM. It can, therefore, be hypothesized that the inherent binding properties of the FFSBM and SBM differed, suggesting that the inherent binding property of the mixture should be considered before the addition of KDF to extruded feeds.

In conclusion, this study showed that expander pretreatment of FFSBM and increasing extrusion temperatures of SBM significantly improved the digestibility of most major nutrients and amino acids. The addition of KDF to FFSBM and SBM diets did generally not affect digestibility of nutrients. Moreover, the digestibility of FFSBM diets measured in mink confirmed the results obtained for Atlantic salmon. Inclusion of SBM improved the physical quality of extruded feeds as measured by hardness, expansion, durability and WSI compared with FFSBM. Expander pretreatment and increasing temperatures during extrusion gave greater expansion of pellets and reduced hardness and durability. The addition of KDF to FFSBM diets, however, reduced expansion of pellets and increased hardness and durability. This effect was less prominent in SBM feeds.

Acknowledgements

The research was financially supported by grant #145949 from The Research Council of Norway, BioMar AS and Addcon Nordic AS. Thea Morken was supported by a PhD-grant from The Norwegian University of Life Sciences (UMB, Ås, Norway). The authors acknowledge Ismet Nikqi, Centre for Feed Technology, for skilful technical assistance during the feed production. We also thank Dr. Gibson Gaylord for expertise and help in carrying out the analysis of trypsin inhibitor activity.

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Paper II

Aquaculture 317 (2011) 138–145

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

Sodium diformate and extrusion temperature affect nutrient digestibility and physical quality of diets with fish meal and barley protein concentrate for rainbow trout (*Oncorhynchus mykiss*)

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ARTICLE INFO

Article history:
Received 20 February 2011
Received in revised form 9 April 2011
Accepted 12 April 2011
Available online 21 April 2011

Keywords:
Barley protein concentrate
Digestibility
Extrusion
Physical quality
Rainbow trout
Sodium diformate

ABSTRACT

The objective of the experiment was to evaluate the effects of ingredient, extrusion temperature, and the conjugated acid salt sodium diformate (HCOOH··HCOO-Na; NaDF) on apparent nutrient digestibility and physical quality of diets for rainbow trout. The experiment had a 2^3 factorial design with two dietary ingredient sources (fish meal [FM] and barley protein concentrate [BPC]), two extruder temperatures (110 and 141 °C), and two levels of NaDF supplementation (0 and 10.6 g kg^{-1}). The results from the digestibility trial showed that inclusion of BPC significantly increased the digestibility of crude protein and several non-essential amino acids (NEAAs), but significantly reduced the digestibility of crude lipid, starch, and all essential amino acids (EAAs). Extrusion at 141 °C significantly increased the digestibility of starch, crude protein, arginine, and several NEAAs, but significantly reduced the digestibility of phenylalanine compared to extrusion at 110 °C. Addition of NaDF significantly increased the digestibility of most major nutrients and individual amino acids. Diets with BPC had significantly higher hardness, expansion ratio, and durability, but significantly lower water stability compared to diets based on FM. Extrusion temperature only affected pellet durability, which was significantly higher for the highest temperature. Addition of NaDF to diets significantly increased the expansion ratio, durability, and water stability compared to diets without NaDF. This experiment showed that BPC is a promising alternative protein source to FM with regard to nutrient digestibility and physical quality of extruded diets. The use of high extrusion temperature (141 °C) and inclusion of NaDF improved both nutrient digestibility and physical quality of diets for rainbow trout.

1. Introduction

Cereal grains are commonly included in diets for carnivorous fish to facilitate binding in extruded diets (Sørensen et al., 2010), but the inclusion level is limited by low nutrient density and the poor ability of these fish to digest and metabolize starch (Krogdahl et al., 2005). Barrows et al. (2007a) reported that barley (*Hordeum vulgare* L.) is a potential ingredient in high energy diets for salmonids. The major components of barley are starch (600 g kg^{-1}), fiber (200 g kg^{-1}) and protein (110 g kg^{-1}) (Åman et al., 1985; Oscarsson et al., 1996), but the chemical composition varies among different barley cultivars and processing conditions (Andersson et al., 2000). Production of ethanol from barley will yield large quantities of protein as a co-product, which can be used in fish diets (Gatlin et al., 2007). Barley contains various antinutritional factors (ANFs) that may interfere with nutrient utilization in fish, including mixed-linked (1→3) (1→4)-β-glucans (Lee et al., 1997) and phytic acid (*myo*-inositol hexaphosphate) or its salts (Reddy, 2002). The variety and content of ANFs present in barley may, however, be considered less prominent compared to other plant-derived materials (Francis et al., 2001; Reddy, 2002). Inclusion of plant protein ingredients in fish diets may be useful for improving physical quality of extruded pellets (Kraugerud et al., 2011; Øverland et al., 2009), due to the structure-forming ability of plant proteins during denaturation (Arêas, 1992; Sørensen et al., 2009).

Feed processing conditions affect the nutritional value of diets for carnivorous fish. Extrusion leads to chemical and physicochemical changes in the raw materials, which may impact digestibility of nutrients (Camire, 2001) and physical quality of the extruded diet (Sørensen et al., 2011). Extrusion at moderate temperatures (<150 °C) generally improves protein digestibility in fish by exposing enzyme-access sites through disruption of native protein structures

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doi:10.1016/j.aquaculture.2011.04.020

(Barrows et al., 2007b; Camire, 2001; Morken et al., in press). Conversely, excessive heating may reduce protein and individual amino acid digestibility by forming hydrophobic complexes, such as disulphide bonds from sulfhydryl groups (Aslaksen et al., 2006; Ljøkjel et al., 2000; 2004; Opstvedt et al., 1984). Disulphide bonds may, on the other hand, improve physical quality of extruded diets by stabilizing the pellet structure (Sørensen et al., 2009). Other physicochemical reactions, such as starch gelatinization, also improve physical quality of feed due to increased binding between feed particles (Sørensen et al., 2011). It is, therefore, important to control the degree of heating during extrusion processing in order to optimize both nutritional value and physical quality of the diet.

Organic acids and their salts have been reported to promote growth in both terrestrial animals (Øverland et al., 2000; Paulicks et al., 2000) and in fish (Lückstädt, 2008; Ringø, 1991). To date, limited information exists on the effect of acid salts on digestibility of nutrients in salmonids. Storebakken et al. (2010) reported an improvement in amino acid digestibility in Atlantic salmon (*Salmo salar*) when adding 12 g kg^{-1} potassium diformate (KDF) to diets prior to extrusion. However, no studies have evaluated effects of sodium diformate (NaDF) on nutrient digestibility in salmonids fed diets with plant protein ingredients such as barley protein concentrate (BPC). Furthermore, limited information exists on the effect of acid salts on physical quality of extruded diets. The aim of the present experiment was, therefore, to investigate the effects of BPC, extrusion temperature, and NaDF supplementation on nutrient digestibility and physical quality of diets for rainbow trout.

2. Materials and methods

2.1. Experimental design, diets, and diet processing

The experiment had a 2^3 factorial design with two dietary ingredients (fish meal [FM] and BPC), two extruder temperatures (110 and 141 °C), and two levels of NaDF (0 and 10.6 g kg^{-1}). The BPC was produced by an enzymatic method that concentrates barley protein and produces raw material for ethanol production. The proximate chemical composition of the FM and BPC used is shown in Table 1. The FM diets were extruded at two temperatures, whereas the BPC diets were extruded at one additional temperature (126 °C) to investigate possible linear or quadratic relationships of extruder temperature on nutrient digestibility. The NaDF (HCOOH··HCOO-Na; Formi® NDF, Addcon Nordic AS, Porsgrunn, Norway) is a conjugated salt of formic acid (HCOOH) and sodium formate (HCOONa). This acid salt contained 966 g kg^{-1} NaDF, of which 195 g kg^{-1} was sodium, 390 g kg^{-1} formic acid, and 381 g kg^{-1} formate (COOH), while the remaining 34 g kg^{-1} was silicate and water. All diets contained a combination of 0.1 g kg^{-1} yttrium oxide (Y_2O_3) and 1 g kg^{-1} chromic oxide (Cr_2O_3) as indigestible markers (Austreng et al., 2000). The formulation of the experimental diets is shown in Table 2.

The extruded diets were produced at Bozeman Fish Technology Center, U.S. Fish and Wildlife Service (Bozeman, MT, USA). The FM was ground to a particle size of approximately 80 μm using an air-swept pulverizer (Model 18H, Jacobsen, Minneapolis, MN, USA), and mixed with wheat flour and micro ingredients in a single-shaft paddle mixer (Model No. 2010, Marion Mixer, Rapids Machinery Co., Marion, IA, USA) for 5 min. The BPC was ground to pass through a 0.8 mm screen using a hammer mill (Model LM6, Kelly Duplex Mill and Mfg., Springfield, OH, USA) prior to mixing with FM, wheat flour and micro ingredients. The ingredients for each diet were mixed in batch sizes of 40 kg. Following dry mixing, the diets were loaded into a volumetric feeder with a feeding rate of approximately 1140 rpm (Model K2VT35, K-Tron North America, Pitman, NJ, USA) and conveyed through a conditioner with 90 s retention time (Model DCC 6X30, Extru-Tech Inc., Sabetha, KS, USA). The diets were extruded using a Bühler DNDL-44 twin-screw extruder (Bühler AG, Uzwil, Switzerland). The extruder consisted of six barrel sections and was fitted with a 5 mm die, resulting in pellets with a mean length and diameter of 4.6 and 5.1 mm, respectively. Water driven heaters (Model HX4003AQ, Mokon, Buffalo, NY, USA) were used to stabilize the temperature in section numbers 1–5 and 6 of the extruder. The heater for sections 1–5 and 6 had set temperatures of 141 and 99 °C, respectively. The FM diets were extruded at 110 and 141 °C, whereas the BPC diets were extruded at 110, 126, and 141 °C (Table 3). The diets were dried for approximately 12 min in a fluidized bed dryer (Model OTW-50, Bühler AG, Uzwil, Switzerland) with hot air at a temperature of 116 °C at the inlet and 99 °C at the outlet, followed by cooling for 30 min on an air-swept perforated tray (custom made). Fish oil was added to the extrudate by vacuum coating (Phlauer™, A&J Mixing International, Inc., Oakville, Ontario, Canada). The extruded diets were packed in plastic lined paper bags and stored at ambient temperature during the digestibility trial.

Table 1
Proximate chemical composition of the experimental ingredients (g kg^{-1}).

Ingredient	Fish meal (FM)	Barley protein concentrate (BPC)
DM	915.5	908.1
In DM		
Lipid	93.1	126.6
Ash	231.6	38.3
Starch	7.0	42.2
Crude protein	724.3	577.6

Table 2
Formulation of the experimental diets (g kg^{-1}).

	FM diets		BPC diets	
	Without NaDF	With NaDF	Without NaDF	With NaDF
Fish meal[a]	632.0	625.4	351.0	346.3
Barley protein concentrate[b]	0.0	0.0	276.0	273.1
Wheat flour[c]	200.0	197.9	196.0	195.0
Fish oil[d]	158.0	156.4	167.0	165.3
Vitamin premix[e]	5.0	5.0	5.0	5.0
Trace mineral premix[f]	1.0	1.0	1.0	1.0
Stay-C[g]	3.0	3.0	3.0	3.0
Yttrium oxide (Y_2O_3)[h]	0.1	0.1	0.1	0.1
Chromic oxide (Cr_2O_3)[h]	1.0	1.0	1.0	1.0
NaDF[i]	0.0	10.6	0.0	10.6

[a] Menhaden Special Select, Omega Protein Corporation, Houston, TX, USA.
[b] Montana Microbial Products, Butte, MT, USA.
[c] Pendleton Flour Mills L.L.C., Pendleton, OR, USA.
[d] Menhaden Fish Oil, Omega Protein Corporation, Houston, TX, USA.
[e] Trout Nutrition USA, Willmar, MN, USA.
[f] U.S. Department of Agriculture, Agricultural Research Service, Bozeman Fish Technology Center, Bozeman, MT, USA.
[g] ROVIMIX® STAY-C® 35, DSM, Heerlen, Netherlands.
[h] Sigma-Aldrich, St. Louis, MO, USA.
[i] Formi® NDF, ADDCON Nordic AS, Porsgrunn, Norway.

2.2. Fish, husbandry, and sampling

A 24-day digestibility experiment with rainbow trout was carried out at Bozeman Fish Technology Center, U.S. Fish and Wildlife Service (Bozeman, MT, USA). Trout (eggs provided by Trout Lodge, WA, USA) with a mean weight of 520 g at the time of sampling were randomly allocated to 30, 416-l fiberglass tanks with 20 fish/tank. The tanks were supplied with recirculated spring water (15 °C; 19 l min^{-1}) and had light on a 14:10 h diurnal cycle. Each diet was fed in excess to 3 replicate fish tanks for 6 h day^{-1} by automatic belt feeders (Model BFS12, 12 h Belt Feeder, Aquatic Eco-Systems, Inc., Apopka, FL, USA). Before receiving the experimental diets, the fish were fed a commercial diet (ARS Plant 646, Nelson's Silver Cup Fish Feed, Nelson & Sons, Inc., Murray, UT, USA).

Table 3
Processing parameters during extrusion of the experimental diets.

	FM diets				BPC diets			
	Without NaDF		With NaDF		Without NaDF		With NaDF	
	110 °C	141 °C	110 °C	141 °C	110 °C	141 °C	110 °C	141 °C
Throughput [kg h^{-1}]	63	63	63	63	63	63	63	63
Screw speed [%]	85	55	54	52	53	52	52	52
Torque [%]	17	20	20	20	24	21	24	21
Water [l h^{-1}]	13	14	13	14	14	13	14	14
Temperature [°C]								
Section 2	53	59	68	46	49	41	64	41
Section 3	112	135	112	136	110	134	111	136
Section 4	109	141	109	142	109	141	110	141
Section 5	86	121	86	121	86	128	87	121
Section 6	101	137	104	138	103	131	104	137
Die	71	64	77	52	66	58	73	52
Die pressure [bar]	23	26	25	24	31	31	30	28

Feces were collected from all fish within each tank 3 times during the experimental period (days 8, 14, and 24) in order to obtain sufficient amount of fecal material. The fecal stripping was performed 12–18 h postprandial as described by Gaylord et al. (2010). The fecal samples were stored individually at −30 °C until freeze drying and chemical analysis. In order to evaluate potential variation in digestibility among the three different strippings, one random tank was selected and material from each stripping was analyzed for crude protein and yttrium oxide.

2.3. Chemical and physical analyses

Ingredients (FM and BPC), diets and freeze-dried feces were analyzed for dry matter (DM) (Dir 71/393/EEC), ash (Dir 71/250/EEC), crude protein (N × 6.25) using the Dumas method as described by Denstadli et al. (2006), crude lipid by HCl hydrolysis followed by diethyleter extraction (Dir 98/64/EC), and starch (AOAC enzymatic method 996.11). The diets were defatted by acetone prior to the starch analysis. Amino acids in diets and feces were analyzed according to the EC method (Dir 98/64/EC) on a Biochrom 30 amino acid analyzer (Biochrom Ltd., Cambridge, UK). Reactive lysine in the diets was determined using the guanidination method as described by Moughan and Rutherfurd (1996). In brief, triplicate samples were incubated in 0.6 M *O*-Methylisourea at room temperature for 3 days (determined as optimal incubation time), with a reagent-to-lysine ratio >1000. The samples were dried and hydrolyzed as described for the amino acid analysis. Homoarginine was analyzed on a Biochrom 30 Amino acid analyzer against external standards. The homoarginine was then transformed to reactive lysine on a molar basis using the molecular weights of homoarginine and lysine. The available lysine in the diets was reported as the reactive lysine in percentage of total lysine in the diet. Samples for analysis of yttrium oxide and sodium was prepared as described by Denstadli et al. (2006) and analyzed with an ICP-AES Perkin Elmer Optima 5300 DV (Perkin Elmer Inc., Shelton, CT, USA). The pH value of the diets was measured as described by Gao et al. (2011).

Physical quality of the diets was evaluated by measuring hardness, expansion ratio, durability, and water stability index (WSI). All physical quality measurements were undertaken at room temperature and performed in triplicate on fat coated diet samples unless otherwise stated. Hardness was determined on 30 pellets with a Texture Analyzer TA-XT2 (SMS Ltd., Surrey, UK), equipped with a 5 kg load cell and a compression speed of 1 mm s^{-1} as described by Øverland et al. (2009). Pellet diameter was measured by the Texture Analyzer in order to calculate the expansion ratio. Durability was measured with a Doris tester (AKVAsmart, Bryne, Norway) as described by Hansen et al. (2010). The pellet collector was emptied on a 3.55 mm screen, and sieved for 60 s at amplitude of 0.5 in a Retsch AS 200 Control sieving machine (Haan, Germany). Durability was calculated as the percentage of whole pellets remaining on the 3.55 mm screen. The WSI was evaluated *in situ* as percent DM recovered from feeding 160 g of extruded diets in tanks containing no fish, using similar water flow and temperature as employed in the digestibility experiment. The WSI was recorded daily in the course of three days, using triplicate tanks per diet.

2.4. Calculations and statistical analysis

Expansion ratio (%) of pellets was calculated as: $100 \times ((\text{diameter of extrudate} - \text{die diameter}) \times \text{die diameter}^{-1})$. The WSI (%) was calculated as the recovery of DM of waste feed: $100 \times ((W \times W_{DM}) \times (F \times F_{DM})^{-1})$, where W is the weight of waste feed collected (g), W_{DM} is the DM content of waste feed (%), F is the weight of feed (g), and F_{DM} is the DM content of feed (%) (Helland et al., 1996). Apparent digestibility (%) of individual nutrients was calculated as: $100 - (100 \times (M_D \times M_F^{-1} \times N_F \times N_D^{-1}))$, where M and N are marker and nutrient concentrations, and the subscripts $_D$ and $_F$ represent diet and feces, respectively (Austreng, 1978).

Analysis of variance was conducted using the General Linear Model (GLM) procedure of the SAS 9.1 computer software (SAS Institute Inc., Cary, NC, USA). Effects of ingredient (FM or BPC), extrusion temperature (110 or 141 °C), and level of NaDF supplementation (0 or 10.6 g kg^{-1}) were tested on data for digestibility in rainbow trout (n = 3) and physical quality (n = 3), using the following model:

$$Y_{ijkl} = \mu + \tau_i + \beta_j + \delta_k + (\tau\beta)_{ij} + (\tau\delta)_{ik} + (\beta\delta)_{jk} + (\tau\beta\delta)_{ijk} + \varepsilon_{ijkl},$$

where μ is the overall mean; τ_i is the main effect of level i (= 1, 2) for the factor ingredient (I); β_j is the main effect of level j (= 1, 2) for the factor extrusion temperature (T); δ_k is the main effect of level k (= 1, 2) for the factor NaDF supplementation (N); $(\tau\beta)_{ij}$ is the interaction effect between level i for factor I and level j for factor T; $(\tau\delta)_{ik}$ is the interaction effect between level i for factor I and level k for factor N; $(\beta\delta)_{jk}$ is the interaction effect between level j for factor T and level k for factor N; $(\tau\beta\delta)_{ijk}$ is the interaction effect between level i for factor I, level j for factor T, and level k for factor N; ε_{ijkl} is the random error; Y_{ijkl} is the observed response variable at level i for factor I, level j for factor T, and level k for factor N.

Results are presented as means for each treatment, and variance is expressed as the pooled standard error of means (S.E.M.). The level of significance was set at $p<0.05$, while tendencies were set at $0.05 \le p < 0.10$. Significant differences among means were ranked by the Ryan–Einot–Gabriel–Welsch multiple range test. Orthogonal polynomials were used to test linear and quadratic responses of increasing the extruder temperature from 110 to 126 and 141 °C on

nutrient digestibility for the BPC diets. Correlation coefficients were determined using the Pearson correlation coefficient procedure.

3. Results

3.1. Ingredients and diets

The proximate chemical composition of the tested FM and BPC ingredients is given in Table 1. The BPC contained less crude protein and ash, but more lipid and starch than the FM. The residual between the analyzed proximal nutrient content and the dry matter content (residual = 1000 − [crude protein + lipid + starch + ash]) was higher for the BPC than the FM, indicating that BPC may contain other compounds, such as non-starch polysaccharides (NSP) (Sørensen et al., 2009). Chemical analysis showed low variation in proximate chemical composition and amino acid composition among the diets (Table 4). The BPC diets contained more NSP; diluting the content of ash, crude protein, and essential amino acids (EAAs) compared to the FM diets. The percentage available lysine in the diets, given as reactive lysine in percentage of total lysine in the diet, increased with increasing extrusion temperature for both the FM and BPC diets (Table 4). Percentage available lysine in the diet was positively correlated ($r = 0.57$, $p = 0.013$) to apparent lysine digestibility for the diets containing BPC.

3.2. Nutrient digestibility

The standard error of the mean apparent digestibility of crude protein among the three different strippings was found to be low (<0.01); therefore, a decision was made to pool the remaining fecal samples for each tank. The main effects of ingredient, extrusion temperature, and NaDF supplementation on apparent digestibility of major nutrients and individual amino acids in rainbow trout are shown in Table 5. There were significant differences between ingredients on the digestibility of all major nutrients and amino acids with the exception of total amino acids and ash. Inclusion of BPC to diets gave a significantly lower digestibility of lipid, starch, all the EAAs, and the non-essential amino acids (NEAAs) alanine, aspargine, glycine, and serine, but increased the digestibility of crude protein and the NEAAs cysteine, glutamine, proline, and tyrosine compared to diets based on FM.

Extrusion processing at high temperature (141 °C) gave a significantly higher digestibility of starch, crude protein, the EAA arginine, and the NEAAs alanine, glycine, proline, and tyrosine compared to extrusion at low temperature (110 °C), whereas the digestibility of the EAA phenylalanine was significantly higher for diets extruded at low temperature. For the BPC diets, increasing extrusion temperatures from 110 to 126 and 141 °C gave a significant linear increase in digestibility of lipid ($p = 0.016$), starch ($p = 0.002$), crude protein ($p < 0.001$), the EAA arginine ($p = 0.008$), and the NEAAs alanine ($p = 0.009$), glutamine ($p = 0.023$), glycine ($p = 0.012$), proline ($p = 0.005$), and ash ($p = 0.04$). No quadratic responses were observed for extrusion temperature on the digestibility of any major nutrients or individual amino acids for fish fed the BPC diets.

Supplementation of NaDF to diets significantly improved the digestibility of all major nutrients and individual amino acids with the exception of starch and the EAA phenylalanine, which were not affected. The apparent digestibility of Na in rainbow trout significantly increased with NaDF supplementation in both the FM and BPC diets (results not shown).

There were significant interactions between ingredient and extrusion temperature for the digestibility of lipid, the EAAs isoleucine and phenylalanine, and the NEAAs cysteine, tyrosine, and ash. Interactive effects of ingredients and increasing extrusion temperatures on digestibility of major nutrients and amino acids in rainbow trout are shown in Table 6. Increasing the extrusion temperature from 110 to 141 °C improved the digestibility of lipid, isoleucine, phenylalanine, cysteine, and ash for the BPC diets, whereas

Table 4
Proximate chemical composition, amino acid composition and available lysine of the experimental diets (g kg^{-1}, n = 3).

Diet	FM diets				BPC diets			
	Without NaDF		With NaDF		Without NaDF		With NaDF	
	110 °C	141 °C	110 °C	141 °C	110 °C	141 °C	110 °C	141 °C
DM	971.8	976.5	974.1	971.9	969.8	975.5	974.8	972.4
In DM								
Lipid	204.6	213.4	210.0	211.7	230.6	231.9	230.0	230.1
Ash	140.6	139.6	143.9	143.3	89.7	89.0	93.4	93.2
Starch	139.9	138.3	136.2	133.8	137.4	138.9	136.6	137.6
Crude protein	489.0	487.1	476.5	482.3	444.5	444.6	439.4	442.4
Total amino acids	371.8	361.9	371.6	360.3	345.4	311.5	327.3	309.7
Essential amino acids (except tryptophan)								
Arginine	24.9	24.1	25.0	24.2	21.4	19.1	20.2	19.0
Histidine	9.3	9.0	9.3	9.0	8.4	7.6	8.0	7.6
Isoleucine	15.6	15.0	15.5	15.0	14.0	12.4	13.0	12.2
Leucine	28.5	27.5	28.0	27.1	25.5	23.0	24.2	22.8
Lysine	28.9	27.9	29.0	28.1	21.4	19.2	20.3	19.0
Methionine	11.1	11.0	11.2	10.8	8.7	7.8	8.2	7.7
Phenylalanine	15.9	9.1	9.4	9.0	9.1	8.3	8.7	8.2
Threonine	17.6	17.1	17.4	17.0	15.2	13.5	14.2	13.4
Valine	18.3	17.8	18.2	17.7	17.1	15.1	15.9	15.0
Non-essential amino acids								
Alanine	23.5	22.9	23.6	22.8	18.4	16.6	17.4	16.5
Aspargine	36.5	35.3	36.3	35.1	28.7	25.7	27.0	25.5
Cysteine[a]	3.7	3.6	3.6	3.5	4.6	4.2	4.4	4.2
Glutamine	62.2	60.1	61.5	59.8	70.4	64.1	67.1	64.0
Glycine	26.6	25.9	26.8	25.9	19.5	17.5	18.5	17.4
Proline	20.6	20.0	20.5	20.1	26.3	24.5	25.6	24.5
Serine	17.2	16.7	17.0	16.5	16.3	14.6	15.2	14.5
Tyrosine	11.4	18.9	19.4	18.7	20.3	18.4	19.3	18.2
Available lysine (%)[b]	82.4	85.5	85.4	87.6	84.1	98.5	92.7	98.0

[a] Cystine and cysteine.
[b] Reactive lysine in percentage of total lysine in the diet.

Table 5
The main effects of ingredient, extrusion temperature, and NaDF supplementation on apparent digestibility (%) of major nutrients and individual amino acids in rainbow trout (n = 3).

	Ingredient		Extrusion temperature		NaDF		S.E.M.†	*p*-values‡						
	FM	BPC	110 °C	141 °C	Without	With		I	T	N	I×T	I×N	T×N	I×T×N
Lipid	94.6[a]	93.7[b]	94.0	94.3	93.5[y]	94.8[x]	0.20	0.005	0.319	<0.001	0.002	0.840	0.011	0.306
Ash	4.9	3.9	3.4	5.4	2.2[y]	6.6[x]	1.0	0.535	0.173	0.007	0.021	0.422	0.775	0.954
Starch	98.7[a]	98.2[b]	98.0[n]	98.9[m]	98.4	98.5	0.12	0.007	<0.001	0.497	0.392	0.182	0.392	0.936
Crude protein	82.4[b]	84.2[a]	82.6[n]	84.1[m]	82.8[y]	83.8[x]	0.21	<0.001	<0.001	0.006	0.207	0.642	0.452	0.839
Total AA	88.2	88.2	88.0	88.4	87.6[y]	88.7[x]	0.21	0.991	0.149	0.002	0.249	0.682	0.224	0.109
Essential amino acids														
Arginine	90.9[a]	90.1[b]	90.2[n]	90.9[m]	89.9[y]	91.1[x]	0.17	0.004	0.010	<0.001	0.678	0.397	0.486	0.056
Histidine	88.4[a]	86.7[b]	87.5	87.7	87.1[y]	88.0[x]	0.22	<0.001	0.524	0.007	0.110	0.775	0.209	0.213
Isoleucine	92.5[a]	91.6[b]	92.0	92.0	91.7[y]	92.3[x]	0.14	<0.001	0.910	0.017	0.038	0.512	0.153	0.181
Leucine	92.5[a]	91.5[b]	92.0	92.0	91.7[y]	92.3[x]	0.14	<0.001	0.725	0.018	0.051	0.859	0.197	0.247
Lysine	91.1[a]	88.6[b]	89.7	90.0	89.5[y]	90.2[x]	0.18	<0.001	0.405	0.007	0.177	0.916	0.273	0.132
Methionine	89.5[a]	88.7[b]	88.9	89.3	88.6[y]	89.7[x]	0.21	0.013	0.149	0.002	0.311	0.706	0.262	0.159
Phenylalanine	85.5[a]	83.7[b]	85.3[m]	83.8[n]	84.9	84.3	0.27	<0.001	0.001	0.143	<0.001	<0.001	0.017	0.006
Threonine	88.3[a]	85.5[b]	86.8	87.0	86.4[y]	87.4[x]	0.23	<0.001	0.588	0.005	0.192	0.940	0.386	0.169
Valine	91.2[a]	90.0[b]	90.5	90.7	90.3[y]	90.9[x]	0.16	<0.001	0.523	0.014	0.075	0.488	0.233	0.088
Non-essential amino acids														
Alanine	87.9[a]	86.6[b]	86.8[n]	87.8[m]	86.5[y]	88.1[x]	0.24	0.002	0.012	<0.001	0.510	0.571	0.320	0.083
Aspargine	80.2[a]	78.1[b]	79.1	79.2	78.5[y]	79.8[x]	0.37	<0.001	0.776	0.019	0.148	0.772	0.281	0.166
Cysteine[¤]	76.7[b]	78.1[a]	77.5	77.4	76.7[y]	78.2[x]	0.36	0.014	0.751	0.010	0.025	0.392	0.733	0.085
Glutamine	91.3[b]	92.7[a]	91.9	92.2	91.7[y]	92.3[x]	0.15	<0.001	0.196	0.007	0.219	0.823	0.182	0.105
Glycine	78.5[a]	77.0[b]	76.8[n]	78.7[m]	76.3[y]	79.2[x]	0.43	0.022	0.007	<0.001	0.720	0.706	0.438	0.076
Proline	83.7[b]	89.0[a]	85.8[n]	86.9[m]	85.4[y]	87.3[x]	0.26	<0.001	0.009	<0.001	0.876	0.128	0.188	0.112
Serine	87.5[a]	86.5[b]	86.8	87.2	86.4[y]	87.5[x]	0.22	0.005	0.282	0.004	0.166	0.755	0.315	0.169
Tyrosine	94.4[b]	95.3[a]	94.4[n]	95.3[m]	94.2[y]	95.5[x]	0.11	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

†Pooled standard error of the mean for the main effects ingredient, extrusion temperature, and NaDF.
‡*p*-values are denoted by: I = ingredient; T = extrusion temperature; N = NaDF; I×T = the interaction between ingredient and extrusion temperature; I×N = the interaction between ingredient and NaDF; T×N = the interaction between extrusion temperature and NaDF; I×T×N = the interaction between ingredient, extrusion temperature, and NaDF. Significant ($p<0.05$) differences among means within ingredient are indicated by superscripts [a, b], significant differences among means within extrusion temperature are indicated by superscripts [m, n], and significant differences among means within NaDF are indicated by superscripts [x, y].
¤Cystine and cysteine.

these digestibilities were reduced for the FM diets. There was also a significant interaction between extrusion temperature and NaDF supplementation for the digestibility of lipid. Higher digestibility of lipid was observed for fish fed diets with NaDF extruded at 141 °C (95.4%) compared to fish fed diets containing no NaDF, extruded at either 110 °C (93.8%) or 141 °C (93.3%).

Table 6
The effect of the interaction between ingredient and extrusion temperature on apparent digestibility (%) of major nutrients and individual amino acids in rainbow trout (n = 3).

Treatment†	FM diets		BPC diets		S.E.M.‡
	110 °C	141 °C	110 °C	141 °C	
Lipid	95.0[a]	94.3[ab]	93.1[b]	94.4[ab]	0.44
Ash	5.7	4.0	1.1	6.8	1.7
Starch	98.4[b]	99.1[a]	97.7[c]	98.7[ab]	0.17
Crude protein	81.8[c]	82.9[bc]	83.3[b]	85.2[a]	0.34
Total amino acids	88.1	88.2	87.8	88.6	0.39
Essential amino acids					
Arginine	90.6[ab]	91.2[a]	89.7[b]	90.5[ab]	0.37
Histidine	88.6[a]	88.3[ab]	86.3[c]	87.0[bc]	0.37
Isoleucine	92.7[a]	92.2[ab]	91.3[c]	91.8[bc]	0.24
Leucine	92.7[a]	92.3[ab]	91.3[c]	91.8[bc]	0.23
Lysine	91.2[a]	91.0[a]	88.3[b]	88.9[b]	0.30
Methionine	89.5	89.6	88.3	89.1	0.38
Phenylalanine	87.5[a]	83.5[b]	83.2[b]	84.2[b]	0.66
Threonine	88.4[a]	88.2[a]	85.2[b]	85.8[b]	0.39
Valine	91.3[a]	91.1[ab]	89.7[c]	90.3[bc]	0.28
Non-essential amino acids					
Alanine	87.6[ab]	88.3[a]	86.0[b]	87.2[ab]	0.50
Aspargine	80.5[a]	79.9[a]	77.6[b]	78.5[ab]	0.60
Cysteine[¤]	77.4[ab]	76.0[b]	77.6[ab]	78.7[a]	0.61
Glutamine	91.3[b]	91.3[b]	92.4[a]	93.0[a]	0.26
Glycine	77.7	79.3	75.9	78.0	0.89
Proline	83.2[b]	84.2[b]	88.4[a]	89.6[a]	0.58
Serine	87.5	87.4	86.1	86.9	0.39
Tyrosine	93.6	95.2	95.2	95.4	0.52

†Significant ($p<0.05$) differences among means are indicated by superscripts [a, b, c].
‡Pooled standard error of the mean.
¤Cystine and cysteine.

3.3. Physical quality

The main effects of ingredient, extrusion temperature, and NaDF supplementation on physical quality of extruded diets are shown in Table 7. Inclusion of BPC gave a significantly higher hardness, expansion ratio, and durability of the pellets, but reduced the WSI and pH compared to diets with FM. Increasing the extrusion temperature from 110 to 141 °C significantly improved pellet durability. Supplementation of NaDF to diets gave a significantly higher expansion ratio, durability, and WSI, but reduced the pH of the extruded diets compared to diets without NaDF.

Significant interactions were found between ingredient and NaDF supplementation for pH of the extruded diets. Diets containing BPC (pH = 6.17) had a significantly lower pH than diets with FM (pH = 6.45) when no NaDF was added. Addition of NaDF resulted in a significantly lower pH of diets with BPC (pH = 5.72) compared to FM (pH = 6.07). There were also significant interactions between extrusion temperature and NaDF supplementation for pH of the extruded diets. Diets extruded at both 110 (pH = 6.32) and 141 °C (pH = 6.30) containing no NaDF had significantly higher pH compared to diets with NaDF. The reduction in pH when adding NaDF to diets was higher when extruding the diets at 141 (pH = 5.87) than 110 °C (pH = 5.93).

Significant three-way interactions among ingredient, extrusion temperature, and NaDF supplementation were found for expansion ratio, durability, and WSI (Table 8). The highest expansion ratio was found for the BPC diet extruded at 110 °C with NaDF, whereas BPC extruded at 110 °C without NaDF resulted in the lowest expansion ratio. The best durability was given by BPC extruded at 141 °C with NaDF, while the FM extruded at 110 °C without NaDF resulted in the

Table 7
The main effects of ingredient, extrusion temperature, and NaDF supplementation on physical quality and pH of extruded diets (n = 3).

	Ingredient		Extrusion temperature		NaDF		S.E.M.†	*p*-values‡						
	FM	BPC	110 °C	141 °C	Without	With		I	T	N	I×T	I×N	T×N	I×T×N
Hardness [N]	24.2^{b}	26.9^{a}	26.1	25.1	25.0	26.2	0.46	<0.001	0.123	0.085	0.216	0.899	0.573	0.939
Expansion ratio [%]	1.5^{b}	2.5^{a}	2.1	1.9	1.3^{y}	2.7^{x}	0.23	0.006	0.420	<0.001	0.017	0.009	<0.001	<0.001
Durability [%]	91.5^{b}	95.2^{a}	92.5^{n}	94.1^{m}	91.7^{y}	94.9^{x}	0.30	<0.001	0.002	<0.001	0.002	0.879	0.449	0.005
Water stability index [%]	91.3^{a}	85.6^{b}	89.0	87.9	85.9^{y}	91.1^{x}	0.42	<0.001	0.086	<0.001	0.003	<0.001	0.002	0.002
pH	6.3^{a}	5.9^{b}	6.1^{m}	6.1^{n}	6.3^{x}	5.9^{y}	0.01	<0.001	<0.001	<0.001	0.339	0.002	0.021	0.289

†Pooled standard error of the mean for the main effects ingredient, extrusion temperature, and NaDF.
‡*p*-values are denoted by: I = ingredient; T = extrusion temperature; N = NaDF; I×T = the interaction between ingredient and extrusion temperature; I×N = the interaction between ingredient and NaDF; T×N = the interaction between extrusion temperature and NaDF; I×T×N = the interaction between ingredient, extrusion temperature, and NaDF. Significant ($p<0.05$) differences among means within ingredient are indicated by superscripts $^{a, b}$, significant differences among means within extrusion temperature are indicated by superscripts $^{m, n}$, and significant differences among means within NaDF are indicated by superscripts $^{x, y}$.

poorest durability. The significantly lowest WSI of the pellets was found for BPC diets extruded at both 110 and 141 °C without NaDF. Hardness of pellets was positively correlated to durability (r = 0.64, $p<0.001$), and pH of the extruded diets was negatively correlated to hardness (r = −0.61, $p=0.002$), expansion (r = −0.49, $p=0.016$), and durability (r = −0.85, $p<0.001$).

4. Discussion

Information on the effect of BPC as a protein source for salmonids is sparse. The present experiment demonstrated that the inclusion of BPC in diets for rainbow trout increased the digestibility of crude protein, but did not affect the digestibility of total amino acids compared with FM diets. The improvement in crude protein digestibility with inclusion of BPC in diets for rainbow trout is in agreement with Gaylord et al. (2008), who observed an apparent protein digestibility of 92 and 90% for BPC and FM, respectively. Barrows et al. (2007a) also reported a higher protein digestibility for diets based on a combination of FM and barley meal compared to diets solely based on plant concentrates or plant meals for rainbow trout. The overall crude protein digestibility for both the BPC (83–85%) and FM (82–83%) diets was, however, low in the present experiment compared to previous observations with rainbow trout when using the fecal stripping technique (Krogdahl et al., 2004; Øverland et al., 2006; Sørensen et al., 2005). This may indicate that the crude protein digestibility was underestimated in both the BPC and FM diets due to the presence of indigestible nitrogenous compounds, including neutral insoluble nitrogen in barley (Silke and Udén, 2006) or irreversibly denatured protein complexes in the BPC or FM (Opstvedt et al., 2003).

Feeding diets with BPC or FM to rainbow trout gave similar digestibility values for total amino acids, indicating that the amino acids were readily digested in both diets. The systematically lower digestibility of individual EAAs and higher digestibility of individual NEAAs in fish fed BPC diets is in line with Gaylord et al. (2010), who reported lower digestibility of most individual EAAs in rainbow trout fed BPC compared to those fed soy protein concentrate, corn gluten meal, or wheat gluten. This could be explained by the lower concentration of EAAs in the BPC compared to FM and that apparent digestibility of a nutrient usually is underestimated at lower concentrations in the diet (Gaylord et al., 2008). The lower lipid digestibility in trout fed the diets containing BPC is most likely explained by the higher content of carbohydrates in the BPC compared to FM. The similar starch level among the diets indicates a higher content of NSP in the BPC, such as mixed-linked (1→3) (1→4)-β-glucans associated with the aleurone and endosperm layers of the grain (Yu et al., 2002). As a consequence, the lipid digestibility could have been reduced (Gatlin et al., 2007), as seen in broiler chickens (Refstie et al., 1999).

The present experiment showed that increasing extrusion temperatures improved the digestibility of starch, protein, and individual amino acids. The high starch digestibility observed for diets extruded at 141 °C is close to previously reported values for rainbow trout (Krogdahl et al., 2004). Literature concerning the effect of extrusion temperature on protein and amino acid digestibility in salmonids or in terrestrial monogastric carnivores is, however, inconsistent. Ljøkjel et al. (2004) reported reduced protein and individual amino acid digestibility in mink (*Mustela vison*) when increasing the extrusion temperature from 100 to 125 and 150 °C. Sørensen et al. (2002) observed no effect of increasing the extrusion temperature from 100 to 125 and 150 °C on protein and individual amino acid digestibility in rainbow trout. Sørensen et al. (2005) also found no effects of increasing the extrusion temperature from 103 to 137 °C on protein digestibility in rainbow trout. On the other hand, Barrows et al. (2007b) reported a slight, but not significant, increase in protein digestibility in rainbow trout when increasing the extrusion temperature from 93 to 127 °C. Although it is recognized that excessive heat treatment may reduce protein and individual amino acid digestibility (Opstvedt et al., 1984), the present experiment showed that increasing the extrusion temperature from 110 to 126 and 141 °C did not exceed the heat threshold required to reduce protein digestibility in rainbow trout. This was also evidenced by the increasing content of available lysine in the diets

Table 8
The effect of the three-way interaction among ingredient, extrusion temperature, and NaDF supplementation on physical quality and pH of extruded diets (n = 3).

Treatment†	FM diets				BPC diets				S.E.M.‡
	110 °C		141 °C		110 °C		141 °C		
	Without NaDF	With NaDF	Without NaDF	With NaDF	Without NaDF	With NaDF	Without NaDF	With NaDF	
Hardness [N]	24.0^{b}	24.7^{ab}	23.3^{b}	24.9^{ab}	27.4^{ab}	28.4^{a}	25.2^{ab}	26.8^{ab}	0.92
Expansion ratio [%]	1.9^{bc}	2.1^{bc}	0.6^{cd}	1.2^{cd}	−0.7^{d}	5.1^{a}	3.4^{ab}	2.3^{bc}	0.47
Durability [%]	87.8^{d}	92.0^{c}	92.0^{c}	94.2^{bc}	94.4^{bc}	95.9^{ab}	92.8^{c}	97.6^{a}	0.59
Water stability index [%]	90.5^{a}	91.2^{a}	91.4^{a}	92.2^{a}	84.6^{b}	89.8^{a}	77.1^{c}	91.0^{a}	0.84
pH	6.5^{a}	6.1^{c}	6.4^{a}	6.0^{d}	6.2^{b}	5.7^{e}	6.2^{bc}	5.7^{e}	0.01

†Significant ($p<0.05$) differences among means are indicated by superscripts $^{a, b, c, d, e}$.
‡Pooled standard error of the mean.

when increasing the extrusion temperature, as well as the positive correlation between digestible lysine and available lysine for the BPC diets.

Supplementation of NaDF to diets improved the digestibility of lipid, ash, crude protein, total and individual amino acids, as previously seen in pigs fed diets supplemented with KDF (Roth et al., 1998). The increase in ash digestibility with NaDF supplementation can be explained by the high digestibility and absorption of Na from NaDF in the diet. Similarly, Mroz et al. (2002) reported an increase in ash and K digestibility with the addition of KDF to pig diets. The inclusion of salts of formic acid in fish diets has shown to improve the performance of Nile tilapia (*Oreochromis niloticus*) and salmonids, as recently reviewed by Ng and Koh (2011). In line with the present results, Lückstädt (2008) reported improved protein digestibility in Atlantic salmon when 13.5 g kg^{-1} KDF was included to raw fish prior to FM production or prior to diet extrusion. Storebakken et al. (2010) also showed an increase in individual amino acid digestibility in Atlantic salmon when diets were supplemented with 12 g kg^{-1} KDF. Limited information is, however, available on the effects of adding Na salts of formic acid in fish diets. Ringø (1992) found no significant effect of adding 10 g kg^{-1} Na-formate on the digestibility of protein or lipid in diets for Arctic charr (*Salvelinus alpinus* L.). The positive effects of dietary organic acids or their salts on nutrient digestibility in pigs has mainly been attributed a lowering of gastric pH, resulting in increased activity of proteolytic enzymes, prolonged gastric retention time, increased antimicrobial activity, and/or stimulation of pancreatic secretions (Partanen and Mroz, 1999). In the present experiment, addition of 10.6 g kg^{-1} NaDF corresponded to a formic acid inclusion of 4.2 g kg^{-1}, and resulted in a pH drop equivalent of 0.4 U in the extruded diets. The improved nutrient digestibility could, therefore, partially be explained by a reduction in gastric pH in fish fed diets containing NaDF.

The improved physical quality by use of BPC is in line with previous studies reporting effects of partially replacing FM with legumes or oilseeds (Kraugerud et al., 2011; Sørensen et al., 2009; Øverland et al., 2009). This may be explained by differences in functional properties of the protein sources used, as discussed by Sørensen et al. (2009). The structure-forming properties of proteins are believed to result from the denaturation, dissociation, and disulfide cross-linkage of globular protein structures during extrusion, resulting in a network of proteins which stabilize the pellet structure (Li and Lee, 1996). Plant ingredients often serve as a source of non-denatured, globular protein (Øverland et al., 2009). Thus, the present results indicate that the protein present in the BPC had more inherent binding properties, and hence, greater structuring capability than FM. Further, the higher level of cysteine in the BPC diets than the FM diets may also explain the improved physical quality of pellets with BPC due to a higher formation of stabilizing disulfide linkages during extrusion (Øverland et al., 2009; Sørensen et al., 2009). The content of NSPs and the composition of the NSP fraction in BPC could also have affected physical quality of the diets, as discussed in previous studies (Hansen and Storebakken, 2007; Kraugerud et al., 2011; Sørensen et al., 2009; Øverland et al., 2009).

Extrusion at 141 °C yielded pellets that were more durable than pellets extruded at 110 °C, whereas pellet hardness, expansion ratio, and water stability were not influenced by extrusion temperature. In contrast, Aarseth et al. (2006) observed a decrease in durability of pellets when increasing the extruder temperature from 100 to 140 °C. The inconsistency between these studies could be explained by differences in diet formulation, extruder hardware, or processing parameters.

Supplementation of NaDF to diets gave more durable and water stable pellets with a slightly larger expansion ratio than diets without NaDF. The pH of the experimental diets was lowered when adding NaDF, as seen when adding KDF to plant-based diets (Ng et al., 2009). The negative correlation between pH and durability and expansion indicates that the improved durability and expansion of pellets in the present experiment was a result of the 0.4 U reduction in pH by the addition of NaDF. These results suggest that NaDF affect the physical quality of diets, even at the low dietary concentration of 10.6 g kg^{-1}. Expansion and durability are usually negatively correlated parameters (Hansen and Storebakken, 2007; Sørensen et al., 2009). The present experiment showed, however, that NaDF increased both pellet durability and expansion. This is in contrast with the findings of Morken et al. (in press), who observed reduced expansion and increased pellet hardness when supplementing 12 g kg^{-1} KDF to extruded fish diets containing soybean meal. The improved physical quality of diets with NaDF could, therefore, also be due to an improved structuring capability of proteins in the presence of Na, as seen when adding NaCl to wheat flour dough (Chiotelli et al., 2004). This may have changed the functional properties of the protein, resulting in altered textural properties of the extrudate.

5. Conclusion

This experiment has demonstrated that inclusion of BPC reduced the digestibility of some nutrients in rainbow trout, improved digestibility of others and improved the physical quality of pellets when compared to diets with FM as the sole protein ingredient. Extrusion at 141 °C improved the digestibility of several nutrients and increased the durability of pellets compared to extrusion at 110 °C. Supplementation of NaDF increased the digestibility of nutrients, as well as improving physical quality of extruded diets, demonstrating a potential for the application of NaDF in diets for rainbow trout. This knowledge should be further supplemented with studies on mechanisms of diformate acid salts to obtain a better understanding of the mode of action of NaDF on the digestibility of nutrients in fish and physical quality of extruded diets.

Acknowledgments

This work was supported by grant # 145949 from the Research Council of Norway and partly from a grant by Addcon Nordic AS. Thea Morken was supported by a PhD-grant and a travel grant ("Småforsk") from the Norwegian University of Life Sciences (UMB, Ås, Norway). We would like to thank Jason Frost at the Bozeman Fish Technology Center, U.S. Fish and Wildlife Service, for excellent technical assistance with the experiment.

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Paper III

Effects of heat treatment and sodium diformate on amino acid composition, *in vivo* digestibility in mink and *in vitro* bioavailability using digestive enzymes from Atlantic salmon

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Abstract

The objective of the present experiment was to investigate effects of heat treatment by autoclaving and addition of the acid salt sodium diformate (NaDF) to diets on amino acid (AA) composition, *in vivo* coefficient of total tract apparent digestibility (CTTAD) in mink (*Neovison vison*) and *in vitro* bioavailability using crude enzyme extracts from Atlantic salmon (*Salmo salar*). The chemical and physical properties of the diets were also evaluated. The experimental treatments were organized according to a 4×2 factorial design with four temperatures (100, 110, 120 and 130 ºC) and two levels of

NaDF supplementation (0 and 10.6 g/kg). *In vivo* CTTAD was determined for all experimental treatments, whereas *in vitro* bioavailability was estimated for the NaDF treatments at 100 and 130 ºC. The results showed that heat treatment at 130 ºC reduced ($P<0.05$) the dietary content of several AA, in particular Arg, Cys, Lys and Met. The *in vivo* CTTAD of crude protein and all individual AA and the *in vitro* bioavailability of AA were reduced ($P<0.01$) with increasing temperatures. Heat treatment also reduced the amount of reactive and available Lys ($P<0.001$), protein solubility ($P<0.01$) and increased browning of the diets as measured by the CIE *L* a* b** color model ($P<0.001$). Inclusion of NaDF reduced ($P<0.05$) the dietary content of several indispensable and dispensable AA. The *in vivo* CTTAD of crude protein and AA in mink was not affected by supplemental NaDF ($P>0.1$), but improved ($P<0.05$) the bioavailability of AA in the *in vitro* assay. The presence of NaDF also reduced the amount of reactive Lys ($P<0.05$), protein solubility ($P<0.05$) and increased browning of the diets ($P<0.001$). In conclusion, the present experiment has demonstrated negative effects of heat treatment on both *in vivo* CTTAD of protein in mink and *in vitro* bioavailability of AA. This could be explained by heat-induced changes in the protein, as shown by the increased browning and reductions in available Lys and protein solubility. Supplemental NaDF did not affect *in vivo* CTTAD in mink, but improved the *in vitro* bioavailability of AA when using digestive enzymes from Atlantic salmon.

Keywords: Autoclave; Digestibility; In vitro; Mink; Sodium diformate; Temperature.

Abbreviations: BPC, barley protein concentrate; CTTAD, coefficient of total tract apparent digestibility; AA, amino acid; GIM, gastrointestinal model; KDF, potassium diformate; NaDF, sodium diformate.

1. Introduction

Animal feeds are processed with the use of heat treatment for hygienization and improvement of nutritional and physical quality (Thomas et al., 1997). Feed ingredients are also subjected to heat treatment to evaporate solvent residues and to inactivate heat-labile antinutritional factors (Storebakken et al., 2000). In both cases, thermal treatment leads to physicochemical and chemical changes which affect the protein quality (Camire, 1998). Apart from denaturation, amino acids (AA) may react with other AA or undergo various side-chain reactions with lipids and their oxidation products, polyphenols, vitamin B_6, various chemical additives and reducing sugars (Cheftel, 1986; Moughan, 2003). These reactions leads to disruption and reformation of strong covalent bonds in the amino acid structure, which render the protein less susceptible to hydrolysis by digestive proteases (Phillips, 1989). Such unwanted heat-induced chemical reactions may also impair the ability of AA to serve as substrates for protein synthesis in the animal (Moughan, 2003).

Structural changes in proteins during heat treatment are a function of temperature, moisture, time and presence of reducing substances in the feed (Papadopoulos, 1989). The extent of structural changes is generally greater when applying dry heat compared to moist heat (Ljøkjel et al., 2000; 2004; Sørensen et al., 2002; Opstvedt et al., 2003), especially at increased treatment times (Arndt et al., 1999; Fontaine et al., 2007). Lysine is particularly exposed to structural changes and may thus serve as an indicator of protein damage during heat treatment (Björck and Asp, 1983; Singh et al., 2007). However, conventional AA analysis does not discriminate between lysine that has not undergone binding with reducing sugars (reactive lysine) and bound lysine (unreactive lysine) from the early stages of the Maillard reaction (Pahm et al., 2008). Thus, it is preferable to

measure reactive lysine to evaluate lysine availability in feeds that have undergone heat treatment (Moughan and Rutherfurd, 1996; Moughan, 2003; Rutherfurd et al., 2007; Singh et al., 2007). Application of *in vitro* models to evaluate changes in bioavailability of AA has also been shown to be useful tools for fish feeds and ingredients (Rungruangsak-Torrissen et al., 2002; Morales and Moyano, 2010). *In vitro* methods have the advantage of being rapid and inexpensive, in addition to reduce the number of experimental animals needed. In this respect, the amount of bioavailable AA refers to the proportion of an ingested nutrient which is available for intestinal absorption and physiological utilization in the animal (Parada and Aguilera, 2007).

Protein digestibility can be improved by organic acid salts, as demonstrated in our recent experiment (Morken et al. 2011a). Positive effects of sodium diformate (NaDF) were observed on digestibility of crude protein (CP) and AA in rainbow trout (*Oncorhynchus mykiss*) fed diets extruded at different temperatures. However, no interactions were observed between heat treatment and acid salts when NaDF or potassium diformate (KDF) were added to diets for rainbow trout and Atlantic salmon (*Salmo salar*), respectively (Morken et al., 2011a; b). There is a gap in the knowledge about the potential protective effect of NaDF on proteins during heat treatment. The objective of the present experiment was, therefore, to investigate the effects of heat treatment at long retention times and the acid salt NaDF on AA composition, *in vivo* digestibility in mink (*Mustela vison*) and *in vitro* bioavailability using crude enzyme extracts from Atlantic salmon. In addition, the chemical and physical properties of the diets were also evaluated.

2. Materials and methods

2.1 Experimental design and diets

The dietary treatments were organized according to a 4×2 factorial design with four temperatures (100, 110, 120, and 130 ºC) and two levels of NaDF supplementation (0 and 10.6 g/kg). Diets with the nutritional characteristics for salmonid fish (Morken et al., 2011a) were formulated with fish meal, barley protein concentrate (BPC), and wheat flour as the main ingredients. The proximate chemical composition of the main ingredients is shown in Table 1. Diet 1 (control) contained no feed additives and Diet 2 (control + NaDF) was formulated by adding 10.6 g/kg NaDF (Table 2). The NaDF (Formi® NDF, ADDCON Nordic AS, Porsgrunn, Norway) is a conjugated salt of formic acid and sodium formate (HCOOH··HCOO-Na). The Formi® NDF contained 970 g/kg NaDF, of which 195.6 g/kg was sodium, 391.5 g/kg formic acid, and 382.9 g/kg formate, while the remaining 30 g/kg was a mixture of vegetable fat (palmitic and stearic acid) and silicic acid (SiO_2). The addition of NaDF was calculated to be equivalent to the formic acid content of 12 g/kg potassium diformate (KDF) (Øverland et al., 2000).

2.2 Heat treatment of experimental diets

The experimental diets were heat treated at Nofima (Ås, Norway), using a Getinge GE 2606 EC (Getinge AB, Getinge, Sweden) laboratory autoclave. The chamber of the autoclave had a height and width of 672 mm, dept of 660 mm, and a volume of 298 L. The temperature sensor within the autoclave chamber was fitted in a glass bottle containing 200 mL of sterilized water at 22 ºC. Individual batches of ground and mixed dry feed ingredients, weighing 2.5 kg each, were added 200 g/kg water (wt/wt) during continuous mixing and put in plastic autoclave bags. The autoclave bag was placed on an

aluminum tray and the material was spread in a thin layer prior to folding the open end of the bag. A second autoclave bag was subsequently threaded over the first bag. Individual batches of each diet was autoclaved using a wet-cycle program with set-temperatures of 100, 110, 120, or 130 ºC and 20 min treatment time at the targeted temperature. The total autoclave run times varied and were on average 56, 68, 77, and 84 min for the set-temperatures of 100, 110, 120, and 130 ºC, respectively. The differences in run times were due to the time needed to reach the desired pressure and set-temperature and subsequent reduction in pressure at the end of an autoclave cycle, and could not be adjusted manually. Although the total treatment time varied among the different heat treatments, set-temperature was constant (20 min) and, therefore, not included in the statistical model. Following autoclaving, the diets were immediately sealed in plastic buckets and stored at –22 ºC. The frozen diet samples were ground to pass through a 1 mm screen using a Retsch SM 100 cutting mill (Retsch Gmbh, Haan, Germany) and stored at –22 ºC until used in the animal experiment.

2.3 Color measurement

Color evaluation of the autoclaved and ground diets without fish oil was undertaken by reflectance spectrophotometry according to the CIE L^* a^* b^* color model. This model is a three-dimensional colorimetric system where L^* ranges from 0 (black) to 100 (white) and indicates lightness, a^* and b^* are the chromaticity co-ordinates indicating the color in the red ($+a^*$ at 0º) to the green ($-a^*$ at 180º) direction as well as the yellow ($+b^*$ at 90º) to the blue ($-b^*$ at 270º) direction (Masoud and Jakobsen, 2003). CIE L^* a^* b^* values of the diet samples were obtained using a HunterLab LabScan® XE colorimetric spectrophotometer (Hunter Associates Laboratory Inc., Reston, VA, USA) with a D65 illuminant, a 10º observer angle, and a viewing port of 1”. Each diet sample

150 was measured in triplicate, and the mean value was recorded based on three parallel
151 readings from each replicate. The *a** and *b** values were transformed to hue angle, *h* =
152 arctan (*b**/*a**), in order to express the color shade (Masoud and Jakobsen, 2003).
153
154 *2.4 In vitro bioavailability assay*
155 The *in vitro* assay involved a two-step gastrointestinal model (GIM) designed to
156 simulate the continuous removal of digestion products during hydrolysis of substrates in
157 the stomach and intestinal phases of digestion (Hamdan et al., 2009). Crude enzyme
158 extracts were collected from Atlantic salmon and *in vitro* bioavailability of total AA was
159 estimated on both levels of NaDF at two temperatures (100 and 130 ºC). The *in vitro*
160 assays were carried out using autoclaved and ground diets added fish oil.
161 Atlantic salmon with 658 g average body weight (n=40; SD=98) were kept in a
162 2.5 m^3 fiberglass tank supplied with seawater at an average water temperature of 6 ºC at
163 the Norwegian Institute for Water Research (Solbergstrand, Norway). Fish were fed a
164 commercial diet (587 CP/kg DM) two times a day (9:00 and 14:30 hours) for 9 days at a
165 level representing on average 0.7% of the body weight. Fish were sampled at different
166 time intervals (1.5 h, 6.5 h and 3 days postprandial) in order to obtain enzyme extracts
167 with a maximum activity. During sampling, fish were anesthetized with 60 mg/L tricaine
168 methanesulfonate (MS-222™, Pharmaq, Hampshire, UK) and killed (head trauma). The
169 sacrificed fish were immediately chilled on ice and stored at −20 ºC prior to dissection of
170 the organs (stomach and proximal intestine including pyloric ceca). Crude extracts from
171 the organ samples and determination of acid and alkaline protease activities were
172 prepared as described by Morales and Moyano (2010) and stored at −20 ºC until used in
173 the *in vitro* assay.

A preliminary assay was performed to assess protein solubility in each diet by reproducing the pH profile to be used during the acid and alkaline phases of the hydrolysis. This assay also served to adjust the initial amount of diet to include in the enzyme assays. In brief, 100 mg diet was suspended in water and stirred, and sampled at different time intervals (Figure 1). The total reaction time was 240 min, of which the acid and alkaline phases constituted 60 and 120 min, respectively. Soluble protein was determined in duplicate for each diet after centrifugation (4,000 rpm, 3 °C, 15 min) as described by Bradford (1976).

The acid phase of the hydrolysis in the GIM was simulated in a closed chamber; 150 mg of each diet were suspended and stirred in water for 60 min to initiate solubilization. The pH was then adjusted to 4.0 by adding 0.5 M HCl followed by addition of crude enzymatic extract from fish stomach in order to reach an enzyme:substrate (E:S) ratio of 50 U/mg protein. Reaction products were not removed during this phase (120 min). The reaction mixture was transferred to a semi-permeable membrane reactor adapted from Savoie and Gauthier (1986). The device was formed by an inner reaction chamber separated from an outer chamber by a dialysis membrane of 3,500 Da MWCO (SpectraPor 6, Spectrum Medical Industries, Los Angeles, CA, USA). The pH was gradually raised to 8.5 by pumping 0.1 M borate buffer prior to addition of crude extract from pyloric ceca providing alkaline proteases to reach an E:S ratio of 27 U/mg protein. The alkaline hydrolysis was maintained for 240 min. The AA released during this digestion phase passed across the membrane and were continuously removed from the outer chamber by the continuous flow of buffer controlled by a peristaltic pump. Both reaction chambers were placed in a thermal water bath which maintained reaction temperature at 25 °C. Although this is higher than the physiological temperature of salmonids, it was selected in order to increase the enzyme activity and to reduce the time

199 required for each analysis, in addition to improve the discriminating power of the assay
200 (Hamdan et al., 2009).

201 The amount of total AA released during hydrolysis was determined at different
202 time intervals during alkaline digestion in both dialysates and the inner chamber. In the
203 latter case, measures were performed on small samples (50 μL) after precipitation of
204 protein by 200 g/L trichloroacetic acid (TCA) followed by centrifugation (4,000 rpm, 3
205 °C, 15 min) of the supernatant solution. The content of AA was determined using the *o*-
206 phthaldialdehyde method of Church et al. (1983). Total amount of AA produced at each
207 sampling moment was estimated from the sum of those measured in the dialysate and
208 those present at such moment in the inner chamber. The *in vitro* assay was run in
209 triplicate for each diet.

210

211 *2.5 Feeding trial with mink*

212 Two mink experiments were carried out at the Department of Animal and
213 Aquacultural Sciences (UMB, Ås, Norway). A total of 16 adult male mink of the
214 genotype standard dark, with an average initial weight of 2161 g (SD = 248), were used in
215 each experiment. The animals were kept in individual cages equipped for controlled
216 feeding and fecal collection. All autoclaved diets were mixed with 167 g/kg fish oil and
217 water, weighed into cups and stored at –22 ºC until one day before feeding. The
218 individual wet daily rations contained 66 g DM (SD = 0.8) and weighed 150 g. Each of
219 the 8 experimental diets was fed to a group of 4 animals for 7 days, of which feces were
220 quantitatively collected for each animal once a day the last 4 days. The animals had free
221 access to water during the feeding trials. Individual fecal samples were frozen at –22 ºC
222 immediately after sampling. At the end of the experiment, the feces were pooled, freeze-
223 dried, ground, and sieved for removal of hair prior to chemical analysis. The CTTAD in

224 mink was calculated as (($a - b$) / a), where a is the nutrient intake and b is the amount of
225 nutrients in feces (Skrede et al., 2001).
226
227 *2.6 Chemical analyses*
228 Main ingredients (fish meal, BPC and wheat flour), freeze-dried diets and feces
229 were analyzed for dry matter (Dir. 71/393/EEC), ash (Dir. 71/250/EEC), CP as Kjeldahl
230 nitrogen (Dir. 93/28/EEC) × 6.25, crude lipid by HCl hydrolysis followed by diethyleter
231 extraction (Dir. EU 98/64), and starch (AOAC enzymatic method 996.11). Diet samples
232 were defatted with acetone prior to starch analysis. Individual AA in diets and feces were
233 analyzed according to the EC method (Dir. 98/64EC) on a Biochrom 30 AA analyzer
234 (Biochrom Ltd., Cambridge, UK). Tryptophan was not analyzed in the present
235 experiment and all AA figures are presented in dehydrated form. Reactive lysine in diets
236 were determined using the guanidination method according to Moughan and Rutherfurd
237 (1996), as previously described by Morken et al. (2011a). The AA and reactive lysine
238 assays were performed with diet samples containing no fish oil and the analyzed contents
239 were corrected for fish oil addition to represent AA and reactive lysine in the complete
240 diets. The pH value of the diets was measured in triplicate as described by Gao et al.
241 (2011). Determination of total formic acid content in diet samples was conducted by
242 ADDCON Europe GmbH (Bitterfeld-Wolfen, Germany). In brief, triplicate samples of
243 diets without fish oil were homogenized and 5–15 g of each diet was weighed in a glass
244 bottle together with 0.3 g sodium butyrate (internal standard). The samples were diluted
245 with 150 mL water prior to extraction in an ultrasonic water bath for 30 min at 40 ºC. The
246 extracts were filtered at 0.45 μm prior to analysis by HPLC on a column designed for
247 separation of organic acids (Rezex™ 8 μm ROA-Organic Acid H^+ [8%], dimension 300 ×
248 7.8 mm, Phenomenex, CA, USA) at 30 ºC with 0.01 N sulfuric acid as effluent and 0.5

mL/min flow. Formic acid was then detected by an RI-refractometer. The column used to separate formic acid did not discriminate between formic acid (HCOOH) and formate ($HCOO^-$). The content of NaDF was calculated for all diets based on values corrected for fish oil addition as $((a \times b) / c)$, where a is the total analyzed content of dietary formic acid and formate, b is the percentage of NaDF in Formi® NDF (97%) and c is the percentage of formic acid and formate in pure NaDF (77.44%).

2.7 Statistical analyses

A four by two ANOVA was performed on data from chemical analyses, color analysis and *in vivo* CTTAD of nutrients in mink using the general linear model procedure of the SAS 9.2 computer software (SAS Institute Inc., Cary, NC, USA). Effects of temperature level (100, 110, 120, or 130 ºC) and NaDF level (0 or 10.6 g kg^{-1}) were tested using the following model: $Y_{ijk} = \mu + \tau_i + \beta_j + (\tau\beta)_{ij} + \varepsilon_{ijk}$, where μ = general mean; τ_i = main effect of level i (= 1, 2, 3, 4) for the factor temperature; β_j = main effect of level j (= 1, 2) for the factor NaDF; $(\tau\beta)_{ij}$ = interaction effect between level i for the factor temperature and level j for the factor NaDF; ε_{ijk} = random effect. Orthogonal polynomials were used to test linear, quadratic and cubic responses of increasing autoclave temperatures on chemical and physical properties of the diets and CTTAD of individual nutrients in mink. Data analysis for protein solubilization and total amount of AA released under the *in vitro* test was performed using a two by two factorial ANOVA by applying the same model as described above, but with two temperature levels (100 and 130 ºC). The results are presented as means for each treatment, and variance is expressed as the pooled standard error of means (SEM). Differences between means were ranked using the Ryan-Einot-Gabriel-Welsch multiple range test. The level of significance was set at $P<0.05$, while tendencies were set at $0.05 \leq P < 0.10$.

3. Results

3.1 Characterization of ingredients and experimental diets

The proximate chemical composition of the main dietary ingredients is given in Table 1. The BPC contained less CP and ash, but more lipid and starch than the fish meal. The residual was higher for BPC than for the other main dietary ingredients. The analyzed proximate chemical compositions of diets are shown in Table 2. The proximate composition showed minor variations among the diets, except for crude lipid which ranged from 254 to 283 g/kg DM.

The interactive effects of temperature and NaDF on the analyzed contents of individual AA are shown in Table 3. The contents of AA appeared to be temperature dependent and were ranked according to temperature within the NaDF treatments. In diets without NaDF, the contents of Arg, Lys, Met, Cys, Glu, Tyr, total indispensable AA and total dispensable AA were reduced ($P<0.05$) after heat treatment at 120 ºC and Arg, Met and Cys were further reduced at 130 ºC ($P<0.05$). In diets with NaDF, the contents of Met, Lys and Cys were reduced ($P<0.05$) after heat treatment at 130 ºC. Addition of NaDF significantly reduced the contents of total AA and the majority of indispensable AA (Arg, His, Leu, Lys, Met and Thr) and dispensable AA (Ala, Asp, Cys, Gly and Ser).

The main effects of temperature and NaDF supplementation on the contents of reactive and available Lys, formic acid, NaDF, and pH of the diets are shown in Table 4. Increasing temperatures during autoclaving gave a linear decrease ($P<0.001$) in the amount of reactive and available Lys. Temperature did not affect the dietary levels of formic acid and NaDF, whereas diet pH was significantly higher for diets heat treated at 100 and 110 ºC than 120 and 130 ºC. Supplementation of NaDF significantly reduced the content of reactive Lys, but did not affect the amount of available Lys in the diets. Diets

containing NaDF had a significantly higher content of formic acid compared to non-supplemented diets, which contained small amounts of formic acid and formate from the dietary ingredients. Consequently, the calculated content of NaDF was similar to the added amount of 10.6 g/kg NaDF when correcting for inherent formic acid and formate contents of the non-supplemented diets. Addition of NaDF also resulted in a significant 0.5 U reduction in pH. Significant interactions between temperature and NaDF were observed on the pH of the diets (Table 5). Diet pH was significantly lower in diets treated at 120 or 130 ºC when no NaDF was added, while addition of NaDF showed the lowest pH at 120 ºC.

3.2 Color of experimental diets

The color of the autoclaved diets, as determined by the CIE L^* a^* b^* coordinates and hue angle (h), were significantly affected by temperature, NaDF as well as the interaction between these (Table 4). Temperature explained the greatest proportion (Type I SS) of the total variation in the model for L^* (98.4%), a^* (96.5%), b^* (47.2%) and hue angle (98.9%). Increasing temperatures increased color development, as shown by the linear ($P<0.001$) reduction in L^*. Concurrently, a^* increased (linear $P<0.001$) in the red direction and b^* decreased (quadratic $P<0.001$) in the yellow direction. The hue angle also decreased (linear $P<0.001$) from 74 to 66º with increasing temperature towards a visually brownish shade. Addition of NaDF significantly increased the color development of diets, as shown by the reduction of L^* and hue angle with the increase of a^* and b^* values.

There were significant interactions between temperature and NaDF supplementation on the CIE L^* a^* b^* coordinates and hue angle (Table 5). The linear reduction of L^* with increasing temperatures was evident for both NaDF treatments, but

*L** decreased more rapidly at 120 and 130 ºC in diets containing NaDF compared to diets without acid salt. The increase in *a** with increasing temperatures was overall higher in diets with NaDF and resulted in increased redness at 130 ºC compared to diets containing no NaDF. Higher *b** values were observed for diets with NaDF processed at 110 and 120 ºC compared to diets without NaDF. The hue angle was similar for both NaDF treatments processed at 110 and 130 ºC, but were significantly lower for diets processed at 100 and 120 ºC when NaDF was added.

3.4 In vitro bioavailability assay

The results from the preliminary assay on protein solubilization in diet samples during acid and alkaline pH conditions are shown in Figure 1. Changes in the amount of solubilized protein were closely related to changes in pH induced after the initial period of stirring in distilled water. For all diets, soluble protein increased when pH was changed from 4.0 to 8.0. Protein solubility decreased from 97.2 to 55.7 μg when temperature was increased from 100 to 130 ºC (P=0.001) and from 85.8 to 67.1 μg when NaDF was supplemented to the diets (P=0.024). The reduction in protein solubilization with supplemental NaDF tended to be most prominent when heating at 100 ºC (P=0.116).

The main effects of temperature and NaDF supplementation on the *in vitro* release of AA during hydrolysis by salmon proteases are shown in Table 6. Both temperature and NaDF significantly affected the hydrolysis of protein by salmon proteases performed *in vitro*. Increasing the autoclave temperature from 100 to 130 ºC gave a significant reduction in the release of AA, while a significant increase was observed when supplementing diets with NaDF. The results indicated a positive interaction (P=0.07) between temperature and NaDF on the amount of AA released during hydrolysis as demonstrated by the higher release at 130 ºC compared to 100 ºC (Figure 2).

3.4 In vivo total tract digestibility in mink

The main effects of temperature and NaDF supplementation on CTTAD of CP and individual AA in mink are shown in Table 6. Significant effects of temperature were observed on the CTTAD of CP, total AA and all individual AA. Increasing the autoclave temperature from 100 to 130 ºC gave a significant linear reduction ($P \leq 0.01$) on the CTTAD of CP and total AA. The CTTAD of the majority of indispensable and dispensable AA decreased at treatment temperatures above 110 ºC. The CTTAD of Met was significantly reduced at 120 ºC and further reduced at 130 ºC. Tyr was significantly reduced at 130 ºC. This was also shown by the significant linear ($P<0.001$) reduction in CTTAD of individual AA in response to increasing autoclave temperatures. Increasing the temperature from 100 to 130 ºC caused the most severe reduction in the CTTAD of Thr (8.4%), Cys (16.7%) and Asp (17.1%), and the least severe reduction in the CTTAD of Pro (5.1%), Phe (5.0%) and Arg (4.4%). There were no significant effects of NaDF level on CTTAD of CP and individual AA. Also, no significant interactions between temperature and NaDF were observed on the CTTAD of any major nutrients or individual AA. There were no significant correlations among *in vivo* CTTAD of Lys, reactive and available Lys.

4. Discussion

The heat treatment applied in the present experiment was targeted to induce thermal stress by autoclaving. The goal was further to investigate if acid salts have a protective effect on proteins during heat treatment due to the reported beneficial effect of NaDF on protein digestibility in rainbow trout fed diets extruded at different temperatures (Morken et al., 2011a).

374 *4.1 Chemical and physical properties of the experimental diets*

375 The reduction of individual AA in response to heat treatment agrees with previous
376 observations where diets have been autoclaved at temperatures ranging from 130 to
377 135 ºC (Skrede and Krogdahl, 1985; Ljøkjel et al., 2000). Loss of AA in feeds due to heat
378 treatment may result from changes in the AA structure caused by interactions between
379 proteins and reducing compounds, such as the Maillard reaction (Cheftel, 1986;
380 Papadopoulos, 1989). Amadori compounds are intermediates resulting from the early
381 stages of the Maillard reaction, and typically involve Lys or Arg in the presence of
382 carbohydrates or their degradation products (Belitz et al., 2009). The Amadori
383 compounds can be regenerated to Lys during acid hydrolysis, while the advanced
384 Maillard products are acid-stable and may give a reduced total Lys content (Moughan,
385 2003; Pahm et al., 2008). Thus, the consistency between reactive and total Lys level in
386 response to NaDF treatment in the present experiment suggests that the protein underwent
387 more severe structural changes when subjected to the acid salt (Moughan, 2003). This is
388 consistent with the higher color development of feed containing NaDF, as the formation
389 of advanced Maillard products is typically shown by an increase in yellow/brown
390 pigmentation (Bates et al., 1994). The increase in browning due to NaDF was unexpected
391 in view of the general assumption that acidic conditions disfavor the Maillard browning
392 reaction (Ashoor and Zent, 1984; Renn and Sathe, 1997; Ajandouz and Puigserver, 1999).
393 However, these results are similar to those reported by Bates et al. (1994) where acidic
394 pH favored Maillard browning, as measured in a model system consisting of wheat
395 starch, glucose and Lys. Thus, the reduced content of reactive Lys in diets with NaDF
396 could be an effect of lowered diet pH, which in turn, aggravated heat-induced changes in
397 the AA structure.
398

399 4.2 *In vitro bioavailability assay*

400 Susceptibility of different proteins to enzymatic hydrolysis by digestive proteases
401 depends on a number of factors such as their solubility, structural complexity and AA
402 composition (de Jonge et al., 2009; Sáenz de Rodrigáñez et al., 2011), as well as external
403 factors like thermal treatment and chemical compounds present in the solution
404 (Bassompierre et al., 1998). In the present work, protein solubilization represented the
405 first step in assessing potential differences among experimental treatments at the pH
406 conditions used to simulate protein hydrolysis in the salmon gut. The final result
407 depended on the type and relative proportion of different protein fractions in the main
408 dietary ingredients (fish meal, BPC and wheat flour). Fish meal is a source of water-
409 soluble albumins and salt-soluble fibrillar protein such as actin, myosin and globulins
410 (Belitz et al., 2009; Hayakawa et al., 2009), whereas barley and wheat contains high
411 amounts of acid/alkali-soluble glutelin (Shewry, 1993; Sathe et al., 2005). This explains
412 the increased amount of soluble protein during the acid phase and the subsequent rapid
413 increase during alkalization as shown in Figure 1. Furthermore, it has been reported that a
414 previous acid digestion stage improves further solubilization and hydrolysis by alkaline
415 proteases (Seymour et al., 1994; Hamdan et al., 2009; Morales and Moyano, 2010).

416 The reduced protein solubility with NaDF supplementation can be explained by a
417 negative effect of increased Na concentrations from NaDF on solubility of barley protein
418 (Bilgi and Celik, 2004), however, it is more reasonable that this observation was a result
419 of increased thermal degradation when adding NaDF to diets, as discussed previously.
420 Heat-induced changes to proteins with subsequent loss of hydrophilic character were
421 most likely the cause of the reduced protein solubility in diets heat treated at 130 ºC.
422 Similar results were reported by Bassompierre et al. (1997; 1998) where protein solubility
423 was consistent with differences in fish meal quality.

Total amount of AA released from the different experimental feeds after the alkaline phase of the hydrolysis demonstrated a clear effect of thermal treatment on the susceptibility of feed protein to be hydrolysed by salmon proteases. This reduction can be explained by a combination of the decreased availability of soluble protein and a lower accesibility of proteases to their target AA as a result of chemical cross-links and/or conformational changes in the protein molecules (Phillips, 1989). Reduction in the availability of Lys as a result of the Maillard reaction may have a considerable effect on the hydrolysing potential of trypsin, and thus, reduce the release of AA as previously described for rainbow trout (Plakas et al., 1985).

4.3 In vivo total tract digestibility in mink

In vivo total tract digestibility was measured in mink to enable biological evaluation of a heat treated mash in order to diminish possible interactive effects of pellet structure on digestive processes in the animal (Hilton et al., 1981; Venou et al., 2009; Aas et al., 2011). Mink is often used as a model animal to simulate digestion of protein and AA in salmonid fish because digestibility coefficients of protein and AA are highly correlated with salmon values (Romero et al., 1994; Skrede et al., 1998; Øverland et al., 2006).

The reduced digestibility of protein and individual AA due to heat treatment by autoclaving is in line with previous results with mink (Skrede and Krogdahl, 1985; Ljøkjel et al., 2000). The latter authors showed that heat treatment gave the largest reduction in the digestibility of Cys and the lowest reduction in the digestibility of Arg, which is in agreement with the present results. These findings can be explained by the loss of AA, and in particular Cys, when diets were treated at high temperatures and long treatment times. The digestibility results coincided with the reduction of available Lys,

449 which is in agreement with our previous findings where rainbow trout was fed diets
450 extruded at 110 and 141 ºC (Morken et al., 2011a). However, in the latter study there was
451 an increase in both digestibility and available Lys with higher extrusion temperatures. It is
452 likely that the reduced digestibility of protein and AA during autoclaving was caused by
453 the formation of Maillard products, as shown by the increased browning with increasing
454 autoclave temperatures. The adverse effect of heating can be regarded a direct effect of
455 the combination of high temperature at long treatment times (Papadopoulos, 1989),
456 because studies on heat treatment by extrusion have not shown similar negative effects on
457 AA contents and protein digestibility in mink (Ljøkjel et al., 2004; Morken et al., 2011b).
458 Supplementation of NaDF to diets did not affect the digestibility of CP and
459 individual AA in mink, as previously observed when using KDF (Morken et al., 2011b).
460 Limited information is available regarding the effects of organic acid salts on protein
461 digestibility in mink, however, positive effects have been reported for growing pigs (Roth
462 et al., 1998) and salmonid fish species (Storebakken et al., 2010; Morken et al., 2011a).
463 Improved protein digestion in pigs fed organic acid salts is considered to be an effect of
464 reduced gastric pH due to increased proteolytic activity and gastric retention time
465 (Partanen and Mroz, 1999). The ability of organic acids to reduce gastric pH depends on
466 the intraluminal digesta acidity and the dissociation properties of the acid (Mroz et al.,
467 2006). Thus, the inconsistency between the present results obtained in mink compared to
468 NaDF for rainbow trout (Morken et al., 2011a) could be attributed to differences in
469 gastric pH between mammals and fish. In general, mink has a higher gastric acidity (pH
470 ~2) than rainbow trout (pH ~4.0) kept in freshwater (Sugiura et al., 2004; 2006; Vhile et
471 al., 2007). The acid dissociation constant of NaDF (pK_a = 3.75) is higher than the gastric
472 pH of mink, indicating that the majority of the acid molecules would be present in the
473 undissociated form. Undissociated forms of short-chain organic acids can rapidly diffuse

474 across the stomach wall, at which they are transported to the liver and oxidized to CO_2
475 and H_2O, while the remaining proportion is excreted as formates via the kidneys
476 (Partanen and Mroz, 1999). Also, at pH conditions lower than the pK_a of NaDF, the
477 NaDF would act as a buffer rather than an acidifying agent. Consequently, the majority of
478 the ingested NaDF would not contribute to reduce gastric pH in mink. It can, therefore, be
479 hypothesized that the effectiveness of NaDF is lower in mink as opposed to rainbow trout
480 due to differences in gastric pH, which suggest that the chemical properties of the acid
481 should be considered in relation to the biological conditions at which it is intended to be
482 used.
483
484 *4.4 Comparison of results obtained in vivo and in vitro*
485 To our knowledge, this is the first experiment comparing the effect of NaDF and
486 heat treatment on *in vivo* digestibility in mink in relation to *in vitro* bioavailability of AA
487 using crude enzyme extracts from Atlantic salmon. The present experiment showed that
488 both methods confirmed a reduction in available protein following heat treatment
489 (reduced digestibility of protein in the *in vivo* assay, a reduction in the total amount of
490 bioavailable AA in the *in vitro* assay). The main difference between these assays was that
491 the *in vitro* method showed a greater sensitivity to the changes in protein caused by NaDF
492 compared to the *in vivo* assay. This greater sensitivity may be explained by use of
493 solubilized protein and use of semi-purified enzyme extracts from the target species
494 (Alarcón et al., 2002). In addition, the *in vitro* assay was carried out under similar pH
495 conditions as in live salmon (Austreng et al., 2000; Nordrum et al., 2000). This *in vitro*
496 method has not previously been used with digestive enzymes obtained from Atlantic
497 salmon. The comparison between *in vivo* digestion in mink and *in vitro* method with use

498 of digestive enzymes from Atlantic salmon showed that the *in vitro* assay can be used to
499 estimate bioavailability of AA in Atlantic salmon.
500
501 **5. Conclusion**
502
503 The present experiment has demonstrated that heat treatment at increasing
504 temperatures by autoclaving showed negative effects on both *in vivo* CTTAD of nutrients
505 in mink and *in vitro* bioavailability of AA. The adverse effect of heat treatment on protein
506 quality was explained by thermal changes to proteins, as observed by reductions in
507 available Lys and protein solubility, as well as increased browning. The addition of NaDF
508 to diets did not affect *in vivo* CTTAD of protein in mink, but improved the total amount
509 of bioavailable AA in the *in vitro* assay. Further, these results indicated that NaDF did not
510 prevent protein damage during heat treatment by autoclaving.
511
512 **Acknowledgements**
513
514 This experiment was supported by grant # 145949 from the Research Council of
515 Norway. Thea Morken was supported by a PhD-grant from the Norwegian University of
516 Life Sciences (UMB, Ås, Norway). The authors gratefully acknowledge Bjørg Leistad,
517 Hans Blom and Grete Skrede at Nofima AS for assistance during heat treatment and color
518 analysis of the diets, Marianne Bratberg Skarra and Øystein Ahlstrøm at UMB for
519 assistance during the mink trial, Milka Kosanovic in AgriMarin Nutrition for supplying
520 the fish meal, Rune Christiansen in ADDCON Nordic AS for supplying the sodium
521 diformate (Formi®-NDF) and for analyzing the formic acid content of the diets and
522 Anders Skrede for a critical review of the manuscript.
523

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782
783

784 Table 1. Proximate chemical composition of the main dietary ingredients (g/kg).

785

	Fish meal	BPC [1]	Wheat flour
Dry matter (DM)	922	944	865
In DM			
Crude protein	713	568	138
Crude lipid	99	138	2
Starch	6	19	820
Ash	173	25	5
Residue [2]	9	250	36

[1] Barley protein concentrate

[2] Residue = 1000 – (protein + lipid + starch + ash), includes analytical errors from the analysis of the respective nutrients.

786

787 Table 2. Formulation and proximate composition of the experimental diets.

788

Formulation (g/kg)	Diet 1	Diet 2
	Without NaDF	With NaDF
Fish meal [1]	350.9	340.3
Barley protein concentrate [2]	276.0	276.0
Wheat flour [3]	197.0	197.0
Fish oil [4]	167.0	167.0
Vitamin and mineral premix [5]	6.0	6.0
Stay-C [6]	3.0	3.0
Y_2O_3 [7]	0.1	0.1
Sodium diformate (NaDF) [8]	0.0	10.6

Composition (g/kg, n=3)	Without NaDF				With NaDF			
	100 °C	110 °C	120 °C	130 °C	100 °C	110 °C	120 °C	130 °C
Dry matter (DM) [9]	969.4	970.4	970.9	971.2	966.1	967.5	967.5	966.2
In DM								
Crude protein	417.8	419.3	411.8	421.9	410.7	414.2	412.5	412.3
Crude lipid	265.8	271.8	269.8	254.1	274.2	275.4	282.8	262.5
Starch	137.6	138.3	138.6	144.3	142.2	140.8	138.1	141.6
Ash [9]	70.4	69.7	70.5	70.4	73.5	74.4	73.7	74.0

[1] NorsECO-LT, Egersund Sildoljefabrikk AS, Egersund, Norway.

[2] Montana Microbial Products, Butte, MT, USA.

[3] Dehulled and ground wheat flour, Norgesmøllene AS/Møllerens, Bergen, Norway.

[4] NorSalmOil, Egersund Sildoljefabrikk AS, Egersund, Norway.

[5] Mineral and vitamin premix for fish, Normin AS, Hønefoss, Norway.

[6] Rovimix ® Stay-C ®, DSM, Herleen, Netherlands.

[7] Metal Rare Earth Limited, Shenzhen, China. The Y_2O_3 was added to allow digestibility determinations by use of the indicator method, although not used in the present experiment.

[8] Formi ® NDF, ADDCON Nordic AS, Porsgrunn, Norway.

[9] The mean for DM and ash is based on two values (n=2).

789

790

791 Table 3. Interactive effects of temperature and sodium diformate (NaDF) supplementation on contents of total and individual amino acids (AA)
792 in the experimental diets (g/kg DM, n=3).
793

Treatment	Without NaDF				With NaDF				S.E.M. [1]	P-values [2]		
	100 °C	110 °C	120 °C	130 °C	100 °C	110 °C	120 °C	130 °C		T	N	T×N
Total AA	350.4 ^{AB}	352.3 ^{A}	343.7 ^{B}	344.0 ^{B}	344.0	344.5	345.1	339.5	1.93	0.015	0.006	0.126
Indispensable AA												
Arg	21.4 ^{A}	21.6 ^{A}	20.8 ^{B}	20.3 ^{C}	20.8	20.7	20.7	20.2	0.13	<0.001	<0.001	0.023
His	9.5	9.5	9.3	9.3	9.3	9.3	9.3	9.2	0.06	0.021	0.037	0.249
Ile	16.8	16.9	16.5	16.6	16.6	16.7	16.7	16.5	0.12	0.176	0.426	0.407
Leu	26.9	27.2	26.5	26.7	26.6	26.6	26.6	26.4	0.17	0.180	0.038	0.214
Lys	22.4 ^{A}	22.2 ^{A}	21.6 ^{B}	21.1 ^{B}	21.8 ^{a}	21.5 ^{ab}	21.2 ^{ab}	20.8 ^{b}	0.18	<0.001	0.001	0.567
Met	8.8 ^{A}	8.7 ^{A}	8.4 ^{B}	8.2 ^{C}	8.4 ^{ab}	8.5 ^{a}	8.4 ^{ab}	8.2 ^{b}	0.06	<0.001	0.006	0.014
Phe	17.7	17.9	17.4	17.5	17.5	17.5	17.6	17.3	0.12	0.088	0.128	0.116
Thr	14.0	14.1	13.8	13.8	13.4	13.5	13.6	13.6	0.09	0.460	<0.001	0.044
Val	19.6	19.7	19.2	19.3	19.3	19.4	19.4	19.2	0.12	0.248	0.279	0.200
Total	157.1 ^{A}	157.7 ^{A}	153.4 ^{B}	152.7 ^{B}	153.8	153.7	153.6	151.4	0.99	0.005	0.008	0.182
Dispensable AA												
Ala	18.0	18.1	17.7	17.8	17.6	17.6	17.7	17.5	0.12	0.405	0.003	0.284
Asp	28.4	28.5	27.8	27.8	27.5	27.7	27.7	27.7	0.19	0.216	0.003	0.114
Cys [3]	4.8 ^{A}	4.8 ^{A}	4.7 ^{B}	4.3 ^{C}	4.6 ^{a}	4.7 ^{a}	4.7 ^{a}	4.4 ^{b}	0.02	<0.001	0.003	<0.001
Glu	72.9 ^{AB}	73.5 ^{A}	71.9 ^{B}	73.1 ^{AB}	72.7	72.9	73.2	71.8	0.33	0.175	0.426	0.006

Gly	17.6	17.8	17.5	17.4	17.3	17.3	17.4	17.2	0.12	0.230	0.003	0.469
Pro	26.5	26.7	26.1	26.2	26.2	26.4	26.5	25.7	0.21	0.050	0.228	0.218
Ser	14.1	14.3	14.0	14.1	13.4	13.5	13.6	13.6	0.09	0.241	<0.001	0.029
Tyr	11.0 ^{A}	10.8 ^{AB}	10.6 ^{B}	10.6 ^{B}	10.7	10.8	10.7	10.3	0.13	0.018	0.239	0.283
Total	193.3 ^{AB}	194.6 ^{A}	190.3 ^{B}	191.3 ^{AB}	190.2	190.8	191.5	188.2	0.97	0.043	0.005	0.068

[1] Pooled standard error of the mean for all diets.

[2] P-values are indicated by: T = temperature; N = NaDF; T×N = the interaction between temperature and NaDF. Significant differences ($P<0.05$) among means within treatments without NaDF are indicated by different superscripts $^{A, B, C}$, whereas significant differences among means within treatments with NaDF are indicated by different superscripts $^{a, b, c}$.

[3] Cystine and cysteine.

794

795

796 Table 4. Main effects of temperature and sodium diformate (NaDF) supplementation on chemical and physical properties of diets (n=3).

797

Treatment	Temperature				S.E.M. [1]	NaDF		S.E.M. [2]	P-values [3]		
	100 °C	110 °C	120 °C	130 °C		Without	With		T	N	T×N
Reactive Lys (g/kg)	18.0 [a]	16.0 [b]	15.6 [b]	14.7 [c]	0.13	16.3 [A]	15.9 [B]	0.09	<0.001	0.019	0.647
Available Lys (%)	84.2 [a]	75.7 [b]	75.3 [b]	72.2 [c]	0.64	76.7	77.0	0.45	<0.001	0.604	0.539
Formic acid (g/kg)	5.0	5.0	4.7	4.8	0.09	0.6 [B]	9.1 [A]	0.07	0.138	<0.001	0.399
Calculated NaDF (g/kg)	6.2	6.2	5.9	6.0	0.12	0.8 [B]	11.4 [A]	0.08	0.138	<0.001	0.399
Diet pH	5.1 [a]	5.1 [a]	5.0 [b]	5.0 [b]	0.01	5.3 [A]	4.8 [B]	0.01	<0.001	<0.001	<0.001
Color analysis											
CIE *L**	53.2 [a]	50.1 [b]	46.4 [c]	41.3 [d]	0.15	48.1 [A]	47.4 [B]	0.10	<0.001	<0.001	0.003
CIE *a**	7.3 [d]	8.6 [c]	10.0 [b]	11.1 [a]	0.03	9.0 [B]	9.5 [A]	0.02	<0.001	<0.001	0.001
CIE *b**	25.3 [c]	25.6 [b]	26.0 [a]	24.8 [d]	0.07	25.1 [B]	25.8 [A]	0.05	<0.001	<0.001	0.001
Hue angle (*h*, °)	73.8 [a]	71.5 [b]	69.0 [c]	65.8 [d]	0.07	70.2 [A]	69.8 [B]	0.05	<0.001	<0.001	0.001

[1] Standard error of the mean for the main effect temperature.

[2] Standard error of the mean for the main effect NaDF.

[3] P-values are indicated by: T = temperature; N = NaDF; T×N = the interaction between temperature and NaDF. Significant ($P<0.05$) differences among means within temperature are indicated by superscripts [a, b, c, d] and significant differences among means within NaDF supplementation are indicated by superscripts [A, B].

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800 Table 5. Interactive effects of temperature and sodium diformate (NaDF) supplementation on chemical and physical properties of diets (n=3).

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Treatment [1]	Without NaDF				With NaDF				S.E.M. [2]
	100 °C	110 °C	120 °C	130 °C	100 °C	110 °C	120 °C	130 °C	
Reactive Lys (g/kg)	18.2 [a]	16.3 [b]	15.6 [bc]	14.9 [cd]	17.9 [a]	15.8 [b]	15.6 [bc]	14.4 [d]	0.19
Available Lys (%)	83.7 [a]	75.6 [b]	74.7 [b]	72.8 [b]	84.8 [a]	75.8 [b]	75.9 [b]	71.6 [b]	0.91
Formic acid (g/kg)	0.6 [b]	0.7 [b]	0.6 [b]	0.6 [b]	9.3 [a]	9.2 [a]	8.8 [a]	8.9 [a]	0.13
Calculated NaDF (g/kg)	0.8 [b]	0.9 [b]	0.7 [b]	0.8 [b]	11.6 [a]	11.5 [a]	11.1 [a]	11.2 [a]	0.17
Diet pH	5.3 [a]	5.3 [a]	5.2 [b]	5.2 [c]	4.8 [de]	4.9 [d]	4.8 [e]	4.8 [d]	0.01
Color analysis									
CIE *L**	53.3 [a]	50.1 [b]	47.2 [c]	41.8 [e]	53.1 [a]	50.2 [b]	45.5 [d]	40.9 [f]	0.21
CIE *a**	7.2 [h]	8.4 [f]	9.6 [d]	10.9 [b]	7.5 [g]	8.8 [e]	10.4 [c]	11.4 [a]	0.05
CIE *b**	25.2 [bc]	25.2 [c]	25.7 [b]	24.2 [d]	25.4 [bc]	26.1 [a]	26.3 [a]	25.5 [bc]	0.10
Hue angle (*h*, º)	74.1 [a]	71.6 [c]	69.5 [d]	65.8 [f]	73.6 [b]	71.3 [c]	68.5 [e]	65.9 [f]	0.10

[1] Significant differences (P<0.05) among means within a row are indicated by different superscripts [a, b, c, d, e, f, g, h].

[2] Pooled standard error of the mean.

802

803 Table 6. Main effects of temperature and sodium diformate (NaDF) supplementation on *in vitro* bioavailability measured as the amount of amino
804 acids (AA) released during alkaline hydrolysis by digestive enzymes from Atlantic salmon (n=3) and *in vivo* coefficient of total tract apparent
805 digestibility (CTTAD) of crude protein and AA in mink (n=4).
806

Treatment	Temperature				S.E.M. [1]	NaDF		S.E.M. [2]	P-values [3]		
	100 °C	110 °C	120 °C	130 °C		Without	With		T	N	T×N
In vitro											
AA released (mg) [4]	25.7 [a]	N.A.	N.A.	18.2 [b]	1.41	18.7 [B]	25.3 [A]	1.41	0.005	0.011	0.07
In vivo											
Crude protein	0.845 [a]	0.838 [a]	0.799 [b]	0.786 [b]	0.0083	0.823	0.811	0.0059	<0.001	0.158	0.748
Total AA	0.875 [a]	0.870 [a]	0.834 [b]	0.814 [b]	0.0084	0.850	0.846	0.0059	<0.001	0.634	0.829
Indispensable AA											
Arg	0.918 [a]	0.917 [a]	0.891 [b]	0.878 [b]	0.0062	0.900	0.902	0.0044	0.001	0.838	0.933
His	0.853 [a]	0.847 [a]	0.809 [b]	0.788 [b]	0.0094	0.827	0.822	0.0066	0.001	0.597	0.834
Ile	0.908 [a]	0.905 [a]	0.877 [b]	0.858 [b]	0.0072	0.887	0.887	0.0051	<0.001	0.982	0.875
Leu	0.908 [a]	0.904 [a]	0.874 [b]	0.857 [b]	0.0072	0.887	0.885	0.0051	<0.001	0.809	0.901
Lys	0.885 [a]	0.876 [a]	0.840 [b]	0.816 [b]	0.0088	0.859	0.850	0.0062	<0.001	0.352	0.881
Met	0.903 [a]	0.898 [a]	0.866 [b]	0.838 [c]	0.0074	0.878	0.874	0.0052	<0.001	0.594	0.789
Phe	0.888 [a]	0.886 [a]	0.860 [b]	0.844 [b]	0.0075	0.871	0.869	0.0053	0.001	0.752	0.914
Thr	0.802 [a]	0.800 [a]	0.760 [b]	0.735 [b]	0.0109	0.780	0.769	0.0077	<0.001	0.311	0.865
Val	0.894 [a]	0.893 [a]	0.862 [b]	0.841 [b]	0.0076	0.873	0.872	0.0054	<0.001	0.964	0.831
Total indispensable	0.889 [a]	0.886 [a]	0.854 [b]	0.834 [b]	0.0078	0.867	0.864	0.0055	<0.001	0.702	0.884
Dispensable AA											

Ala	0.880 [a]	0.874 [a]	0.844 [b]	0.825 [b]	0.0078	0.858	0.854	0.0055	<0.001	0.558	0.896
Asp	0.754 [a]	0.740 [a]	0.671 [b]	0.625 [b]	0.0151	0.706	0.690	0.0107	<0.001	0.292	0.843
Cys [5]	0.712 [a]	0.712 [a]	0.650 [b]	0.593 [c]	0.0162	0.672	0.662	0.0114	<0.001	0.541	0.863
Glu	0.911 [a]	0.902 [a]	0.870 [b]	0.856 [b]	0.0076	0.886	0.884	0.0054	<0.001	0.858	0.775
Gly	0.838 [a]	0.833 [a]	0.802 [b]	0.779 [b]	0.0079	0.817	0.810	0.0056	<0.001	0.413	0.819
Pro	0.905 [a]	0.901 [a]	0.874 [b]	0.859 [b]	0.0064	0.885	0.884	0.0045	<0.001	0.987	0.476
Ser	0.818 [a]	0.815 [a]	0.771 [b]	0.752 [b]	0.0105	0.796	0.782	0.0074	<0.001	0.179	0.875
Tyr	0.872 [a]	0.868 [ab]	0.836 [bc]	0.818 [c]	0.0096	0.848	0.849	0.0068	0.001	0.983	0.813
Total dispensable	0.864 [a]	0.857 [a]	0.818 [b]	0.797 [b]	0.0089	0.836	0.832	0.0063	<0.001	0.590	0.773

[1] Standard error of the mean for the main effect temperature.

[2] Standard error of the mean for the main effect NaDF supplementation.

[3] P-values are indicated by: T = temperature; N = NaDF; T×N = the interaction between temperature and NaDF. Significant ($P<0.05$) differences among means within temperature are indicated by superscripts [a, b, c, d] and significant differences among means within NaDF supplementation are indicated by superscripts [A, B].

[4] Treatment effects of temperatures 110 and 120 ºC were not analyzed for total AA released during alkaline hydrolysis. Thus, results were subjected to a 2×2 factorial ANOVA.

[5] Cystine and cysteine.

Figure captions:

Figure 1. Protein solubilization in diets without (C) and with NaDF (N) processed at two temperatures (100 and 130 ºC) under similar pH conditions as applied in the *in vitro* assay. Values are presented as means (n=2) ± SD for each time interval.

Figure 2. Total amount of amino acids (AA) released from diets without (C) and with NaDF (N) processed at two temperatures (100 and 130 ºC) during the alkaline phase of the *in vitro* hydrolysis. Values are presented as means (n=3) ± SD for each time interval.

817 Figure 1.

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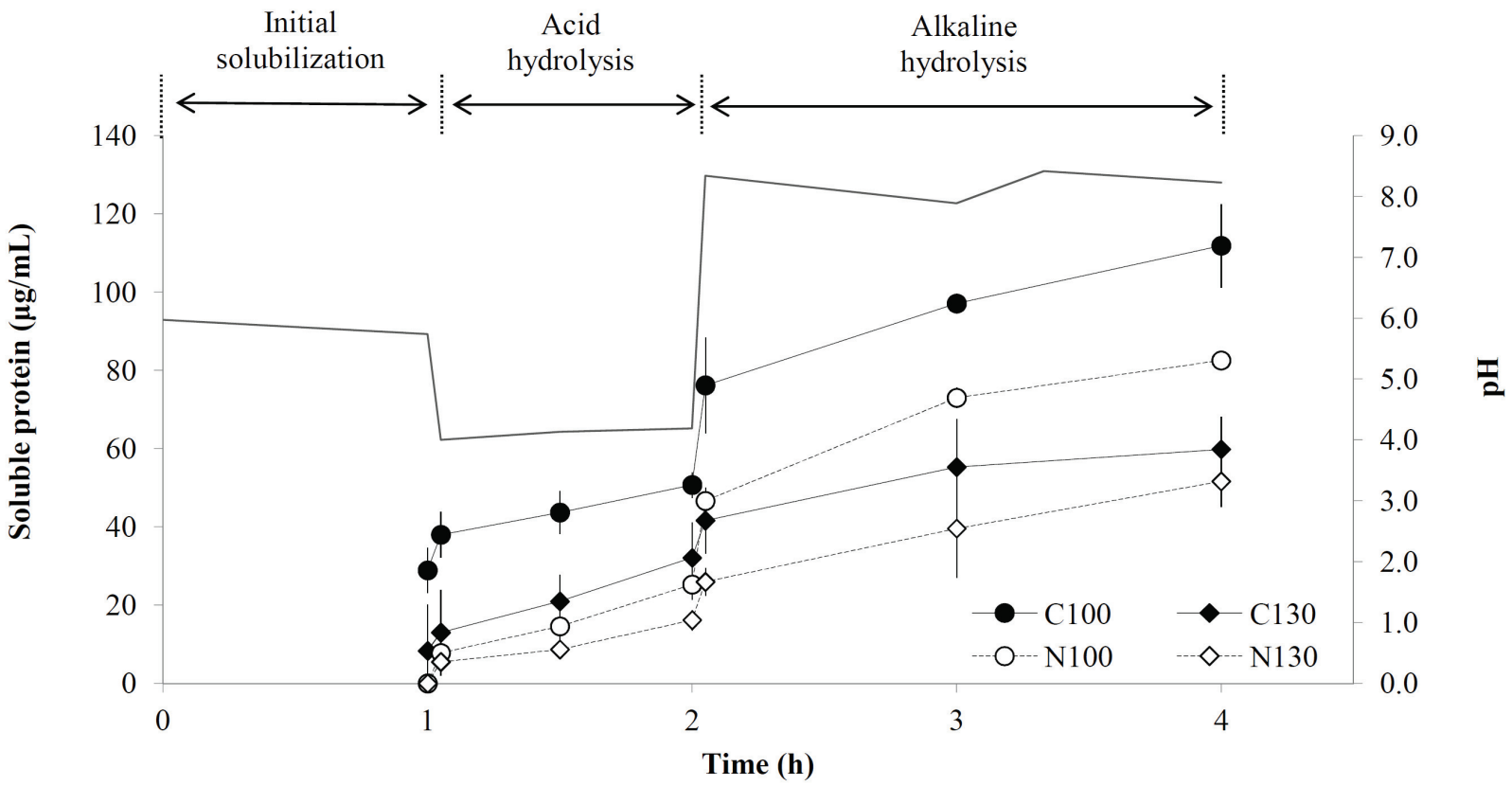

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821 Figure 2.

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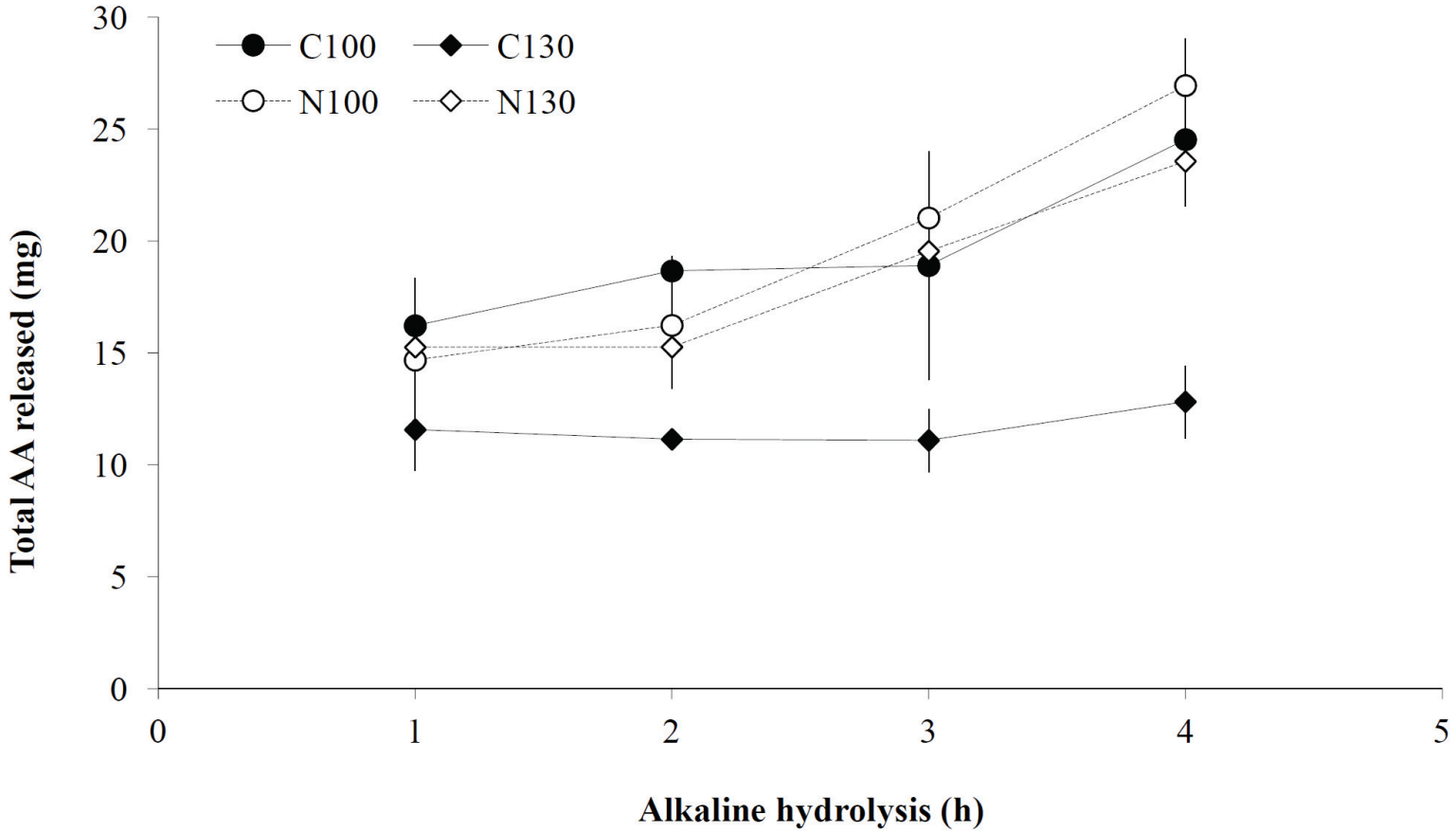

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