Hydrolysis of Atlantic salmon (*Salmo salar*) rest raw materials

Influence of process conditions and evaluation of hydrolysate in diets for broiler chickens and piglets

Hydrolyse av restråstoff fra Atlantisk laks (*Salmo salar*)

Effekt av prosessbetingelser og evaluering av hydrolysat i fôr til kylling og smågris

Philosophiae Doctor (PhD) Thesis

Margareth Opheim

Department of Animal and Aquacultural Sciences

Norwegian University of Life Sciences

Trondheim/Ås 2015

Thesis number 2015:85

ISSN 1894-6402

ISBN 978-82-575-1320-7

Table of contents

Acknowledgements

This study was a part of a collaborative project between the Department of Animal and Aquacultural Sciences (IHA) at Norwegian University of Life Sciences (NMBU), SINTEF Fisheries and Aquaculture, Nutrimar, and Felleskjøpet Fôrutvikling (FKF), and I am grateful for the financial support from by the Regional Research Found Mid-Norway, the National Research Council of Norway, and Felleskjøpet Fôrutvikling.

Firstly, I would like to thank my supervisors Nils Petter Kjos (IHA, NMBU), Margareth Øverland (IHA, NMBU), and Hallgeir Sterten (FKF) for their support, insight and guidance throughout this study. Their sharing of experience, knowledge and support are highly appreciated. A special thanks goes to all of my colleagues at FKF, for all the discussions and support. In particular, Knut Røflo for giving me the opportunity to perform this study, Anne Stine Ekker for sharing her statistical aptitude with me and our many useful, joyous and developing statistical discussions, and Hallgeir Sterten, for all possible support and the sharing of his unique knowledge on animal nutrition with me.

My gratitude also goes to Dag Kristoffer Forberg (NMBU), Linda Andreassen (NMBU), and Benny Berg at the Centre for Poultry Research (SPR) for their support and the excellent handling of the animals during the feeding experiments and sampling. I also wish to thank Frank Sundby (NMBU) for his positive and enthusiastic attitude for making new tasks workable in a short time-frame.

All co-authors of the papers, Rasa Šližytė, Ivar Storrø, Fiona Provan, Eivind Larssen, and Mikael Lenz Strube, are gratefully appreciated for their contribution and for sharing their experience in scientific writing with me. A special thanks goes to Rasa Šližytė (SINTEF) for her “all-round” support and insight in motivation and Ivar Storrø for his many valuable suggestions, feedback and challenging questions during this time. I also want to thank Tore Remman (Nutrimar) for his positive attitude and rapid and accurate handling when production of hydrolysates was needed. Without his help and positive attitude, this work and thesis would not have been possible.

I also want to thank my friends, especially Lise and Jorid for their emotional support and encouragement throughout this study period.

My special thanks goes to my parents, Jorunn and Ole Morten. You are always supportive, giving me opportunities, challenging me, believing in me and unconditionally loving me. I also thank my parents-in-law, Olaug and Kjell, for always being supportive, helpful and positive.

Last, but not least, my thanks and gratitude goes to Kurt Ole and our lovely children, Aleksander, Kristian Magnus, Sigurd and Iben Marie for your love, patience, support, and understanding and for putting things into perspective whenever needed.

Margareth Opheim

Trondheim, September 2015

Abstract

Annually Norway produces 1.3 million tons of Atlantic salmon and the levels of production are still rising. After withdrawal of eviscerated fish and filet, 336 000 tons of rest raw material are available for further processing. About 50% of the rest raw material from aquaculture in Norway is used to produce a semisolid fluid called salmon-silage supplying the animal feed industry, where it is known to be rich in marine proteins and oil. It is however possible to increase the value of this rest raw material by milder processes e.g. enzymatic hydrolysis, allowing to recover proteins and extract oil in order to supply the market with higher value products with desired features. The general goal when utilizing rest raw material from aquaculture is to exploit valuable marine proteins and oil, and increase the sustainability in both aquaculture and livestock production.

The enzymatic hydrolysis process is achieved by activity of enzymes at certain temperature for a defined time. During the hydrolysis process, smaller peptides and free amino acids are formed and solubilized in an aqueous phase called salmon protein hydrolysate (SPH). Hydrolysates containing peptides are shown to be more efficiently absorbed in the small intestine in comparison to both intact protein and free amino acids. In addition, hydrolysates from marine sources have good functional and bioactive properties.

Broiler chickens and piglets are more prone to small intestinal dysfunction linked to retarded growth and enteric diseases the first 14 days, post-hatch and post-weaning respectively. Traditionally in Norway, fishmeal is used during these periods, as the content of easily digestible protein with a well-balanced amino acid composition is high in fishmeal. The content of peptides in the hydrolysates are thought to improve intestinal function and intestinal health that are important for high utilization of feed, improved growth and disease resistance.

The overall aim of this study was to develop protein feed ingredient(s) from Atlantic salmon rest raw materials with higher nutritional value than fishmeal for inclusion in feed formulations for broiler chickens and piglets. One experiment evaluated the influence of process conditions during hydrolysis of salmon rest raw material on hydrolysate yield and composition, while two other experiments evaluated the influence of salmon protein hydrolysate in diets for broiler chickens and piglets on growth performance, intestinal morphometry, and ileal microbiota (piglets only).

This study has shown that unstable and traditionally low value viscera-containing raw materials are possible to utilize in an effective way when care is taken during the whole process from slaughter to hydrolysis. Two different starting materials were used as substrates for enzymatic hydrolysis, in addition, initial oil withdrawal and initial heat inactivation of the endogenous enzymes were performed on one starting material. We found that the composition of starting material reflected the proximal composition of the hydrolysate, as high lipid content in the raw material tended to increase the lipid content in the hydrolysate at the expense of crude protein content. Initial heat inactivation of the endogenous enzymes dramatically influenced the yield, as no hydrolysis seemed to take place after the addition of papain and bromelain. Mild thermal treatment (40°C) to facilitate oil withdrawal before hydrolysis of viscera with addition of papain and bromelain, resulted in high quality of the withdrawn oil, and no significant difference in SPH yield or protein recovery. Small differences were observed in the SPH on yield, protein recovery, the degree of hydrolysis, amino acid composition, nutritional value or number of different bioactive peptides detected when the commercial enzymes, Protamex and papain plus bromelain, were used on the same starting material. Additionally, hydrolysates from hydrolysis of viscera with only endogenous enzymes performed similarly to the afore-mentioned measures, but contained more lipids and was more bitter in comparison to hydrolysates from viscera obtained by addition of commercial enzymes.

In the broiler chicken experiment, two hydrolysates from viscera were produced, one by use of only endogenous enzymes and one with the addition of the commercial enzymes papain plus bromelain in mixture. The hydrolysates were included with 5% and 10% (50% and 100% exchange for fishmeal protein) in both the starter and grower diets for broiler chickens. In addition, one fishmeal diet (FM) and one plant protein-based diet (PP) were included as controls. Inclusion of SPH in the starter diet significantly increased the average daily gain (ADG) in comparison to the control diets (FM and PP). Morphometric analysis, at day 10, showed increased duodenal villus height and tendencies to increased ileal villus height and villus absorption area when marine proteins were included in the diets in comparison to the plant protein-based diet. The improved small intestinal development may favor growth performance at older ages, which corresponds well to the observed increase of ADG in broiler chickens fed marine protein diets in comparison to the PP diet during the grower period.

In the piglet experiment, two hydrolysates were produced without addition of commercial enzymes, one from viscera and one from a mixture of viscera, heads and frames. Two diets were included 10% SPH in exchange for fishmeal protein, one diet was included fishmeal (FM)

and one diet was based on plant protein (PP). Piglets did not show differences in growth performance during the first 11 days or for the overall period for any of the diets. In addition, only small differences were detected in the ileal microbiota community, although, an inverse correlation was shown between the genera *Turicibacter* and *Lactobacillus*, which together comprised most of the ileal bacterial genera. The piglets fed diets containing marine proteins showed increased duodenal villus absorption area and tendencies to increased duodenal villus height at day 11, in comparison to piglets fed the PP diet.

In both broiler chickens and piglets we found a significant positive correlation between duodenal villus height and ADG during the first 10 days post-hatch and 11 days post-weaning, respectively, indicating the importance of intestinal development and maintenance in both broiler chickens and piglets for growth performance.

In conclusion, enzymatic hydrolysis seem to be a feasible way to extract nutritional valuable proteins and peptides from Atlantic salmon rest raw materials. Salmon protein hydrolysates are found to be an excellent novel source of proteins with high nutritional value and potentially positive effects on intestinal development and maintenance.

Sammendrag

Norge har en økende produksjon av Atlantisk laks og produserer årlig 1,3 millioner tonn. Etter sløying og filetering er 336 000 tonn restråstoff tilgjengelig for videre bearbeidelse. Omtrent 50% av restråstoffet som oppstår ved slakting av laks i Norge blir syrekonservert til et produkt kalt lakseensilasje, som hovedsakelig brukes som råvare i fôrindustrien. Imidlertid er det mulig å øke verdien av lakserestråstoffet og produsere mer høyverdige produkter ved mer skånsom og styrt prosessering som f.eks. enzymatisk hydrolyse. Det overordnede målet ved utnyttelse av restråstoff fra lakseoppdrettsnæringen er å utvinne verdifulle marine proteiner og olje, samt å øke bærekraften i både akvakulturnæringen og i husdyrproduksjonen.

Enzymatisk hydrolyse oppnås med enzymatisk aktivitet ved en gitt temperatur over en definert tid. Under hydrolyseprosessen blir proteinet brutt ned til små peptider og frie aminosyrer i en vandig løsning kalt lakseproteinhydrolysat (SPH). Det er vist at hydrolysater som inneholder peptider blir absorbert mer effektivt i tarmen sammenlignet med både intakt protein og frie aminosyrer. I tillegg har marine peptider gode funksjonelle og bioaktive egenskaper.

Kylling og smågris er mer utsatt for fordøyelsesforstyrrelser og redusert vekst i løpet av de første 14 dagene etter henholdsvis klekking og avvenning. Tradisjonelt er fiskemel brukt i fôret i disse periodene da innholdet av lettfordøyelig protein med en velbalansert aminosyre-sammensetning er høyt i fiskemel. Generelt mener en at marine proteinhydrolysater og dermed peptider i dyrefôr kan bedre både tarmfunksjon og tarmhelse som igjen er viktig for en høy utnyttelsesgrad av fôret, økt tilvekst og motstandskraft mot sykdom.

Målet med denne studien var å utvikle proteinråvare(r) basert på restråstoff fra lakseoppdrett med høyere næringsverdi enn fiskemel til bruk i fôr til kylling og smågris. Ett eksperiment evaluerte effekten av ulike prosessbetingelser under hydrolyse av lakserestråstoff med tanke på utbytte og sammensetning av hydrolysatet, mens to andre eksperimenter undersøkte effekten av hydrolysat i fôr til kylling og smågris med tanke på vekstparametre, tarmmorfometri og ileal mikrobiota (kun smågris).

Studiet har vist at ustabilt restråstoff med tradisjonelt lav verdi kan utnyttes på en effektiv måte ved kontrollert håndtering under hele prosessen fra slakt til hydrolyse. To ulike restråstoff ble brukt som substrat for enzymatisk hydrolyse. På ett av restråstoffene ble det i tillegg ekstrahert olje og gjennomført varmeinaktivering av de endogene enzymene. Sammensetningen av

restråstoffet reflekterte sammensetningen i hydrolysatet, ettersom høyt fettinnhold i restråstoffet tenderte til høyere fettinnhold i hydrolysatet på bekostning av proteininnholdet.

Varmeinaktivering av de endogene enzymene hadde stor påvirkning på utbytte av hydrolysatet ettersom det ikke så ut til å skje noen hydrolyse etter tilsetting av papain pluss bromelain. Moderat oppvarming (40 °C) av slo for å lette ekstraksjon av olje før hydrolyse med papain og bromelain, resulterte i høy kvalitet på den ekstraherte oljen og ingen signifikant forskjell i utbytte av hydrolysat eller gjenfinning av protein i hydrolysatet.

Små forskjeller ble observert med hensyn til utbytte, gjenfinning av protein, hydrolysegrad, aminosyresammensetning, næringsverdi eller antall forskjellige bioaktive peptider i hydrolysatet når de kommersielle enzymene, Protamex og papain pluss bromelain, ble brukt på samme restråstoff. Hydrolyse av slo med kun endogene enzymer ga tilsvarende verdier i hydrolysatet på de ovenfor nevnte parameterne, men inneholdt mere fett og var mer bitter sammenlignet med hydrolysater produsert fra slo med kommersielle enzymer.

I kyllingforsøket ble det brukt to ulike hydrolysater fra slo, ett produsert med kun endogene enzymer og ett med tilsetning av papain og bromelain. Hydrolysatene ble tilsatt med 5% og 10% (50% og 100% i bytte mot fiskemelprotein) i både start- og vekstfôr. I tillegg inngikk ett fôr med fiskemel (FM), og ett planteproteinbasert fôr (PP) som kontrollfôr. Kylling som ble fôret med startfôr inneholdende SPH hadde signifikant høyere gjennomsnittlig daglig tilvekst (ADG) sammenlignet med kylling fôret med kontrollfôr (FM og PP). Morfometrisk analyse, på dag 10, viste økt villi høyde i duodenum og tendenser til økt villi høyde og villi absorpsjonareal i ileum når marine proteiner inngikk i fôret sammenlignet med det planteproteinbaserte fôret. Den forbedrede tynntarmsutviklingen kan bidra til økt tilvekst senere, noe som samsvarer godt med den observerte økningen i tilvekst hos slaktekylling gitt fôr inneholdende marint protein i forhold til det planteproteinbaserte fôret under vekstperioden.

I forsøket med smågris ble det brukt to ulike hydrolysater produsert uten bruk av kommersielle enzymer, ett fra slo og ett fra en blanding av slo, hode og rygg. To forsøksfôr inneholdt 10% SPH (100% i bytte mot fiskemelprotein), ett fôr inneholdt fiskemel (FM) og ett fôr var basert på planteprotein (PP). Det ble ikke funnet forskjeller mellom smågris gitt de forskjellige forsøksfôrene med hensyn til vekstparameterne, hverken i løpet av de første 11 dagene eller for hele forsøksperioden. I tillegg ble kun små forskjeller påvist i den ileale bakteriefloraen, selv om et ble funnet en signifikant invers korrelasjon mellom bakterieslektene *Turicibacter* og

Lactobacillus, som sammen utgjorde størsteparten av bakterieslektene i ileum. Smågris som ble fôret med marine proteiner hadde større villi absorpsjonsareal og tendens til høyere villi i duodenum på dag 11, sammenlignet med smågris gitt det planteproteinbaserte fôret.

Både hos kylling og gris ble det funnet en signifikant positiv korrelasjon mellom vill høyde i duodenum, og gjennomsnittlig daglig tilvekst i løpet av de første 10 dagene etter klekking og 11 dagene etter avvenning hos henholdsvis kylling og smågris. Dette indikerer betydningen av tarmutvikling og tarmmorfologi hos både kylling og smågris for tilvekst.

På bakgrunn av dette studiet kan det konkluderes med at enzymatisk hydrolyse er en velegnet metode for å ekstrahere ernæringsmessige verdifulle proteiner og peptider av restråstoff fra Atlantisk laks. Lakseproteinhydrolysat synes å være en velegnet proteinkilde for kylling og gris med høy ernæringsmessig verdi og potensielle positive effekter på tarmutvikling og morfometri.

List of papers

The presented thesis is based on the following papers. Papers will be referred to by their Roman numerals in the text:

I Šližytė, R., Opheim, M., Storrø, I., Hallgeir Sterten (2015). Simple technologies for converting rest raw materials of Atlantic salmon (*Salmo salar*) into valuable tasty feed ingredients. *Submitted.*

II Opheim, M., Šližytė, R., Sterten, H., Provan, F., Larssen, E. & Kjos, N.P. (2015) Hydrolysis of Atlantic salmon (*Salmo salar*) rest raw materials – Effect of raw material and processing on composition, nutritional value, and potential bioactive peptides in the hydrolysates. *Process Biochemistry* (50), 1247-1257.

III Opheim, M., Sterten, H., Øverland, M. & Kjos, N.P. (2015) Atlantic salmon (*Salmo salar*) protein hydrolysate - Effect on growth performance and intestinal morphometry in broiler chickens. *Submitted.*

IV Opheim, M., Strube, M.L., Sterten, H., Øverland, M. & Kjos, N.P. (2015) Atlantic salmon (Salmo salar) protein hydrolysate - Effect on growth performance, intestinal morphometry and microbiota composition in weaning piglets. *Submitted.*

1. Introduction

1.1. Atlantic salmon farming in Norway

Atlantic salmon farming is a large and growing industry in Norway and annually about 1.3 million tons of salmon are produced along Norway's coastline (Olafsen *et al.*, 2014). Rest raw material arises when the eviscerated salmon and salmon filet, which are the main products, are withdrawn. The term "rest raw material" has recently become more frequently used than "by-product" or "co-product" in both industrial and scientific language. Moreover, Norwegian national regulations define "by-products" as products not intended for human consumption (Lovdata, 2014). However, as long as the rest raw materials are treated and processed in a proper way and according to the regulations, the final products are well suited and potentially applicable for both animal and human consumption.

The rest raw material consists of viscera, heads, frames, skin, blood and trimmings and about 336 000 tons wet weight are annually available for further processing (Olafsen *et al.*, 2014). From the amount of available rest raw material that arises in Norwegian Atlantic salmon farming, 89 % is utilized into different feed ingredients (87%), human consumption (13%) and a small part as biogas/energy (Olafsen *et al.*, 2014). As viscera constitutes 40% of the available rest raw material (Olafsen *et al.*, 2014), it is important to include this fraction in subsequent processes. Despite rest raw material composition is dependent on the market of the primary product(s), and hence may change over time, viscera probably will still account for a considerable part of the available rest raw material.

Traditionally about 50% of the Atlantic salmon rest raw materials in Norway are minced and acidified to reach a pH < 4, producing a semisolid fluid called salmon-silage (RUBIN, 2012) and used as a raw material in animal feed production (Olafsen *et al.*, 2014). Although, the feed industry can use fish-silage as a feed ingredient, fish-silage often varies in stability and composition. Production of the traditional silage is a time-consuming process (Liaset *et al.*, 2000) that lasts over several days (Gildberg, 1993) or weeks (Mackie, 1974) and the process is difficult to control (Gildberg, 1993; Kristinsson and Rasco, 2000b). In addition, several amino acids, such as cysteine, tryptophan, methionine, histidine and proline can be reduced or lost (Dapkevičius *et al.*, 1998; Ramasubburayan *et al.*, 2013) together with undesired formation of biogenic amines during the ensilaging process (Dapkevicius *et al.*, 2000). The marine rest raw materials contains nutritionally-valuable lipids and proteins (Šližytė *et al.*, 2005a; Picot *et al.*,

2010; Rustad *et al.*, 2011) and there is a need for the development of a milder and more controllable process in order to further increase the utilization of this valuable material.

The animal livestock production is always in search of sustainable protein sources and increasing feed costs and fishmeal prices causes nutritionists to consider alternative protein sources. Due to the amount of available rest raw materials from Atlantic salmon farming and knowledge of the high nutritional values of these proteins, new technologies are highly sought after in order to produce a sustainable, composition-stable and high nutritional-value protein hydrolysate. In addition, many of the isolated peptides from marine protein hydrolysates have shown promising effects on health by exhibiting antioxidative, antihypertensive, antibacterial, anticancer and immune-modulating properties (Kim and Mendis, 2006; Thorkelsson *et al.*, 2008; Ewart *et al.*, 2009; Harnedy and FitzGerald, 2012; Ngo *et al.*, 2012).

1.2. Hydrolysis of rest raw materials

Protein hydrolysis is a technological processing method used to extract and modify protein from underutilized raw materials and is achieved by use of chemicals or enzymes that attacks and cleave peptide bonds in the presence of water. During the hydrolysis process, proteins are broken into peptides of smaller molecular size and the increase of ionizable amino and carboxyl groups may potentially enhance the solubility of the protein (Panyam and Kilara, 1996). The increased protein solubility allows proteins to dissolve in an aqueous phase and the extraction of lipids, (emulsion), hydrolysate (aqueous phase) and sediments/sludge becomes possible by phase-separation (Figure 1). Depending on the desired application, the degree of hydrolysis and other process parameters may differ, although it is generally a goal to achieve a hydrolysate with high protein content with smaller peptides and less free amino acids and intact proteins. In addition, the hydrolysate preferable has a low content of lipids to avoid lipid oxidation (Gildberg, 1993; Kristinsson and Rasco, 2000b) and the formation of unpleasant flavors (Gildberg, 1993).

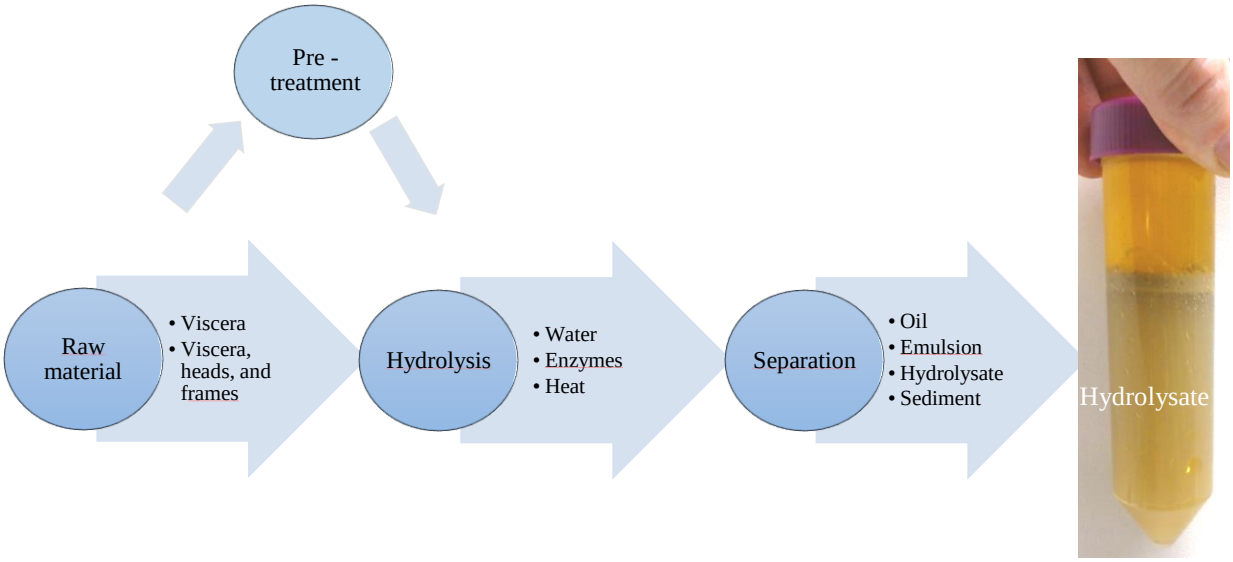


Figure 1

General outline of the enzymatic hydrolysis process

1.2.1. Raw material

Hydrolysis is applied in order to increase the value of the starting material and different rest raw materials can be used. As rest raw material from Atlantic salmon farming arises on different sites during the slaughter and filleting processes the rest raw materials are diverse and may be divided into different fractions. Although viscera are a major constituent of the rest raw material from Atlantic salmon farming (Olafsen *et al.*, 2014) many studies have used rest raw materials without viscera (Kristinsson and Rasco, 2000c; Liaset *et al.*, 2002; Liaset *et al.*, 2003; Aksnes *et al.*, 2006a; He *et al.*, 2012). The high enzymatic activity (Kristinsson and Rasco, 2000c; Sovik and Rustad, 2005) and loss of integrity immediately after slaughter causes viscera to be considered as an unstable raw material difficult to handle. However, the feature of high enzymatic activity of viscera can be exploited in subsequent processes and reduce the requirements for commercial enzymes during hydrolysis - especially under the controlled conditions in slaughterhouses for Atlantic salmons. Viscera consists of different integrated parts not easily sortable, which may decrease the possible usage of the hydrolysates. For instance, it is shown that gall bladder in the starting material for hydrolysis increases the bitterness in the hydrolysates (Dauksas *et al.*, 2004) which can hinder the successful application of hydrolysates

in food and feed formulations. Conversely, viscera may contain other beneficial components and exhibit valuable bioactive properties in comparison to other parts of the rest raw materials.

1.2.2. Pretreatments

Pretreatments are often applied in order to achieve a stable starting material for hydrolysis i.e. inactivate endogenous enzymes, extract valuable components before hydrolysis, and/or increase the yield of all parts of the rest raw material together with cost-benefit considerations.

One way to stabilize and control the enzymatic reactions is to inactivate the endogenous enzymes present in the raw material. Peptidases in viscera, liver and cut-offs in cod, have their maximum proteolytic activity at 50-65°C at pH 7 (Sovik and Rustad, 2005), and the temperature has to be at >70°C for minimum 10 minutes in order to inactivate the endogenous enzymes naturally present in Atlantic salmon viscera (Five, 2013). Several researchers have reported a reduced yield of protein hydrolysate (Mutilangi *et al.*, 1996; Slizyte *et al.*, 2004a) and reduced nutritional value when excess heating before hydrolysis is applied. In an industrial production this extra heating is a costly process that increases the price of the final product(s); therefore the cost benefit considerations of such treatment has to be evaluated.

Oil separation before hydrolysis changes the starting material composition before hydrolysis and lowers the amount of enzymes needed for hydrolysis, as enzymes often are often added at raw material wet weight basis. The oil extracted at temperatures <70°C is often of higher quality and contains less free fatty acids than oil separated after hydrolysis (Carvajal *et al.*, 2014). During hydrolysis, the temperature is maintained when lipases present in the raw material are still active, which might facilitate lipolysis and formation of free fatty acids.

1.2.3. Enzymes

As chemical hydrolysis has several drawbacks (Lahl and Braun, 1994; Kristinsson and Rasco, 2000b), a more controllable hydrolysis can be achieved by using enzymes. The enzymes are endogenous enzymes present in the raw material, commercial enzymes added to the reaction or a combination thereof. The endogenous enzymes are primarily trypsin, chymotrypsin, and pepsin present in fish viscera and digestive tract together with some activity of the proteases present in fish muscle cells (Kristinsson and Rasco, 2000b). The endogenous enzymes are shown to be efficient in viscera-containing material (Pastoriza *et al.*, 2004; Aspmo *et al.*, 2005)

and can be more efficient in the hydrolysis process compared to commercial enzymes (Pastoriza *et al.*, 2004).

Enzymes that cleave peptide bonds are called peptidases and essentially cleave proteins in two different ways. Endopeptidases, which preferably attacks peptide bonds away from termini of the polypeptide chain and exopeptidases that acts only near the N- or C-terminus of the polypeptide chain. Due to these properties, exopeptidases tends to give hydrolysates rich in free amino acids, and endopeptidases tends to give rise to hydrolysates rich in peptides and with less free amino acids. In addition, different enzymes have different specificity to both cleavage sites and protein conformation, and, depending on temperature, pH, enzyme to substrate level, and time, have different activities. The choice of enzyme(s) also depends on the raw materials as lean and fatty raw materials may favor use of different enzymes. Because of these features, the enzymes have an important key role in controlling the enzymatic process (Liaset *et al.*, 2000; Aspmo *et al.*, 2005) and in maximizing the outcome in respect to yield, its bitter taste, peptide, and amino acid composition of the hydrolysates.

1.2.4. Process conditions

Temperature and time of hydrolysis depends on the choice of enzyme. Cost-benefit considerations are of importance when planning industrial hydrolysis and the reaction-criteria like temperature and time should be optimized at the plant during production establishment. To stop the enzymatic hydrolysis process, the enzymes are most often heat inactivated. Protamex, papain, bromelain, and endogenous enzymes from Atlantic salmon are all inactivated at 90°C for 10 minutes (Five, 2013). Regulations for hygienization of the products to ascertain hygienic secure products often include a heating process, hence the heat inactivation of the enzymes and hygienization are often combined.

Following the hydrolysis process, a separation step is applied in order to extract the different fractions that evolves during hydrolysis. Separation can be achieved by membranes, decanter, tricanter, and by centrifugation, resulting in oil, a possible emulsion phase, an aqueous protein rich phase (the protein hydrolysate), and a sediment layer. After phase separation, different techniques may be applied to further refine the products. The use of a filtration step on the hydrolysate in order to detect the impact of filtration on the peptide populations (Bourseau *et al.*, 2009) and to extract proteins with a desired molecular weight is increasing. The filtration of hydrolysate has been applied to detect the size of the peptides and molecules within the

hydrolysates inhabiting the desired growth performance properties detected in Atlantic cod and in rainbow trout (Aksnes *et al.*, 2006a; Aksnes *et al.*, 2006b) and possible bioactive properties (Picot *et al.*, 2010).

1.3. Protein hydrolysates

1.3.1. Nutritional evaluation

The nutritional value of protein is often determined by protein content and amino acid composition. The "ideal protein" concept is based on the ideal situation, where amino acid supply precisely matches the amino acid requirements and was developed more than 50 years ago (van Milgen and Dourmad, 2015). To determine the requirements of the animal, the tissue (and milk) amino acid composition has been used to develop an ideal amino acid profile in the diet. Protein evaluated as having a high quality, i.e. fishmeal protein, therefore has an amino acid profile resembling the tissue profile of the animal. In animal nutrition, feed formulations are designed to supply sufficient amounts of the indispensable limiting amino acid to support the requirements for growth, maintenance and production in the animal. A well-balanced amino acid composition hinders the supply of amino acids in excess that have to be metabolized and excreted by the animal. Furthermore, the environmental impact also increases when nutrients are being excreted instead of utilized within the animal. Consequently, novel ingredients in their simplest form in order to design and formulate tailor-made diets for used in critical phases during livestock production is a perishable property of feed ingredients.

A widely used method to evaluate the nutritional value is to apply different equations based on the concentration of several indispensable amino acids. Many different equations for protein efficiency ratio (PER) have been developed for use in human nutrition (Alsmeyer *et al.*, 1974; Lee *et al.*, 1978). However, recently the protein digestibility corrected amino acid score (PDCAAS) (WHO/FAO/UNU, 2007) and digestible indispensable amino acid score (DIAAS) (FAO, 2013) have been recommended for protein quality evaluation by the Food and Agriculture Organization (FAO). This ratios and scores are developing and the latest recommendations from FAO (2013) allows correction for ileal digestibility of the different indispensable amino acids. In order to be able to compare different foods, FAO (2013) recommends the use of a reference amino acid pattern for a preschool child. However, the reference pattern has direct influence on the score, and is therefore not necessarily transmissible to animal feeding in different species and life stages.

Fishmeal is known to have high nutritional value and a complementary amino acid profile to plant based protein sources for use in monogastric animals as reviewed by (Cho and Kim, 2011). The small water-soluble peptides and free amino acids that are present in the hydrolysates might be favorable to animals in some challenging periods, where highly digestible and well-balanced diets are necessary to achieve a healthy development and a high integrity level in the immature or challenged intestine. Therefore, the formation and size of the peptides are an important feature of hydrolysates as smaller peptides and residues < 4-5 amino acids (about 0.5 kDa) are able to be transported by special peptide transporters in the small intestine (Gilbert *et al.*, 2008).

1.3.2. Bioactive peptides

Bioactive food compounds and bioactive peptides are "hot topics" in today's research as the focus is now changing from feed and food stuffs with an adverse effect on health into health-beneficial components as functional food (Crowe and Francis, 2013). The detection of possible bioactive peptides in fish protein hydrolysates, has evoked the interest of many scientists exploring the opportunity for high-value products from the rest raw material (Slizyte *et al.*, 2009; Kim and Wijesekara, 2010; Rustad *et al.*, 2011; Chalamaiah *et al.*, 2012; Ngo *et al.*, 2012). Many of the isolated peptides are shown to exert different biological activities with promising effects upon health. Most of the studied effects involve modulation of the blood pressure and immune system in addition to anti-cancer, anti-bacterial and anti-oxidative effects (Thorkelsson *et al.*, 2008; Ewart *et al.*, 2009; Harnedy and FitzGerald, 2012). The pharmaceutical industry is also searching for possibilities of using peptides from marine sources as pharmaceuticals and nutraceuticals, and several products are already in the market or included in ongoing trials (Cheung *et al.*, 2015).

Peptides between 3 and 20 amino acid residues (about 0.5-2.1 kDa) are regarded as the most promising in the search for biological active peptides as the chance of crossing the intestinal membrane and exerting a biological effect is higher for the lower molecular weight peptides (Roberts *et al.*, 1999). In addition, bioactive peptides usually contains between 3 and 20 amino acid residues (Pihlanto-Leppälä, 2000).

1.3.3. Sensory and functional properties

In addition to the nutritional value of the hydrolysates, they also inhabit functional properties that may be important for successful market penetration. The protein functional properties are

defined as “Functional properties of proteins for foods connote the physicochemical properties which govern the behavior of protein in foods” (Kinsella and Melachouris, 1976) or “Any property of a food or food ingredient except its nutritional ones that affects its utilization” (Pour-El, 1981). Often these physicochemical and functional properties, summarized in Phillips *et al.* (1994), are valuable for the potential use of the hydrolysates. Some of the most important functional properties in hydrolysates are their water binding capacity, water solubility (Shahidi and Synowiecki, 1997; Kristinsson and Rasco, 2000a), and fat-absorption and emulsifying capacity (Lam and Nickerson, 2013). Depending on the intended use of the hydrolysates, some of the functional characteristics are troublesome for successful penetration of fish protein hydrolysates into the human market. In particular, these characteristics are the sensory properties such as the bitter taste and “fishy” smell in addition to the color, which is often dark brown. In animal feeding the flavor and taste of fish are of minor importance and the flavor and taste of fish might even enhance the palatability of the diets in animals. However, this is difficult to foresee, as there are differences in bitter taste preferences between species. Birds have a low number of taste buds and T2R genes associated with bitter taste in comparison to humans and pigs (Shi and Zhang, 2006; Roura *et al.*, 2013). Although birds do perceive bitter taste for different compounds (Balog and Millar, 1989; Kudo *et al.*, 2010), it is unknown if they can recognize all bitter compounds identified by humans (Roura *et al.*, 2013). In contrast, piglets are known to be sensitive to several components humans find bitter and the inter-individual variation appears to be large for some components (Nelson and Sanregret, 1997). However, piglets have a noticeable preference for sweeteners such as xylitol and sucrose (Hellekant and Danilova, 1999; Glaser *et al.*, 2000) making it possible to conceal the bitter taste and increase the palatability of the diet. As knowledge about how these bitter peptides arises, the methods for concealment or removal of the bitter taste have been studied by several researchers (Dauksas *et al.*, 2004; Leksrisompong *et al.*, 2012).

1.4. Broiler chicken and piglet production in Norway

In Norway, broiler chickens are hatched in a hatchery and transported to the broiler farm as day-old chicks. The annual production was 60 million broilers in 2011 (Landbruksdirektoratet, 2011) reaching a mean slaughter-weight of 1256 g in 31.5 days, with a mortality rate of 2.6% for the entire period (Nortura, 2014). There has been a considerable increase in poultry production worldwide since 1970 (Yegani and Korver, 2008), which is also seen in Norway

(Nortura, 2014). As growth performance improves, the birds' nutrition and healthcare are becoming more demanding and challenging (Choct *et al.*, 1999; Cooper and Songer, 2009).

In addition, the ban on in-feed antibiotics in the European countries (Van Immerseel *et al.*, 2009) further increases the impact of feed formulations and management on the production unit. Norway has a low antibiotic usage profile (Mo *et al.*, 2014); however, the ionophoric coccidiostatic Narasin has been added to the broiler chicken feed on a regular basis, although a phasing out is now ongoing. Narasin is in Norway classified as a coccidiostatic feed additive, while in other countries classified as antibiotic due to an antibacterial effect on gram positive bacteria. In broiler chicken production the coccidiostatic and antibacterial effects against *Clostridium* species seem beneficial in avoiding the development of necrotizing enteritis. As coccidia and *Clostridium perfringens* are known to play a role in the pathogenesis of necrotizing enteritis, there is great concern that the phasing out of Narasin in the Norwegian broiler chicken production will increase the incidence of necrotizing enteritis. Enteric diseases are major factors for economic losses in the broiler chicken production due to reduced growth performance, increased mortality rates and increased medication costs (M'Sadeq *et al.*, 2015). In addition to enteric diseases at the farm, 0.45% of the broiler chickens are found to be unsuited for human consumption due to ascites and heart-related diseases during inspection at the slaughter house (Nortura, 2014). The intensive growth of muscle tissue is a challenge for both the circulatory and skeletal system, demanding a broad approach in the feed formulation satisfying all parts of the broiler chicken development.

The annual production of slaughter pigs in Norway was 1.49 million pigs raised at 2358 production units in 2014 (Animalia, 2014). Weaning is not allowed before the age of 28 days (Lovdata, 2003) and the average weaning age is 32.9 days (Animalia and Norsvin, 2014). At weaning the mean piglet weight is 10.7 kg and from weaning to 41 days post-weaning the mortality rate is 1.7% (Animalia and Norsvin, 2014). Ideally, the sow is moved while the piglets are raised in the farrowing pen until they are approximately 30 kg live weight and moved to a fattening/finishing unit. However, in many instances the newly weaned piglets are moved to another environment, and mixed with unknown piglets, hence experiencing both psychological and environmental disruption in addition to the abrupt loss of sows milk, and care. These multi-factorial stressors often results in intestinal disturbances predisposing for post-weaning digestive disorders in piglets and reduced growth performance (Kim *et al.*, 2012; Heo *et al.*, 2013).

Although the effect of age, sex, weight and different nutritional and feeding strategies at weaning is well known (Pluske *et al.*, 2003; Kim *et al.*, 2012; Heo *et al.*, 2013), weaning diarrhea is still one of the most important factors in economic losses in the swine-production. In a small survey including 61 piglets with disease-related mortality in Norway, the incidence of hemorrhagic enteritis associated with weaning diarrhea in the piglets was 53% (Åkerstedt *et al.*, 2013). Weaning diarrhea causes major economic losses and the increasing incidence coincides with the ban of in-feed antibiotics and growth promotors in the Scandinavian and European countries (Fairbrother *et al.*, 2005). The increased incidence of weaning diarrhea necessitates research into better nutrition and management together with the development of vaccines. Although exact incidence of weaning diarrhea is difficult to obtain, the impression is that better management and nutrition have resulted in a decreased incidence of weaning diarrhea in Norway during the latest years.

1.5. Intestinal changes and challenges in broiler chickens and piglets

The importance of a healthy intestine for productivity and disease resistance is well known and has a role in animal welfare and economic considerations in both commercial broiler chicken (Choct, 2009) and piglet production. Often a highly palatable and digestible feed is needed to stimulate feed intake and support a healthy intestinal growth and maturation. Luminal stimulation and adequate feed intake are important factors for intestinal maintenance, growth and maturation and are well known in broiler chickens, piglets and humans.

Young animals have periods where they are more prone to delayed or retarded growth and to develop diseases. In broiler chickens, the intestine appears immature upon hatching and develops extensively during the first two weeks (Uni *et al.*, 1998; Iji *et al.*, 2001; Batal and Parsons, 2002). In addition, the broiler chicken has to adapt from nutrition from the yolk sac into an exogenous nutrient source to assimilate the nutrient requirement for growth. The villus height increases in all small intestinal segments during the first 15-21 days post-hatch (Uni *et al.*, 1999; Iji *et al.*, 2001) and the protein component is reported to be the most important nutrient for the recovery of intestinal villus after feed withdrawal (Maneewan and Yamauchi, 2004). In addition, the plasma concentration of IgG (IgY) in the chicken is at its lowest one to two weeks after hatching (Hamal *et al.*, 2006). These factors predisposes the broiler chickens for developing diseases especially during the first 14 days post-hatch. As impaired intestinal growth is difficult to remedy later in production (Uni, 1998; Juul-Madsen *et al.*, 2004) stimulating post-hatch intestinal development and absorption capacity are of major importance.

In piglets, weaning diarrhea often appears around two weeks post-weaning. The prompt disruption from a milk-based diet into a grain-based diet, together with the withdrawal of the sow's milk, rich in protective immunoglobulins, challenges the adaptive responses of the piglet's intestines. It has been demonstrated that the proximal small intestinal villus undergoes atrophy post-weaning (Pluske *et al.*, 1997; Hedemann *et al.*, 2003; Vente-Spreeuwenberg *et al.*, 2003), and the aim of the feed formulations is often to maintain the integrity of the intestines and hinder villus atrophy, in addition to supply the animal with sufficient nutrients to support the requirements for maintenance and growth. During the post-weaning period, the feed intake is below their maintenance requirements and the piglets usually lose weight. This period of negative energy balance can last for up to six to nine days post-weaning (Pluske *et al.*, 2005). A substantial amount of research has been performed in order to minimize the growth-check and incidence of post-weaning diarrhea (Pluske *et al.*, 2005; Halas *et al.*, 2007; Heo *et al.*, 2008), as weaning weight and weight gain the initial week post-weaning are reported to explain a large percentage of the variation in body weight at day 20 post-weaning (Pluske *et al.*, 2005).

1.6. Intestinal microbiota

As highlighted by Bauer *et al.* (2006), the intestinal microbiota community have a significant impact on immunity and health, especially in younger animals. There is a symbiotic relationship between the host and the microbiota community that acts on the metabolism of nutrients and contributes to resistance of colonization of exogenous microorganisms (Bauer *et al.*, 2006). This symbiosis is both beneficial and deleterious for the host and the main aim of altering the microbiota community is to increase the benefits and decrease the cost to the host (Richards *et al.*, 2005). Macronutrients like fiber, protein and fat are known to influence the microbiota community (Wu *et al.*, 2011; David *et al.*, 2014) and the microbiota community changes rapidly and in a reproducible manner (David *et al.*, 2014).

The intestines are naturally colonized by microbiota after birth; however, a stable microbial community takes some time to establish (Gaskins, 2001), and therefore the effect of diets and supplements is probably more efficient in younger animals. In general, younger animals are more prone to develop intestinal dysfunction and diarrhea due to intestinal microbial imbalances in comparison to older animals.

1.7. Protein digestion and absorption

Proteins are normally hydrolyzed into smaller peptides and free amino acids in the stomach and intestine. The low pH in the stomach unfolds the proteins and exposes the peptide bonds for pepsin cleavage. Further cleavage of peptide bonds takes place in duodenum, where the proenzymes from pancreas are activated and trypsin, chymotrypsin, and carboxypeptidases cleave the ingested protein into smaller peptides. In addition, different peptidases lining the brush border of the intestine cleave smaller peptides into tri- and dipeptides, and free amino acids. Small peptides below 4-5 amino acid residues in length are able to be transported through specialized peptide transporters, co-transported with other components, and can diffuse through permeable tight junctions (Gilbert *et al.*, 2008). The specialized peptide transporters PepT1, PepT2 and PHT 1 and PHT2 are all proton-coupled and transport their substrates in a species-, tissue-, and development-specific manner (Chen *et al.*, 2005; Zwarycz and Wong, 2013). The peptide transporter PepT1 is widely distributed in the small intestines of broilers (Chen *et al.*, 2005; Zwarycz and Wong, 2013), has low affinity for several peptides (Brandsch *et al.*, 2004), and a high capacity, and can therefore be regarded as important for the regulation of di- and tripeptide absorption even in the immature intestine. In rats, a brief fast or malnutrition due to parasite infection increases the intestinal gene expression of PepT1 (Thamotharan *et al.*, 1999; Barbot *et al.*, 2003), probably increasing the peptide absorption capacity. Furthermore, an increased level and quality of dietary protein is associated with increased gene expression of PepT1 in chickens and rats (Shiraga *et al.*, 1999; Chen *et al.*, 2005).

Hydrolysates contains high levels of smaller peptides and free amino acids that may increase the absorption rates in comparison to intact proteins. The regulatory responses are difficult to predict, however, it has been demonstrated in a dog study by Zhao *et al.* (1997), a dose-dependent slowing of the intestinal transit time and that intact protein reduces the transit time more effectively than the hydrolyzed form. In addition, the protein absorption efficiency was reported to be nearly constant although the protein absorbed in the proximal intestine was significantly higher when protein was given in the hydrolyzed form in comparison to the intact form (Zhao *et al.*, 1997). A more efficient absorption of amino acids into the portal blood from peptides compared to free amino acids and intact proteins is reported in rats (Kodera *et al.*, 2006) and in piglets, the absorption of amino acids is higher, faster and more homogenous after hydrolysate infusion in comparison to a free amino acid infusion (Rerat *et al.*, 1988). In addition, higher mRNA expression of several peptide and amino acid transporters was reported

when a hydrolyzed whey protein source was given to chickens in comparison to both intact whey proteins and free amino acids (Gilbert *et al.*, 2010).

Amino acid transporters with a high affinity to the different amino acids, transport amino acids into the intestinal epithelia. These transporters, are highly specialized, although most amino acids are transported by more than one transporter, providing a backup capacity (Broer, 2008). The amino acid transporters are found to be slower in amino acid absorption and are less energy-efficient in comparison to peptide absorption (Daniel, 2004).

2. Objectives of the study

The main objective of this study was to increase the utilization of Atlantic salmon rest raw materials to produce value added feed ingredients for broiler chickens and piglets with higher nutritional value than traditional fishmeal. Based on this overall aim the specific objectives were:

- To evaluate the influence of process conditions during hydrolysis of Atlantic salmon rest raw material on hydrolysate yield, protein recovery and hydrolysate composition
    - Influence of raw material, pretreatments, enzymes and time of hydrolysis (Study I).

- To evaluate the nutritional value and sensory properties of hydrolysates from Atlantic salmon rest raw materials
    - Influence of raw material, pretreatments, enzymes, and compare industrial versus laboratory production of hydrolysate (Studies I and II).

- To evaluate the influence of salmon protein hydrolysate on growth performance, small intestinal morphometry and microbiota (piglets) when included in diets for broiler chickens (Study III) and piglets (Study IV).

3. Materials and methods

Studies I and II

In studies I and II, two different starting materials for the hydrolysis were used: 1) 100 % viscera (V) and 2) a mixture of 50% viscera, 25% heads, and 25% frames/trimmings (VHF) on a wet weight basis. Regarded as unstable and a difficult material to handle, viscera are often excluded in studies of rest raw material from Atlantic salmon farming. As viscera accounts for 40% of the rest raw material available from Atlantic salmon farming in Norway (Olafsen *et al.*, 2014) and the industrial hydrolysis plant is co-located with a large salmon slaughter factory at Frøya in Norway, this fraction was included in the study. The co-location makes it possible to process the viscera-containing raw material immediately after slaughter and extract the valuable oil and protein present in viscera in a controlled manner. The composition of VHF mixture was similar to the composition of the materials delivered to the industrial hydrolysis plant in 2014.

Four hydrolyses were performed with different starting materials (V and VHF) and enzymes (Protamex and a papain plus bromelain mixture) to obtain the hydrolysates. In addition, three hydrolyses were performed with viscera as starting material: 1) without addition of commercial enzymes, 2) after initial inactivation of endogenous enzymes by heat (70°C, >5 min) and subsequent hydrolysis with a papain plus bromelain mixture and 3) after slight heating (40°C) with oil withdrawal before hydrolysis with a papain plus bromelain mixture. The choice of enzymes and process conditions were taken on the basis of previous knowledge and experience at the laboratory. Three of the laboratory hydrolysates were chosen to be produced in industrial scale based on a total judgement of nutritional value and cost/benefit considerations in both hydrolysate producer and possible end-user in the livestock production.

All hydrolyses were performed at 52°C for up to 120 minutes and the industrial production mimicked the laboratory hydrolysis but ran for 60 minutes. The industrial hydrolyzing plant has equipment designed for industrial production and thus the laboratory versus industrial production dissimilarities on hydrolysate composition were detected. Raw materials, pretreatments, enzymes, hydrolysis, sampling and analysis are described in Papers I and II.

Study III

Six experimental diets were conducted in both the starter and grower periods for broiler chickens. Two different hydrolysates were added the diets at two different levels in a 50% and 100% exchange of fishmeal protein, one diet was included 4% fishmeal, and one diet was based on plant protein. The hydrolysates differed in the enzymes used during hydrolysis and were produced from viscera only. One hydrolysate was produced with only endogenous enzymes, while the other hydrolysate was obtained by use of a papain plus bromelain mixture in addition to the endogenous enzymes present in viscera. The two hydrolysates were the same as two of the industrial produced hydrolysates in study I. Further details of production details of the hydrolysates are described in Papers I and II

Immediately after hatching, 5282 broiler chickens were sorted by sex and allotted in 66 pens in four rooms. The experimental diets were fed to the broiler chickens during the starter (days 0-9) and grower period (days 10-28) and one broiler chicken, per pen, was sampled at day 10 for morphometric analysis. Animal housing, diet composition, recording, sampling, and analysis are described in Paper III.

Study IV

Study IV included four experimental diets, fed to piglets from weaning day to day 32 post-weaning. Two diets were added different SPH' in a 100% exchange of fishmeal protein, one diet included 4% fishmeal and one diet was plant protein-based. The hydrolysates were produced at the same industrial hydrolysis plant as studies I, II, and III, without addition of commercial enzymes and from 100% viscera (V) and a mixture of 50% viscera + 25% heads + 25% frame/trimmings (VHF) (wet weight basis). At weaning, 96 piglets were blocked by sex, litter, and live weight, and allocated to four dietary treatments with four replicate pens. At day 11 post-weaning, two piglets each pen were sacrificed and intestinal samples collected for morphometric and 16S rRNA microbiota analysis. Animal housing, hydrolysate and diet composition, sampling and analysis are described in Paper IV.

4. Results and discussion

4.1. Yield, recovery and protein content in the hydrolysates

The yield of SPH, protein content and amino acid composition in the hydrolysate is an important issue as protein is an expensive feed ingredient in feed formulations. Composition of raw material and process conditions influenced the kinetics of the hydrolysis and composition of the hydrolysates (Papers I and II). The rest raw materials used as starting material for hydrolysis had lipids as the major component of the dry material (68-73% on dry matter basis) (Paper I, Table 1) which is typical for fish rest raw materials containing significant amounts of viscera (Šližytė *et al.*, 2005b; Šližytė *et al.*, 2005c; FAO, 2007). Due to the high content of lipids in the starting material, one hydrolysis was performed after separation of oil (Paper I, Figure 1). On a dry matter basis, the protein concentration varied from 13% in the viscera raw material to 22% in the mixture raw material (Paper I, Table 1).

4.1.1. Raw material

Hydrolysis of the viscera, head and frame mixture yielded more SPH in comparison to hydrolysis of viscera alone after 60 minutes of hydrolysis (Paper I, Table 3). However, due to the high fat content in the starting material, which is inert for protein hydrolysis, direct comparison between these two starting materials may give a biased result. When calculated on a fat-free basis, hydrolysis for 60 minutes produced similar amount of SPH (Paper I, Figure 2) and similar recovery of proteins (Paper I, Table 4) from both rest raw starting materials.

The protein content in the hydrolysates varied depending on the starting material. The "lean" viscera, head and frame mixture starting material generated a higher concentration of protein and less lipids in the hydrolysates in comparison to the more fatty viscera starting material (Paper II, Tables 1 and 2). This tendency is also shown by Šližytė *et al.* (2005a), who reported an inverse linear correlation between lipids in cod rest raw material and protein content in the hydrolysate.

4.1.2. Pretreatments

The effect of excessive heat (>70°C) in order to inactivate the endogenous enzymes and to have increased control over the hydrolysis process had a significant impact on the yield and composition of the hydrolysates (Papers I and II). No hydrolysis appears to take place after the

addition of papain plus bromelain as shown by the yield, protein recovery and degree of hydrolysis (Paper I, Figure 2, Tables 3, 4 and 6). In addition, heat inactivation of the endogenous enzymes significantly reduced the protein content and increased the lipid content in the hydrolysates in comparison to the other hydrolysates from viscera (Paper II, Table 2). The reduced efficiency of hydrolysis after excessive heat-pretreatment is described earlier (Slizyte *et al.*, 2004b; Šližytė *et al.*, 2005c; Duan *et al.*, 2010; Liu and Zhao, 2010) and is hypothesized to be due to conformational changes in the protein structure (Mutilangi *et al.*, 1996; Branden and Tooze, 1999) or formation of lipid-protein complexes in the hydrolysate (Šližytė *et al.*, 2005c) making the proteins more resistant to enzymatic breakdown. In addition, the total amount of active proteolytic enzymes are reduced due to the inactivity of the endogenous enzymes.

Extraction of oil before hydrolysis did not influence the yield of SPH or protein recovery (Paper I, Figure 2, Table 4) which indicates that heating of raw material up to 40°C does not denature proteins, making them more resistant to hydrolysis, or reduce the activity of the endogenous enzymes. Furthermore, the hydrolysate after initial oil extraction had higher protein concentration in comparison to the other viscera hydrolysates (Paper II, Table 2), therefore, providing further evidence of the starting materials' influence on hydrolysate composition. In addition, the oil extracted before hydrolysis had a higher quality compared to oil after enzymatic hydrolysis (Paper I, Table 2) which is also reported in herring by Carvajal *et al.* (2014). On top of the aforementioned benefits of oil separation before hydrolysis, the productivity of the hydrolysis reactor will increase due to decreased hydrolysis volume.

4.1.3. Enzymes

Enzymes used in our study (endogenous enzymes present in viscera, Protamex, and papain plus bromelain mixture) inhabit both endopeptidase and exopeptidase activities and are often used during hydrolysis of marine rest raw materials as they are known to be well suited for the solubilization of marine proteins. The choice of Protamex or papain plus bromelain during hydrolysis, did not significantly influence differences in SPH yield, protein recovery or protein content in the SPH (Paper I, Figure 2, Tables 3 and 4, and Paper II, Table 2). In addition, hydrolysis of viscera with only endogenous enzymes produced similar SPH yield, protein recovery and protein content in the SPH in comparison to hydrolysis of viscera added commercial enzymes (Paper I, Figure 2, Tables 3 and 4, and Paper II, Table 2). The high proteolytic activity of endogenous enzymes in viscera is also shown in hydrolysis of Atlantic

cod (Aspmo *et al.*, 2005) and in pink salmon (Bower *et al.*, 2011). The effect of adding commercial enzymes like Protamex or papain plus bromelain to already existing endogenous enzymes when hydrolyzing viscera to increase SPH yield, protein recovery or protein content of the hydrolysate, is therefore questionable. The addition of Protamex and papain plus bromelain, however, significantly reduced the lipid content in the viscera hydrolysates compared to hydrolysis with only endogenous enzymes (Paper II, Table 2). This might be a significant factor as low lipid content in the SPH is important for the stability and quality of the SPH (Gildberg, 1993; Kristinsson and Rasco, 2000b).

It is important to be aware of the high amount of endogenous enzymes present in the starting materials in this study. Viscera accounted for at least 50% of the starting material and the amount of endogenous enzymes were doubled when viscera were the solely substrate in comparison to the viscera, head, and frame mixture. The total amount of endogenous enzymes and therefore total amount of enzymes during hydrolysis were higher when viscera were the sole substrate compared to the viscera, head, and frame mixture.

4.1.4. Time of hydrolysis, kinetics

The increase in SPH yield was faster during the first 60 minutes of hydrolysis compared to the increase in the following 60 minutes of hydrolysis (Paper I, Table 3). The exceptions were the hydrolysis with the heat-denatured starting material, where no hydrolysis seemed to take place and the viscera hydrolyzed with papain plus bromelain showing a significant increase in amount of SPH (55% increase during 60-120 minutes of hydrolysis). This significant increase of SPH yield during 60-120 minutes after hydrolysis of viscera with papain plus bromelain, indicates a delayed effect of these enzymes on slowly hydrolysable parts of viscera compared to Protamex. On a fat-free basis, hydrolysis of both starting materials for 60 minutes produced similar amounts of SPH and recovered protein independent of enzymes used, while further 60 minutes of hydrolysis (60-120 minutes of hydrolysis) increased the yield and protein recovery when only viscera was used as starting material (Paper I, Figure 2, Table 4). The viscera, head and frame mixture starting material contain more easily degradable muscle proteins compared to viscera only, which contains slowly hydrolysable parts of collagen connective tissue lining the guts.

4.2. Nutritional evaluation and sensory properties of the hydrolysates

The nutritional value of protein is primarily dependent on the ability to fulfil the human or animal requirements for protein and essential amino acids. The concept "ideal protein" is, however, under pressure, as new and novel discoveries about amino acid metabolism and the effects on growth and health are discovered (Wu, 2014). In addition, the knowledge about peptides and their potential bioactive effects is becoming increasingly evident (Kim and Mendis, 2006; Ngo *et al.*, 2012).

4.2.1. Protein quality

Hydrolysates from different raw materials have different amino acid compositions (Paper II, Table 3) which is also shown by (Shahidi *et al.*, 1995). The different protein quality measures like PER, PDCAAS and DIAAS, almost all showed slightly higher values for the hydrolysates produced from viscera in comparison to hydrolysates produced from the viscera, head and frame mixture (Paper II, Table 3). The choice of Protamex, papain plus bromelain, only endogenous enzymes or oil-removal before hydrolysis was of minor importance on PER, PDCAAS, and DIAAS measures, while initial heat-inactivation of viscera before hydrolysis lowered the nutritional value, mostly influenced by lower concentrations of indispensable amino acids like histidine, tryptophan, and leucine (Paper II, Table 3). However, all hydrolysates, except the hydrolysate from initially heat-treated viscera, had DIAAS values exceeding 78 with the highest value being 92 (Paper II, Table 3). In comparison, the DIAAS values of wheat, peas, and whole milk powder are calculated to be 40, 64 and 122 respectively (FAO, 2013). The high protein quality measures imply that hydrolysates from Atlantic salmon viscera-containing rest raw materials are well balanced in accordance to the recommended human amino acid reference pattern from FAO.

4.2.2. Degree of hydrolysis and molecular weight distribution

Hydrolysates obtained from viscera, except the hydrolysate from initial heat-inactivated viscera, showed higher degree of hydrolysis, more free amino acids, and higher amount of smaller peptides (<1kDa) in comparison to hydrolysates obtained from the mixed starting material (Paper II, Tables 4 and 5). Excessive heat treatment of viscera before hydrolysis by papain plus bromelain markedly lowered the degree of hydrolysis, the amount of free amino acids, and percentage of smaller peptides (<1kDa) in the hydrolysates in comparison to the other viscera hydrolysates (Paper II, Tables 4 and 5). This again supports the theory of

conformational changes of proteins during excessive heating making them more resistant to degradation by proteases. The lower efficiency of hydrolysis and larger peptides in the hydrolysates after initial excessive heat-treatment is also described in hydrolysis of blue mackerel (*Decapterus maruadsi*) (Liu and Zhao, 2010) and silver carp (*Hypophthalmichthys molitrix*) (Duan *et al.*, 2010). Oil-removal before hydrolysis increased the degree of hydrolysis and amount of free amino acids in the hydrolysates (Paper II, Table 4) probably due to the time spent before hydrolysis and heating to 40°C to facilitate oil-removal where the endogenous proteases in viscera were active. Different enzymes, including only endogenous enzymes in viscera, had a minor impact on the degree of hydrolysis and amount free amino acids - although Protamex tended to produce hydrolysates with slightly higher degree of hydrolysis, lower amount of free amino acids, and smaller peptides (<1kDa) compared to papain plus bromelain from both rest raw materials (Paper II, Tables 4 and 5). The small differences detected between the enzymes, Protamex and papain plus bromelain, were probably due to the high proteolytic activity of the raw material itself, as it contained at least 50% viscera that may have dominated the proteolytic activity during hydrolysis. The high proteolytic activity from viscera is also reported from a number of other studies (Pastoriza *et al.*, 2004; Aspmo *et al.*, 2005; Sovik and Rustad, 2005; Bower *et al.*, 2011). The observed lower degree of hydrolysis, amount of free amino acids and smaller peptides (<1kDa) in the industrially-produced hydrolysates compared to the corresponding hydrolysates produced in the laboratory (Paper II, Tables 4 and 5) was probably partly due to the shorter time from slaughter to hydrolysis, and time spent to reach 52°C in the industry. A strong positive significant correlation between the degree of hydrolysis and amount free amino acids was detected (Paper II), which is in line with findings of Šližytė *et al.* (2005a) in hydrolysis of cod by-products.

4.2.3. Bioactivity

Different raw materials were expected to give rise to different peptides in the hydrolysates. However, the most striking result of this analysis was the low number of different peptides recognized in the hydrolysates obtained by Protamex in comparison to hydrolysates obtained by use of papain plus bromelain in both viscera and viscera, head, and frame mixture starting materials (Paper II, Table 6). Despite the difference in number of detected peptides, the number of different bioactive motifs detected was at the same level for all the laboratory hydrolysates, regardless of different starting materials, pretreatments, or enzymes (Paper II, Table 6). The majority of recognized bioactive peptides were associated with the cardiosystem (Paper II, Table 6), upon which most of the published data is based.

4.2.4. Bitterness

The viscera, head, and frame mixture starting material had a significantly lower median bitterness compared to the viscera starting material and the same tendency was also observed in the hydrolysates after 60 minutes of hydrolysis (Paper I, Figure 4). Heat inactivation of the endogenous enzymes led to the bitterest sample both before hydrolysis and after 60 minutes of hydrolysis, while separation of oil before hydrolysis did not influence the bitter taste of the hydrolysate after 60 minutes of hydrolysis (Paper I, Figure 4). Hydrolysis of viscera with only endogenous enzymes produced a bitterer hydrolysate in comparison to hydrolysate from viscera with active endogenous enzymes and commercial enzymes added to the hydrolysis reaction (Paper I, Figure 4). It is reported that the formation of the bitter taste is dependent on several factors, such as starting material, enzymes, and the hydrolyzing process itself (Dauksas *et al.*, 2004). Although all mechanisms for formation of bitter compounds are not clearly understood, the exposure of hydrophobic amino acid side-chains during hydrolysis is thought to be important (Kristinsson and Rasco, 2000a), together with the degree of hydrolysis - which is also shown to influence the formation of bitter peptides. Restricting the degree of hydrolysis to 3-5 % (Adler-Nissen, 1984) or increasing the degree of hydrolysis to produce high concentrations of free amino acids, reduces the sensation of bitter compounds in the hydrolysates as it is known that hydrophobic peptides are considerably more bitter than the corresponding mixture of free amino acids (Belitz and Wieser, 1976).

4.3. Hydrolysates as feed ingredient in broiler chickens and piglets

4.3.1. Growth performance

The addition of 10% SPH in broiler chicken diets increased the ADG up to 12 % during the first 10 days post-hatch in comparison with broiler chickens fed the plant protein-based or fishmeal diets. Inclusion of 5% SPH in the broiler chicken diets also significantly increased the ADG during the first nine days post-hatch in comparison to the plant protein-based and fishmeal diets (Paper III, Table 3). The observed increase in ADG is supported by the findings of Wagner and Bregendahl (2007) with salmon protein concentrate and Mateos *et al.* (2014) with porcine mucosa hydrolysate in broiler chicken diets. In our study (Paper III), no differences were detected in feed or energy intake between any of the diets the first 10 days post-hatch, which is in line with Mateos *et al.* (2014) while Wagner and Bregendahl (2007) concluded that the observed increase in weight gain was due to an increased feed intake. In our experiment (Paper

III) the feed utilization, but not the energy utilization, in the 10% SPH diet groups were increased compared to the plant protein-based or fishmeal diet groups during the first 10 days post-hatch. The observed increase in ADG might be due to the content of peptides increasing the absorption of amino acids in the immature intestine, stimulating intestinal growth and development, together with increased amino acids concentration in the portal blood enhancing the amino acids available for growth and/or due to some bioactive properties of the hydrolysates.

During the grower period the difference in daily gain diminished between broiler chickens fed the SPH containing diets and the fishmeal diet, while the broiler chickens fed the plant protein based diet still had the lowest growth rates (Paper III, Table 3). At the end of the experiment, day 28, no difference in live weight was observed between broiler chickens fed the SPH or fishmeal diets, while the broiler chickens fed the plant protein-based diet weighed less in comparison to the other groups (Paper III). During the later phase of the experiment the broiler chickens' intestines are more mature and compensatory regulation of amino acid absorption and metabolism might conceal the effects of feeding SPH on growth performance that were seen during the starter period. In addition, the litter score indicated more moisture in the litter at day 28 in the plant protein diet group in comparison to the marine protein diet groups (Paper III).

The inclusion of 10% SPH in diets for weaning piglets did not have effect on growth performance in piglets during the first 11 days or for the overall period 0-32 days post-weaning (Paper IV, Table 3). This is in line with Tucker *et al.* (2011) who reported no differences in growth performance the first week post-weaning in piglets fed diets which included 1.5% and 3.0% SPH and Nørgaard *et al.* (2012) the first 14 days of the trial in piglets fed diets containing fishmeal, SPH or soy protein concentrate (SPC). Nørgaard *et al.* (2012) reported an increased overall feed intake in piglets fed SPH diets in comparison to a FM diet, but not to a SPC diet, which is in contrast to the experiment in Paper IV - where no differences in feed intake were observed at any time between the experimental diet groups. However, these studies are not directly comparable as Tucker *et al.* (2011) fed diets containing antibiotics that may have influenced the results as infeed antibiotics are known to exhibit growth-promoting effects and Nørgaard *et al.* (2012) introduced the experimental diets one week post-weaning. Growth performance in piglets fed hydrolysates from other sources shows conflicting results as Vente-Spreeuwenberg *et al.* (2004) reported no difference in growth performance in piglets fed hydrolyzed soybean meal and wheat gluten, while Zhou *et al.* (2010) reported increased growth

performance with increasing levels of enzymolytic soybean meal in piglets. Increased daily gain and feed intake is reported at two and three weeks post-weaning in piglets fed hydrolyzed blood cells compared to piglets fed fishmeal or non-hydrolyzed blood cells (Chen *et al.*, 2013).

The conflicting results reported on the effect of hydrolysates compared to intact proteins on growth performance may be due to several factors. When feeding young animals the state of intestinal development and maturity, immunity and species differences are obviously variable. In addition, the hydrolysate composition can be influenced by several factors, as shown in Papers I and II. When feeding diets including hydrolysates, the peptide content and molecular weight distribution is of significance as it is the peptide content and composition that is hypothesized to enhance the protein digestibility and exert the possible biological effects.

4.3.2. Intestinal morphometry

Morphometric analysis has been and is a useful tool to study the development and growth of the intestines in young animals. The intense metabolism and nutrient requirements in addition to the intestine's importance for nutrient absorption, growth and disease resistance makes intestinal morphometry and morphology a useful tool for feed evaluation. Villus height and morphology have been described as important indicators of intestinal maturity, functionality and health (Lallès *et al.*, 2007; Montagne *et al.*, 2007).

The addition of SPH in the diets increased the duodenal villus height in broiler chickens (Paper III, Table 4) and tended to increase the duodenal villus height in piglets (Paper IV, Table 4) in comparison to groups fed the plant protein–based diet, but not when compared to groups fed the FM diet. This indicates that marine protein in the diets improved villus growth in broiler chickens and, in piglets, tended to decrease villus atrophy in the duodenum. The improved intestinal morphometry in duodenum may indicate increased proximal intestinal absorption and growth when peptides are supplied in the diet. However, as no differences were found in duodenal villus height between broiler chickens fed diets which included SPH in comparison to the fishmeal diet, the effect on villus height cannot exclusively explain the observed difference in ADG during the starter period in broiler chickens. A combined effect of peptides on villus height and a more efficient absorption of amino acids into the portal blood may be part of a plausible explanation for the observed effects on ADG in broiler chickens.

It is not known if there are components in the marine protein containing diets that enhance the villus development and maintenance, or if there are factors in the plant protein-based diets that

are detrimental for the villus. Marine protein sources contain nitrogenous components, such as taurine and hydroxyproline, which are not detected in soy protein isolates (Liaset *et al.*, 2003). Both taurine and hydroxyproline are associated with an increased growth performance in Atlantic salmon (Kousoulaki *et al.*, 2009). However, impaired intestinal development and no effect on daily gain or feed intake are reported in broiler chickens fed diets which included taurine (Huang *et al.*, 2014). In contrast, the plant protein-based diets contained more soya in comparison to the other experimental diets and the anti-nutritional factors - such as lectins, oligosaccharides, saponins and trypsin inhibitors, which are known to be present in soya, might have a negative impact on the intestinal development and maintenance. It should however be mentioned that the soya used in the piglet study was from a soya source where the anti-nutritional factors are claimed to be significantly reduced or eliminated.

No differences in growth performance were detected between animal groups fed the fishmeal and plant protein-based diets during the initial periods of the feeding experiments (Papers III and IV). These findings indicates that the effect of marine proteins most probably are through supporting villus development and integrity during the early phases post-hatch and post-weaning as seen by the proximal intestinal morphometry. This coincides with the time of extensive growth and development in the broiler chickens intestines and atrophy and loss of integrity in the piglet intestines, where a healthy intestine is a key factor for the animal growth performance and disease resistance.

A significant positive correlation was found between duodenal villus height and average daily weight gain in both broiler chickens the first 10 days post-hatch ($r^2 = 0.27$, $p=0.03$, data obtained from the experiment in Paper III) and piglets the first 11 days post-weaning (Paper IV, Figure 1). This is in line with Pluske *et al.* (1996), who reported a positive correlation between proximal intestinal villus height and weight gain in piglets fed diets of ewes' milk or a standard starter diet. These findings underline the importance of the intestinal integrity and development in order to exploit the animal's growth potential.

The ileal villus height tended to be increased in broiler chickens fed the SPH diets in comparison to the plant protein-based diet (Paper III, Table 4), although this was not seen in the piglets receiving SPH diets (Paper IV, Table 4). Interestingly, the piglets fed the SPH diets tended to show an increased ileal crypt depth compared to piglets fed both the plant protein-based diet and the fishmeal diet (Paper III, Table 4). The mechanisms for this observation are not known; however, if the SPH diets led to less protein reaching the hindgut and subsequent ileal crypt

hypertrophy, this may be of importance for the microbiota community in the hindgut. Microbiota fermentation is influenced by substrate availability and undigested proteins reaching the hindgut are regarded as unfavorable as the number of proteolytic bacteria might increase (Halas *et al.*, 2007; Opapeju *et al.*, 2009).

4.3.3. Ileal microbiota

The ileal microbiota composition was notably similar between piglets fed the experimental diets (Paper IV), indicating small differences in the substrate available for microbial fermentation in the ileum content. Alteration of the microbiota community is mainly achieved through changes in macronutrients such as fiber, protein and fat (Wu *et al.*, 2011; David *et al.*, 2014) and the results from Paper IV indicates that inclusion of hydrolysates in piglet diets does not alter ileal intestinal microbiota in well-balanced diets. The microbiota community consisted of 30-45% of the genus *Lactobacillus* and 30-40% of the genus *Turicibacter* (Paper IV). Different strains of *Lactobacillus* are shown to suppress or inhibit microbial pathogens (Servin, 2004) and the probiotic effects of *Lactobacillus* are well known, hence the highly significant inverse correlation between *Lactobacillus* and *Turicibacter* described in Paper IV, is therefore of importance. This is in addition to the general importance of correlations between microbiota, as bacterial imbalances are known to be non-beneficial for the host.

5. Conclusion, further perspectives and recommendations

The present study has shown that the composition of raw materials together with process control are the key factors for producing highly valuable feed ingredients from salmon rest raw materials. It is possible, to some extent, to design the proximal composition, the size of the peptides formed, the amino acid composition, and the potential bioactive peptides by combining different raw materials, enzymes, and pretreatments. In general, hydrolysates from salmon rest raw materials contain high levels of proteins, with high nutritional value, and a number of bioactive motifs.

Viscera have high activity of the endogenous enzymes that intensively hydrolyze material immediately post-mortem and during the stages of raw material preparation. The addition of commercial enzymes is not always more efficient and economically beneficial, but can improve the taste or release the oil from the hydrolysate fraction. Initial heat inactivation of the endogenous enzymes increases the bitterness, and reduces the yield and nutritional value of the hydrolysate. On the contrary, the initial separation of oil before hydrolysis offers several advantages for the hydrolysis process, such as less lipids in the hydrolysate compared to corresponding treatment without oil separation, higher quality in oil separated before hydrolysis compared to after hydrolysis and increased productivity of the hydrolysis reactor due to reduction of the hydrolysis volume. In addition, initial separation of oil did not affect the nutritional value of the proteins or amino acid composition.

Salmon protein hydrolysates (SPH) are found to be an excellent novel source of proteins well suited in diets for broiler chickens and weaning piglets. In broiler chickens, the inclusion of SPH in the starter diets increased the growth performance in comparison to both a plant protein-based diet and a fishmeal containing diet. The proximal intestinal development was enhanced by inclusion of SPH in the broiler chicken diets compared to the plant protein-based diet, but not when compared to the fishmeal diet. During the grower period, broiler chickens fed diets with inclusion of SPH or fishmeal had an increased weight gain in comparison to broiler chickens fed the plant protein-based diet.

Piglets fed diets with 10% SPH inclusion had similar growth performance during the first 11 days post-weaning and for the overall period 0-32 days post-weaning as piglets fed diets with fishmeal or a plant protein-based diet. The inclusion of 10% SPH in the diets tended to maintain the proximal intestinal architecture with increased villus height and absorption area in

comparison to the piglets fed the plant protein-based diet. In the ileal microbiota analysis, a highly significant positive correlation was found between the bacterial genera *Lactobacillus* and *Turicibacter*, which is important as the microbiota community have several interrelationships that may be useful when designing feed to influence the intestinal microbiota community. At genus level, only small differences in ileal microbiota were detected between piglets fed the different diets.

A significant positive correlation between the duodenal villus height and average daily gain in the first 10 days post-hatch in broiler chickens and 11 days post-weaning in piglets were found, implying the importance of intestinal architecture for growth performance.

No adverse effects were detected by including SPH in the broiler chicken or piglet diets, and enzymatic hydrolysis of Atlantic salmon rest raw material appears to be a feasible way to extract valuable proteins and peptides from lower value products.

Some perspectives and recommendations for future works:

- Viscera should be included in future studies as it is a major constituent of the available rest raw material arising from fish farming and fishery.

- Mild thermal treatment in order to extract high quality oil before hydrolysis was a feasible solution in the laboratory. Future work should focus on how to implement oil extraction before hydrolysis in an industrial production.

- The sediment/sludge fraction after hydrolysis should be evaluated as possible feed ingredient.

- Several interesting peptides with possible bioactivity was detected in the hydrolysates. Further studies on possible bioactivity should be conducted.

- The functional physical properties, like pelleting-binding capacity, should be better evaluated as this is an important feature in the feed industry.

- To gain more knowledge on changes in carcass composition related to the increased weight gain seen in broiler chickens fed SPH in the starter diets.

- Interrelationships between different bacteria, fungus and viruses in different intestinal compartments and the influence of feed are important and further research warranted.

- The environmental footprint should be included in cost-benefit analysis and regulated to improve and encourage better utilization of all materials arising from the food and feed chain.

- This study has shown that local rest raw material is a highly valuable resource and that interdisciplinary scientific cooperation is important in increasing the sustainability in feed and food production, both locally and from a more global perspective.

6. References

Adler-Nissen, J. (1984). Control of the proteolytic reaction and of the level of bitterness in protein hydrolysis processes. *Journal of Chemical Technology and Biotechnology* 34(3): 215-222.

Aksnes, A., Hope, B., Hostmark, O. & Albrektsen, S. (2006a). Inclusion of size fractionated fish hydrolysate in high plant protein diets for Atlantic cod, Gadus morhua. *Aquaculture* 261(3): 1102-1110.

Aksnes, A., Hope, B., Jonsson, E., Bjornsson, B. T. & Albrektsen, S. (2006b). Size-fractionated fish hydrolysate as feed ingredient for rainbow trout (Oncorhynchus mykiss) fed high plant protein diets. I: Growth, growth regulation and feed utilization. *Aquaculture* 261(1): 305-317.

Alsmeyer, R. H., Cunningham, A. E. & Happich, M. L. (1974). Equations predict PER from amino acid analysis. *Food Technology* 28(7): 34-40.

Animalia (2014). Slaktegris 2014. http://www.animalia.no/Slakt--kjott--og-eggkvalitet/ Klassifisering/Statistikk---Slakt/Dyreslag-GRIS/Statistikk---Gris/.

Animalia & Norsvin (2014). Ingris Årsstatistikk 2014. https://norsvin.no/ Fag/Ingris/ AArsstatistikk.

Aspmo, S. I., Horn, S. J. & H. Eijsink, V. G. (2005). Enzymatic hydrolysis of Atlantic cod (Gadus morhua L.) viscera. *Process Biochemistry* 40(5): 1957-1966.

Balog, J. M. & Millar, R. I. (1989). Influence of the sense of taste on broiler chick feed consumption. *Poultry Science* 68(11): 1519-1526.

Barbot, L., Windsor, E., Rome, S., Tricottet, V., Reynes, M., Topouchian, A., Huneau, J. F., Gobert, J. G., Tome, D. & Kapel, N. (2003). Intestinal peptide transporter PepT1 is over-expressed during acute cryptosporidiosis in suckling rats as a result of both malnutrition and experimental parasite infection. *Parasitology Research* 89(5): 364-370.

Batal, A. B. & Parsons, C. M. (2002). Effects of age on nutrient digestibility in chicks fed different diets. *Poultry Science* 81(3): 400-407.

Bauer, E., Williams, B. A., Smidt, H., Verstegen, M. W. & Mosenthin, R. (2006). Influence of the gastrointestinal microbiota on development of the immune system in young animals. *Current Issues in Intestinal Microbiology* 7(2): 35-51.

Belitz, H. D. & Wieser, H. (1976). [Steric arrangement of sweet and bitter taste of amino acids and peptides (author's transl)]. *Z Lebensm Unters Forsch* 160(3): 251-253.

Bourseau, P., Vandanjon, L., Jaouen, P., Chaplain-Derouiniot, M., Masse, A., Guerard, F., Chabeaud, A., Fouchereau-Peron, M., Le Gal, Y., Ravallec-Ple, R., Berge, J. P., Picot, L., Piot, J. M., Batista, I., Thorkelsson, G., Delannoy, C., Jakobsen, G. & Johansson, I. (2009). Fractionation of fish protein hydrolysates by ultrafiltration and nanofiltration: impact on peptidic populations. *Desalination* 244(1-3): 303-320.

Bower, C., Malemute, C. & Bechtel, P. (2011). Endogenous protease activity in by-products of pink salmon (oncorhynchus gorbuscha). *Journal of Food Biochemistry* 35(2): 628-637.

Branden, C. & Tooze, J. (1999). *Introduction to protein structure*. New York: Garland Publishing.

Brandsch, M., Knütter, I. & Leibach, F. H. (2004). The intestinal H+/peptide symporter PEPT1: structure–affinity relationships. *European Journal of Pharmaceutical Sciences* 21(1): 53-60.

Broer, S. (2008). Amino acid transport across mammalian intestinal and renal epithelia. *Physiological Reviews* 88(1): 249-286.

Carvajal, A. K., Slizyte, R., Storrø, I. & Aursand, M. (2014). Production of high quality fish oil by thermal treatment and enzymatic protein hydrolysis from fresh Norwegian spring spawning herring by-products. *Journal of Aquatic Food Product Technology*: In press.

Chalamaiah, M., Kumar, B. D., Hemalatha, R. & Jyothirmayi, T. (2012). Fish protein hydrolysates: Proximate composition, amino acid composition, antioxidant activities and applications: A review. *Food Chemistry* 135(4): 3020-3038.

Chen, H., Pan, Y., Wong, E. A. & Webb, K. E. (2005). Dietary protein level and stage of development affect expression of an intestinal peptide transporter (cPepT1) in chickens. *The Journal of Nutrition* 135(2): 193-198.

Chen, Q. S., Zhang, H., Zheng, Y. B., Shan, A. S. & Bi, Z. P. (2013). Effects of enzymatically hydrolyzed blood cells on growth performance and intestinal characteristics of newly weaned piglets. *Livestock Science* 157(2-3): 514-519.

Cheung, R., Ng, T. & Wong, J. (2015). Marine Peptides: Bioactivities and Applications. *Marine Drugs* 13(7): 4006.

Cho, J. H. & Kim, I. H. (2011). Fish meal--nutritive value. *Journal of Animal Physiology and Animal Nutrition* 95(6): 685-692.

Choct, M. (2009). Managing gut health through nutrition. *British Poultry Science* 50(1): 9-15.

Choct, M., Hughes, R. J. & Bedford, M. R. (1999). Effects of a xylanase on individual bird variation, starch digestion throughout the intestine, and ileal and caecal volatile fatty acid production in chickens fed wheat. *British Poultry Science* 40(3): 419-422.

Cooper, K. K. & Songer, J. G. (2009). Necrotic enteritis in chickens: A paradigm of enteric infection by Clostridium perfringens type A. *Anaerobe* 15(1–2): 55-60.

Crowe, K. M. & Francis, C. (2013). Position of the academy of nutrition and dietetics: functional foods. *J Acad Nutr Diet* 113(8): 1096-1103.

Daniel, H. (2004). Molecular and integrative physiology of intestinal peptide transport. *Annual Review of Physiology* 66: 361-384.

Dapkevičius, M. D. E., Batista, I., Nout, M. J. R., Rombouts, F. M. & Houben, J. H. (1998). Lipid and protein changes during the ensilage of blue whiting (Micromesistius poutassou Risso) by acid and biological methods. *Food Chemistry* 63(1): 97-102.

Dapkevicius, M. L. N. E., Nout, M. J. R., Rombouts, F. M., Houben, J. H. & Wymenga, W. (2000). Biogenic amine formation and degradation by potential fish silage starter microorganisms. *International Journal of Food Microbiology* 57(1–2): 107-114.

Dauksas, E., Slizyte, R., Rustad, T. & Storro, I. (2004). Bitterness in fish protein hydrolysates and methods for removal. *Journal of Aquatic Food Product Technology* 13(2): 101-114.

David, L. A., Maurice, C. F., Carmody, R. N., Gootenberg, D. B., Button, J. E., Wolfe, B. E., Ling, A. V., Devlin, A. S., Varma, Y., Fischbach, M. A., Biddinger, S. B., Dutton, R. J. & Turnbaugh, P. J. (2014). Diet rapidly and reproducibly alters the human gut microbiome. *Nature* 505(7484): 559-563.

Duan, Z. H., Wang, J. L., Yi, M. H. & Yin, A. Q. (2010). Recovery of proteins from silver carp by-products with enzymatic hydrolysis and reduction of bitterness in hydrolysate. *Journal of Food Process Engineering* 33(5): 962-978.

Ewart, H. S., Dennis, D., Potvin, M., Tiller, C., Fang, L. H., Zhang, R., Zhu, X. M., Curtis, J. M., Cloutier, S., Du, G. H. & Barrow, C. J. (2009). Development of a salmon protein hydrolysate that lowers blood pressure. *European Food Research and Technology* 229(4): 561-569.

Fairbrother, J. M., Nadeau, É. & Gyles, C. L. (2005). Escherichia coli in postweaning diarrhea in pigs: an update on bacterial types, pathogenesis, and prevention strategies. *Animal Health Research Reviews* 6(01): 17-39.

FAO (2007).Salmon by-product proteins (Ed A. Ramirez). Food and Agriculture Organization of the United Nations.

FAO (2013).Dietary protein quality evaluation in human nutrition: Report of an FAO Expert Consultation. 31 March-2 April, 2011 Auckland, New Zealand Food and Agriculture Organization of the United Nations.

Five, K. (2013).Enzymatic hydrolysis of salmon by-products : Effect of process conditions on ACE inhibiting activities of fish protein hydrolysates. urn:nbn:no:ntnu:diva-22411: Norwegian University of Science and Technology, Faculty of Natural Sciences and Technology, Department of Biotechnology.

Gaskins, H. R. (2001).Intestinal bacteria and their influence on swine growth. In *Swine Nutrition 2nd ed.*, 585-608 (Ed A. J. Lewis). Florida: CRC Press LLC.

Gilbert, E. R., Li, H., Emmerson, D. A., Webb, K. E. & Wong, E. A. (2010). Dietary protein composition influences abundance of peptide and amino acid transporter messenger ribonucleic acid in the small intestine of 2 lines of broiler chicks. *Poultry Science* 89(8): 1663-1676.

Gilbert, E. R., Wong, E. A. & Webb, K. E. (2008). Board-invited review: Peptide absorption and utilization: Implications for animal nutrition and health. *Journal of Animal Science* 86(9): 2135-2155.

Gildberg, A. (1993). Enzymic processing of marine raw materials. *Process Biochemistry* 28(1): 1-15.

Glaser, D., Wanner, M., Tinti, J. M. & Nofre, C. (2000). Gustatory responses of pigs to various natural and artificial compounds known to be sweet in man. *Food Chemistry* 68(4): 375-385.

Halas, D., Heo, J. M., Hansen, C. F., Kim, J. C., Hampson, D. J., Mullan, B. P. & Pluske, J. R. (2007). Organic acids, prebiotics and protein level as dietary tools to control the weaning transition and reduce post-weaning diarrhoea in piglets. *CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources* 2(079): 1-13.

Hamal, K. R., Burgess, S. C., Pevzner, I. Y. & Erf, G. F. (2006). Maternal antibody transfer from dams to their egg yolks, egg whites, and chicks in meat lines of chickens. *Poultry Science* 85(8): 1364-1372.

Harnedy, P. A. & FitzGerald, R. J. (2012). Bioactive peptides from marine processing waste and shellfish: A review. *Journal of Functional Foods* 4(1): 6-24.

He, S., Franco, C. & Zhang, W. (2012). Process optimisation and physicochemical characterisation of enzymatic hydrolysates of proteins from co-products of Atlantic Salmon (Salmo salar) and Yellowtail Kingfish (Seriola lalandi). *International Journal of Food Science and Technology* 47(11): 2397-2404.

Hedemann, M. S., Hojsgaard, S. & Jensen, B. B. (2003). Small intestinal morphology and activity of intestinal peptidases in piglets around weaning. *J Anim Physiol Anim Nutr (Berl)* 87(1-2): 32-41.

Hellekant, G. & Danilova, V. (1999). Taste in domestic pig, Sus scrofa. *Journal of Animal Physiology and Animal Nutrition* 82(1): 8-24.

Heo, J. M., Kim, J. C., Hansen, C. F., Mullan, B. P., Hampson, D. J. & Pluske, J. R. (2008). Effects of feeding low protein diets to piglets on plasma urea nitrogen, faecal ammonia nitrogen, the incidence of diarrhoea and performance after weaning. *Archives of Animal Nutrition* 62(5): 343-358.

Heo, J. M., Opapeju, F. O., Pluske, J. R., Kim, J. C., Hampson, D. J. & Nyachoti, C. M. (2013). Gastrointestinal health and function in weaned pigs: a review of feeding strategies to control post-weaning diarrhoea without using in-feed antimicrobial compounds. *Journal of Animal Physiology and Animal Nutrition* 97(2): 207-237.

Huang, C. X., Guo, Y. M. & Yuan, J. M. (2014). Dietary taurine impairs intestinal growth and mucosal structure of broiler chickens by increasing toxic bile acid concentrations in the intestine. *Poultry Science* 93(6): 1475-1483.

Iji, P. A., Saki, A. & Tivey, D. R. (2001). Body and intestinal growth of broiler chicks on a commercial starter diet. 1. Intestinal weight and mucosal development. *British Poultry Science* 42(4): 505-513.

Juul-Madsen, H. R., Su, G. & Sorensen, P. (2004). Influence of early or late start of first feeding on growth and immune phenotype of broilers. *British Poultry Science* 45(2): 210-222.

Kim, J. C., Hansen, C. F., Mullan, B. P. & Pluske, J. R. (2012). Nutrition and pathology of weaner pigs: Nutritional strategies to support barrier function in the gastrointestinal tract. *Animal Feed Science and Technology* 173(1-2): 3-16.

Kim, S. K. & Mendis, E. (2006). Bioactive compounds from marine processing byproducts - A review. *Food Research International* 39(4): 383-393.

Kim, S. K. & Wijesekara, I. (2010). Development and biological activities of marine-derived bioactive perptides: A review. *Journal of Functional Foods* 2(1): 1-9.

Kinsella, J. E. & Melachouris, N. (1976). Functional properties of proteins in foods: A survey. *C R C Critical Reviews in Food Science and Nutrition* 7(3): 219-280.

Kodera, T., Hara, H., Nishimori, Y. & Nio, N. (2006). Amino acid absorption in portal blood after duodenal infusions of a soy protein hydrolysate prepared by a novel soybean protease D3. *Journal of Food Science* 71(7): 517-525.

Kousoulaki, K., Albrektsen, S., Langmyhr, E., Olsen, H. J., Campbell, P. & Aksnes, A. (2009). The water soluble fraction in fish meal (stickwater) stimulates growth in Atlantic salmon (Salmo salar L.) given high plant protein diets. *Aquaculture* 289(1-2): 74-83.

Kristinsson, H. G. & Rasco, B. A. (2000a). Biochemical and functional properties of Atlantic salmon (Salmo salar) muscle proteins hydrolyzed with various alkaline proteases. *Journal of Agricultural and Food Chemistry* 48(3): 657-666.

Kristinsson, H. G. & Rasco, B. A. (2000b). Fish protein hydrolysates: Production, biochemical, and functional properties. *Critical Reviews in Food Science and Nutrition* 40(1): 43-81.

Kristinsson, H. G. & Rasco, B. A. (2000c). Kinetics of the hydrolysis of Atlantic salmon (Salmo salar) muscle proteins by alkaline proteases and a visceral serine protease mixture. *Process Biochemistry* 36(1-2): 131-139.

Kudo, K., Shiraishi, J., Nishimura, S., Bungo, T. & Tabata, S. (2010). The number of taste buds is related to bitter taste sensitivity in layer and broiler chickens. *Anim Sci J* 81(2): 240-244.

Lahl, W. J. & Braun, S. D. (1994). Enzymatic production of protein hydrolysates for food use. *Food Technology* 48(10): 68-71.

Lallès, J.-P., Bosi, P., Smidt, H. & Stokes, C. R. (2007). Weaning — A challenge to gut physiologists. *Livestock Science* 108(1–3): 82-93.

Lam, R. S. H. & Nickerson, M. T. (2013). Food proteins: A review on their emulsifying properties using a structure–function approach. *Food Chemistry* 141(2): 975-984.

Landbruksdirektoratet (2011).Utvikling slakta kyllinger fordelt på fylke. https://www.slf.dep.no/no/11105/utvikling-slakta-kyllinger-fordelt-p%C3%A5-fylke.

Lee, Y. B., Elliott, J. G., Rickansrud, D. A. & Hagberg, E. Y. C. (1978). Predicting protein efficiency ratio by the chemical determination of connective tissue content in meat. *Journal of Food Science* 43(5): 1359-1362.

Leksrisompong, P., Gerard, P., Lopetcharat, K. & Drake, M. (2012). Bitter taste inhibiting agents for whey protein hydrolysate and whey protein hydrolysate beverages. *J Food Sci* 77(8): S282-287.

Liaset, B., Julshamn, K. & Espe, M. (2003). Chemical composition and theoretical nutritional evaluation of the produced fractions from enzymic hydrolysis of salmon frames with Protamex (TM). *Process Biochemistry* 38(12): 1747-1759.

Liaset, B., Lied, E. & Espe, M. (2000). Enzymatic hydrolysis of by-products from the fish-filleting industry; chemical characterisation and nutritional evaluation. *Journal of the Science of Food and Agriculture* 80(5): 581-589.

Liaset, B., Nortvedt, R., Lied, E. & Espe, M. (2002). Studies on the nitrogen recovery in enzymic hydrolysis of Atlantic salmon (Salmo salar, L.) frames by Protamex (TM) protease. *Process Biochemistry* 37(11): 1263-1269.

Liu, T. X. & Zhao, M. M. (2010). Thermal pretreatment and chemical modifications as a means to alter hydrolytic characteristics and prevent bitterness in hydrolysates of fishery by-catch (Decapterus maruadsi) protein. *International Journal of Food Science and Technology* 45(9): 1852-1861.

Lovdata (2003). Forskrift om hold av svin: FOR-2003-02-18-175. https://lovdata.no/dokument/SF/forskrift/2003-02-18-175?q=forskrift+om+hold+av+svin.

Lovdata (2014).Forskrift om animalske biprodukter som ikke er beregnet på konsum https://lovdata.no/dokument/SF/forskrift/2007-10-27-1254.

M'Sadeq, S. A., Wu, S., Swick, R. A. & Choct, M. (2015). Towards the control of necrotic enteritis in broiler chickens with in-feed antibiotics phasing-out worldwide. *Animal Nutrition* 1(1): 1-11.

Mackie, I. M. (1974). Proteolytic enzymes in recovery of proteins from fish waste. *Process Biochemistry* 9: 12-14.

Maneewan, B. & Yamauchi, K. (2004). Intestinal villus recovery in chickens refed semi-purified protein-, fat-, or fibre-free pellet diets. *British Poultry Science* 45(2): 163-170.

Mateos, G. G., Mohiti-Asli, M., Borda, E., Mirzaie, S. & Frikha, M. (2014). Effect of inclusion of porcine mucosa hydrolysate in diets varying in lysine content on growth performance and ileal histomorphology of broilers. *Animal Feed Science and Technology* 187: 53-60.

Mo, S. S., Norström, M., Slettemeås, J. S., Løvland, A., Urdahl, A. M. & Sunde, M. (2014). Emergence of AmpC-producing Escherichia coli in the broiler production chain in a country with a low antimicrobial usage profile. *Veterinary Microbiology* 171(3–4): 315-320.

Montagne, L., Boudry, G., Favier, C., Le Huerou-Luron, I., Lalles, J. P. & Seve, B. (2007). Main intestinal markers associated with the changes in gut architecture and function in piglets after weaning. *British Journal of Nutrition* 97(1): 45-57.

Mutilangi, W. A. M., Panyam, D. & Kilara, A. (1996). Functional properties of hydrolysates from proteolysis of heat-denatured whey protein isolate. *Journal of Food Science* 61(2): 270-275.

Nelson, S. L. & Sanregret, J. D. (1997). Response of pigs to bitter-tasting compounds. *Chemical Senses* 22(2): 129-132.

Ngo, D. H., Vo, T. S., Ngo, D. N., Wijesekara, I. & Kim, S. K. (2012). Biological activities and potential health benefits of bioactive peptides derived from marine organisms. *International Journal of Biological Macromolecules* 51(4): 378-383.

Nortura (2014). Slaktekyllingkontrollen In *Nortura Slaktekyllingkontrollen* https://medlem.nortura.no/fjoerfe/slaktekyllingkontrollen/.

Nørgaard, J. V., Blaabjerg, K. & Poulsen, H. D. (2012). Salmon protein hydrolysate as a protein source in feed for young pigs. *Animal Feed Science and Technology* 177(1-2): 124-129.

Olafsen, T., Richardsen, R., Nystøyl, R., Strandheim, G. & Kosmo, J. P. (2014).Analyse marint restråstoff, 2013. SINTEF Fisheries and Aquaculture.

Opapeju, F. O., Krause, D. O., Payne, R. L., Rademacher, M. & Nyachoti, C. M. (2009). Effect of dietary protein level on growth performance, indicators of enteric health, and gastrointestinal microbial ecology of weaned pigs induced with postweaning colibacillosis. *Journal of Animal Science* 87(8): 2635-2643.

Panyam, D. & Kilara, A. (1996). Enhancing the functionality of food proteins by enzymatic modification. *Trends in Food Science & Technology* 7(4): 120-125.

Pastoriza, L., Sampedro, G., Cabo, M. L., Herrera, J. J. R. & Bernárdez, M. (2004). Solubilisation of proteins from rayfish residues by endogenous and commercial enzymes. *Journal of the Science of Food and Agriculture* 84(1): 83-88.

Phillips, L. G., Whitehead, D. M. & Kinsella, J. (1994).Introduction to functional properties of proteins. In *Structure–Function Properties of Food Proteins*, 107-109 (Ed L. G. P. M. W. Kinsella). Boston: Academic Press.

Picot, L., Ravallec, R., Fouchereau-Peron, M., Vandanjon, L., Jaouen, P., Chaplain-Derouiniot, M., Guerard, F., Chabeaud, A., LeGal, Y., Alvarez, O. M., Berge, J. P., Piot, J. M., Batista, I., Pires, C., Thorkelsson, G., Delannoy, C., Jakobsen, G., Johansson, I. & Bourseau, P. (2010). Impact of ultrafiltration and nanofiltration of an industrial fish protein hydrolysate on its bioactive properties. *Journal of the Science of Food and Agriculture* 90(11): 1819-1826.

Pihlanto-Leppälä, A. (2000). Bioactive peptides derived from bovine whey proteins: opioid and ace-inhibitory peptides. *Trends in Food Science & Technology* 11(9–10): 347-356.

Pluske, J. R., Hampson, D. J. & Williams, I. H. (1997). Factors influencing the structure and function of the small intestine in the weaned pig: a review. *Livestock Production Science* 51(1-3): 215-236.

Pluske, J. R., Kerton, D. K., Cranwell, P. D., Campbell, R. G., Mullan, B. P., King, R. H., Power, G. N., Pierzynowski, S. G., Westrom, B., Rippe, C., Peulen, O. & Dunshea, F.

R. (2003). Age, sex, and weight at weaning influence organ weight and gastrointestinal development of weanling pigs. *Australian Journal of Agricultural Research* 54(5): 515-527.

Pluske, J. R., Payne, R. L., Williams, I. H. & Mullan, B. P. (2005). Early feeding for lifetime performance of pigs. *Recent advances in animal nutrition* 15.

Pluske, J. R., Williams, I. H. & Aherne, F. X. (1996). Maintenance of villous height and crypt depth in piglets by providing continuous nutrition after weaning. *Animal Science* 62: 131-144.

Pour-El, A. (1981).Protein functionality: classification, definition, and methodology. In *Protein Functionality in Foods*, Vol. 147, 1-19: American Chemical Society.

Ramasubburayan, R., Iyapparaj, P., Subhashini, K. J., Chandran, M. N., Palavesam, A. & Immanuel, G. (2013). Characterization and nutritional quality of formic acid silage developed from marine fishery waste and their potential utilization as feed stuff for common carp cyprinus carpio fingerlings. *Turkish Journal of Fisheries and Aquatic Sciences* 13(2): 281-289.

Rerat, A., Nunes, C. S., Mendy, F. & Roger, L. (1988). Amino-acid absorption and production of pancreatic hormones in non-anesthetized pigs after duodenal infusions of a milk enzymic hydrolysate or of free amino-acids. *British Journal of Nutrition* 60(1): 121-136.

Richards, J. D., Gong, J. & de Lange, C. F. M. (2005). The gastrointestinal microbiota and its role in monogastric nutrition and health with an emphasis on pigs: Current understanding, possible modulations, and new technologies for ecological studies. *Canadian Journal of Animal Science* 85(4): 421-435.

Roberts, P. R., Burney, J. D., Black, K. W. & Zaloga, G. P. (1999). Effect of chain length on absorption of biologically active peptides from the gastrointestinal tract. *Digestion* 60(4): 332-337.

Roura, E., Baldwin, M. W. & Klasing, K. C. (2013). The avian taste system: Potential implications in poultry nutrition. *Animal Feed Science and Technology* 180(1-4): 1-9.

RUBIN (2012).Varestrømsanalyse for 2011. Rubin report series: http://www.rubin.no/images/files/documents/varestrm_2011_nettversjon1.pdf.

Rustad, T., Storro, I. & Slizyte, R. (2011). Possibilities for the utilisation of marine by-products. *International Journal of Food Science and Technology* 46(10): 2001-2014.

Servin, A. L. (2004). Antagonistic activities of lactobacilli and bifidobacteria against microbial pathogens. *FEMS Microbiology Reviews* 28(4): 405-440.

Shahidi, F., Han, X.-Q. & Synowiecki, J. (1995). Production and characteristics of protein hydrolysates from capelin (Mallotus villosus). *Food Chemistry* 53(3): 285-293.

Shahidi, F. & Synowiecki, J. (1997). Protein hydrolyzates from seal meat as phosphate alternatives in food processing applications. *Food Chemistry* 60(1): 29-32.

Shi, P. & Zhang, J. (2006). Contrasting modes of evolution between vertebrate sweet/umami receptor genes and bitter receptor genes. *Molecular Biology and Evolution* 23(2): 292-300.

Shiraga, T., Miyamoto, K.-I., Tanaka, H., Yamamoto, H., Taketani, Y., Morita, K., Tamai, I., Tsuji, A. & Takeda, E. (1999). Cellular and molecular mechanisms of dietary regulation on rat intestinal H+/peptide transporter PepT1. *Gastroenterology* 116(2): 354-362.

Šližytė, R., Daukšas, E., Falch, E., Storrø, I. & Rustad, T. (2005a). Characteristics of protein fractions generated from hydrolysed cod (Gadus morhua) by-products. *Process Biochemistry* 40(6): 2021-2033.

Šližytė, R., Daukšas, E., Falch, E., Storrø, I. & Rustad, T. (2005b). Yield and composition of different fractions obtained after enzymatic hydrolysis of cod (Gadus morhua) by-products. *Process Biochemistry* 40(3–4): 1415-1424.

Slizyte, R., Johnny, V. N., Turid, R. & Storrø, I. (2004a). Hydrolysis of cod (Gadus morhua) by-products: influence of initial heat inactivation, concentration and separation conditions. *Journal of Aquatic Food Product Technology* 13(2): 31-48.

Slizyte, R., Mozuraityte, R., Martinez-Alvarez, O., Falch, E., Fouchereau-Peron, M. & Rustad, T. (2009). Functional, bioactive and antioxidative properties of hydrolysates obtained from cod (Gadus morhua) backbones. *Process Biochemistry* 44(6): 668-677.

Slizyte, R., Nguyen, J. V., Rustad, T. & Storro, I. (2004b). Hydrolysis of Cod (Gadus morhud) By-Products. *Journal of Aquatic Food Product Technology* 13(2): 31-48.

Šližytė, R., Rustad, T. & Storrø, I. (2005c). Enzymatic hydrolysis of cod (Gadus morhua) by-products: Optimization of yield and properties of lipid and protein fractions. *Process Biochemistry* 40(12): 3680-3692.

Sovik, S. L. & Rustad, T. (2005). Proteolytic activity in byproducts from cod species caught at three different fishing grounds. *Journal of Agricultural and Food Chemistry* 53(2): 452-458.

Thamotharan, M., Bawani, S. Z., Zhou, X. & Adibi, S. A. (1999). Functional and molecular expression of intestinal oligopeptide transporter (Pept-1) after a brief fast. *Metabolism* 48(6): 681-684.

Thorkelsson, G., Sigurgisladottir, S., Geirsdottir, M., Johannsson, R., Guerard, F., Chabeaud, A., Bourseau, P., Vandanjon, L., Jaouen, P., Chaplain-Derouiniot, M., Fouchereau-Peron, M., Martinez, A. O., LeGal, Y., Ravallec, R., Picot, L., Berge, J. P., Delaonny, C., Jakobsen, G., Johansson, I. & Batista, I. (2008).Mild processing techniques and development of functional marine protein and peptide ingredients. In *Improving seafood products for the consumer*(Ed T. Borresen).

Tucker, J. L., Naranjo, V. D., Bidner, T. D. & Southern, L. L. (2011). Effect of salmon protein hydrolysate and spray-dried plasma protein on growth performance of weanling pigs. *Journal of Animal Science* 89(5): 1466-1473.

Uni, Z. (1998). Impact of early nutrition on poultry: Review of presentations. *Journal of Applied Animal Research* 7: 452-455.

Uni, Z., Ganot, S. & Sklan, D. (1998). Posthatch development of mucosal function in the broiler small intestine. *Poultry Science* 77(1): 75-82.

Uni, Z., Noy, Y. & Sklan, D. (1999). Posthatch development of small intestinal function in the poult. *Poultry Science* 78(2): 215-222.

Van Immerseel, F., Rood, J. I., Moore, R. J. & Titball, R. W. (2009). Rethinking our understanding of the pathogenesis of necrotic enteritis in chickens. *Trends in Microbiology* 17(1): 32-36.

van Milgen, J. & Dourmad, J. Y. (2015). Concept and application of ideal protein for pigs. *J Anim Sci Biotechnol* 6(1): 15.

Vente-Spreeuwenberg, M. A. M., Verdonk, J., Koninkx, J., Beynen, A. C. & Verstegen, M. W. A. (2004). Dietary protein hydrolysates vs. the intact proteins do not enhance mucosal integrity and growth performance in weaned piglets. *Livestock Production Science* 85(2-3): 151-164.

Vente-Spreeuwenberg, M. A. M., Verdonk, J., Verstegen, M. W. A. & Beynen, A. C. (2003). Villus height and gut development in weaned piglets receiving diets containing either glucose, lactose or starch. *British Journal of Nutrition* 90(5): 907-913.

Wagner, C. & Bregendahl, K. (2007).Effect of dietary salmon protein concentrate on growth performance of broiler chickens. In *Animal Industry Report: AS 653,ASL R2220.*, Vol. AS 653http://lib.dr.iastate.edu/ans_air/vol653/iss1/46: Iowa State University.

WHO/FAO/UNU (2007).Protein and amino acid requirements in human nutrition : report of a joint WHO/FAO/UNU expert consultation. In *WHO Technical Report Series*, Vol. 935.

Wu, G. (2014). Dietary requirements of synthesizable amino acids by animals: a paradigm shift in protein nutrition. *Journal of Animal Science and Biotechnology* 5(1): 34-46.

Wu, G. D., Chen, J., Hoffmann, C., Bittinger, K., Chen, Y.-Y., Keilbaugh, S. A., Bewtra, M., Knights, D., Walters, W. A., Knight, R., Sinha, R., Gilroy, E., Gupta, K., Baldassano, R., Nessel, L., Li, H., Bushman, F. D. & Lewis, J. D. (2011). Linking long-term dietary patterns with gut microbial enterotypes. *Science (New York, N.y.)* 334(6052): 105-108.

Yegani, M. & Korver, D. R. (2008). Factors Affecting Intestinal Health in Poultry. *Poultry Science* 87(10): 2052-2063.

Zhao, X.-T., McCamish, M. A., Miller, R. H., Wang, L. & Lin, H. C. (1997). Intestinal transit and absorption of soy protein in dogs depend on load and degree of protein hydrolysis. *The Journal of Nutrition* 127(12): 2350-2356.

Zhou, S. F., Sun, Z. W., Ma, L. Z., Yu, J. Y., Ma, C. S. & Ru, Y. J. (2010). Effect of feeding enzymolytic soybean meal on performance, digestion and immunity of weaned pigs. *Asian-Australasian Journal of Animal Sciences* 24(1): 103-109.

Zwarycz, B. & Wong, E. A. (2013). Expression of the peptide transporters PepT1, PepT2, and PHT1 in the embryonic and posthatch chick. *Poultry Science* 92(5): 1314-1321.

Åkerstedt, J., Oropeza, M., Sunde, M., Tønnesen, R. & Mork, J. (2013).Sykdom og dødsårsaker hos smågris i intensivt jordbruk på Jæren (Smågrisprosjektet RFF VEST). *Husdyrforsøksmøtet 2013* http://www.umb.no/statisk/husdyrforsoksmoter/2013/10_4.pdf.

Papers I-IV

Paper I

Simple technologies for converting rest raw materials of Atlantic salmon (Salmo salar) into valuable and tasty feed ingredients

Rasa Slizyte[a*], Margareth Opheim[b,c], Ivar Storrø[a], Hallgeir Sterten[b],

[a]SINTEF, Fisheries and Aquaculture, Trondheim, Norway

[b]Felleskjøpet Fôrutvikling AS, Trondheim, Norway

[c]Department of Animal and Aquacultural Sciences, Norwegian University of Life sciences, Norway

* Corresponding author, e-mail address: rasa.slizyte@sintef.no

Abstract

Different fresh rest raw materials (viscera, heads and frames) from farmed Atlantic salmon (*Salmo salar*) were differently pretreated and hydrolyzed with the use of commercial (Protamex, Papain/Bromelain mixture) and endogenous enzymes. More viscera in the starting material enhance the bitterness in the hydrolysate, and increase the amount of endogenous enzymes which influences kinetics and extent of hydrolysis. Solubilisation by addition of commercial enzymes is not always more efficient and economically beneficial, but can be used to improve the taste and to release oil from hydrolysate fraction. Denatured proteins are difficult to hydrolyze and obtained hydrolysate taste more bitter compared to other hydrolysis treatments.

Separation of high quality oil before hydrolysis by mild thermal treatment does not influence hydrolysate yield, and decreases the concentration of lipids in hydrolysates. The initial oil separation also increases productivity of the hydrolysis reactor due to reduction of hydrolysis volume.

Keywords: Enzymatic hydrolysis; Salmon; Viscera; Rest raw material; Bitterness, Feed

Introduction

Norway farmed 1.3 million tons of Salmon in 2013 [1] and from that 333 000 ton of rest raw materials (both salmon and trout) were collected. Viscera and trimmings accounted for about 50% of the rest raw materials, while heads and backbones made for about 7% and 8% respectively [1]. Profitable utilization of fish rest raw materials is a hot topic for both research and fishery industry. Many studies on rest raw materials as well as practical industrial

application have been performed during the last decades. The knowledge of potential functional products from the rest raw materials has evoked an interest from a wide range of scientists developing pharmaceuticals and other "high value products" [2, 3]. But still, most of the salmon rest raw materials today are utilized for production of silage (approx. 55%) and oil (approx. 25%) and only approx. 5 % is used for production of protein isolates and hydrolysates [4].

Silage is often processed further into different products, such as oil and protein concentrate and used as feed ingredients for monogastric animals, as pigs, poultry, pets, fur animals and farmed fish. Silage is obtained by reducing pH in the rest raw material to approximately 4.0 and storing the material for a certain time at ambient temperature [5]. In fish silage, the endogenous proteases promote hydrolysis and oil separation [6]. During silage preparation an aqueous phase rich in small peptides and free amino acids is formed. The solubilisation of fish tissue in traditional silage is a time consuming process which lasts over several days [7] or weeks [8]. In addition, it is difficult to control the silage process and the final product has bitter taste [6, 7]. Lipid oxidation leads to undesirable changes in flavor, color, and nutritional value. Due to process conditions and formation of covalent bonds between lipid oxidation products and proteins some amino acids like cysteine, tryptophan, methionine, histidine and proline will be reduced or lost [9-11]. Potential formation of biogenic amines can also be mentioned as undesirable process in silage production over time [12].

Controlled enzymatic hydrolysis with added commercial enzymes is a technological solution, which can be applied in order to recover both valuable fish oil and proteins from rest raw materials in a mild and reproducible way. In addition, enzymatically hydrolyzed proteins often have better bioactive properties compared to silage proteins and can be used in different diet formulations to improve immune function and weight management [13] and exhibit other health beneficial properties [2]. One of the shortcomings of produced fish protein hydrolysates (FPH) is the bitter taste. The differences in bitter taste preferences between species can hinder successful application of hydrolysates in feed formulations. Birds have a low number of taste buds and T2R-genes, associated with bitter taste, compared to humans and pigs [14, 15] and it is unknown if they recognize all bitter compounds identified by humans [14]. It is, however, shown that chickens perceive bitter taste for compounds like quinine hydrochloride [16] and saccharine [17]. Additionally, the sensation of bitter taste varies within commercial chicken breeds [16] making feed refusal difficult to foresee. Piglets are known to be sensitive to several components humans will find bitter and the inter-

individual variation appears to be large for some components [18]. Piglets, however, have large preferences for sweeteners as i.e. sucrose and xylitol [19, 20] making it possible to enhance palatability for piglets in diet formulations.

The possible sources of bitter taste may be the composition of starting material or the hydrolysis process itself [21]. To define the sources and origin of the bitterness and to find the critical technological points influencing the tastes of hydrolysates is a key issue for industrial application when managing taste development. Optimal composition of rest raw material as well as adjusted processing conditions would prevent or reduce undesirable taste in the protein containing fractions. Better sensory properties would allow to use hydrolysates in several applications as it is well documented that hydrolysates have good functional and bioactive [22] properties and have high nutritional value [23, 24]. The non-soluble fraction after hydrolysis can constitute a significant part, which contains a high percentage of protein from the raw material. It is shown to have a higher protein efficiency ratio (PER) than FPH (the soluble part), containing a high amount of lipids (up to 50%), including a high concentration of phospholipids (up to 60% of lipids) and might be a bulk product for further processing and purification of phospholipids, however, it can be an interesting feed ingredient "as it is" [3].

Rest raw materials like heads, bones, cut-offs and damaged fillets can be used for production of fish protein hydrolysates and marine oil for human consumption. However viscera or parts of viscera are often considered as undesirable raw materials for human consumption, mostly due to ethical considerations. Fish viscera contain parts like stomach, liver, kidney, gall bladder, intestines - all parts considered to have a high enzymatic activity. Under uncontrolled conditions and at elevated temperature these enzymes will lead to decomposition and reduced quality immediately after loss of integrity. Due to the mentioned factors, viscera are defined as a perishable raw material difficult to handle. At the same time, viscera can be considered as a raw material rich in nutritionally valuable peptides and amino acids [23] that contain active endogenous enzymes which could be employed for the hydrolysis and solubilizing of proteins. In addition, viscera contain high percentage of valuable lipids which are inert material during protein hydrolysis and may be extracted before hydrolysis.

The main aim of this study was to evaluate kinetics and yields of the hydrolysis process, quality of the oil and hydrolysate when different Atlantic salmon (*Salmo salar*) rest raw materials were hydrolyzed. Different process conditions as well as two different commercial

enzymes were tested. Hydrolysis with and without endogenous enzymes was also investigated.

Material and methods

Rest raw material

Whole fresh salmon, heads and frames (backbone from the fileting line) were taken from an industrial slaughtering line (Salmar ASA, Norway), packed separately in plastic and stored in crushed ice in styrofoam boxes. The raw materials were transported (6 hours) to SINTEF laboratories (SeaLab, Trondheim, Norway) by thermo transport and kept in a dark cold room on ice (4 ^{o}C). The fish, weighing 4.5 to 4.9 kg, heads and frames were delivered every other day. The raw materials were processed either 24 or 48 hours after leaving the slaughtering line. Viscera were collected from the whole salmon immediately before mincing. Viscera, heads and frames were minced separately in a Hobart mincer using 10 mm holes to produce a homogenous batch of each rest raw material. The minced raw materials were kept on ice (max 1 hour) until further processing. Minced heads (50 %, calculated on wet weight basis) and minced frames (50%), was mixed to ensure that the composition of the head and frames would be representative for later processing in a larger scale.

Enzymes and chemicals

Papain FG (powder on maltodextrin, from *Carica papaya*, cysteine protease with broad specificity, 500 TU/mg) and Bromelain (powder on maltodextrin, *Ananas comosus,* sulfhydryl protease with broad specificity, 400 GDU/g) (both from Enzybel Intl.s.a., Belgium) and Protamex (*Bacillus protease from* Novozymes A/S, Bagsvaerd, Denmark) were used for the hydrolysis. Protamex was kindly delivered by Novozymes and comply with the recommended purity specifications for food-grade enzymes given by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the Food Chemicals Codex (FCC). Methanol, chloroform, formaldehyde hexane, diethyleter, and formic acid (Merck, Darmstad, Germany) were used for the chemical analysis. All chemicals used were of reagent grade.

Hydrolysis experiments

Two different rest raw materials were used for hydrolysis: 1) mixture of viscera, heads and frames (VHF): 50:25:25% (based on wet weight basis) and 2) viscera (V) 100% (Figure 1). The hydrolysis was performed in a 4 l closed glass vessel placed in a water bath (52^{o}C) and stirred with a propeller (diameter 10 cm, approx. 80 rpm) to ensure the homogeneity of mixture during the whole hydrolysis. The experiment was performed with adding warm (50

ºC) distilled water (1:1 of raw material mass). The hydrolysis mixture was warmed up (35-45 min) in warm water batch (54°C) until the temperature of the mixture reached 52ºC. Then enzymatic hydrolysis was initiated by adding either 0.1% Protamex (by wet weight of raw material mixture) or 0.05%+0.05% of Papain and Bromelain respectively (Hydrolysis No 1 - 4 in Figure 1). Protamex was chosen as a commonly used enzyme in fish industry and mixture of Papain/Bromelain is shown to give good sensory properties of hydrolysates [25]. All enzymes were dissolved in water to ensure easy and complete addition into the hydrolysis mixture. For hydrolysis kinetic studies representative sample from each hydrolysis were taken after 30, 60, 90 and 120 min of hydrolysis. Enzymatic activity in the samples was inactivated by microwave heating for 5 min at 90ºC. The mixtures were cooled down to room temperature, then placed in 50 ml graduated centrifuge tubes (NUNC) and centrifuged at 6500*g for 10 minutes. After centrifugation, the tubes were put upright in a freezer (-80ºC) and all fractions: oil, emulsion, fish protein hydrolysate (FPH) and sludge were separated by cutting the frozen content of the tubes. The FPH fractions were freeze-dried. Several control hydrolyses were performed with 100% viscera as a raw material. Hydrolysis No 5 (Figure 1) was performed with only endogenous enzymes (without addition of commercial enzymes). In hydrolysis No 6 endogenous enzymes were inactivated by heating viscera up to 70°C and kept at this temperature for 5 min. Then the hydrolysis was performed at 52°C by adding 0.05%+0.05% of Papain/Bromelain. In hydrolysis experiment No 7 most of the oil from viscera was separated before hydrolysis after heating the viscera up to 40°C (20 min to reach and 5 min to keep the temperature of 40°C). Oil was separated by centrifugation (2250×g, 10 min) and rest material was hydrolyzed by adding 0.05%+0.05% of Papain/Bromelain. The hydrolysis process as well as inactivation of enzymes and separation of different fractions were performed as described above. All hydrolysis trials were performed in two replicates.

Chemical analyses

The moisture content in the samples was determined gravimetrically after drying at 105 ^{0}C until constant weight of samples was achieved (typically 24 h). Ash content was determined after heating dry samples at 550^{0}C for 20 h. Total nitrogen (N), determined by CHNS-O elemental combustion system (Costech Instruments ECS 4010) and crude protein was estimated by multiplying total N by a factor of 6.25. The measurements were performed in four parallels. The total lipid content was determined gravimetrically and expressed as g lipid/g sample material wet weight or dry weight by the method of Bligh and Dyer [26]. For

the crude protein fraction, the extraction of lipids was performed on freeze-dried material. The analyses were performed in duplicates.

Peroxide value (PV)

PV in the oil samples was determined by the iodometric titration method described in a titration application issued by Radiometer Analytical SAS (Villeurbanne Cedex, France). The titration end point was assessed potentiometrically, using TitraLab980 automatic titrator (Radiometer Analytical SAS, Copenhagen, Denmark), coupled with a single platinum electrode (M21Pt, Radiometer Analytical ASA, Copenhagen, Denmark) and a reference electrode (REF 921, Radiometer Analytical ASA, Copenhagen, Denmark). The analysis was performed with 4 – 6 parallels, and the results are expressed in meq/kg oil as a mean value ± standard deviation of the mean.

Free fatty acid (FFA)

FFA content in oil samples was analyzed according to a procedure proposed by Bernardez et al. [27]. Isooctane was used as a solvent for lipids, instead of cyclohexane. Each sample was measured in four parallels. Standard curve prepared with oleic acid standard (0 – 20 μmol) was used for FFA content calculation. The results are expressed as % of oleic acid ± standard deviation.

Degree of hydrolysis

The degree of hydrolysis was evaluated as the proportion (%) of α-amino nitrogen with respect to the total N in the sample [28]. Analyses were performed in duplicate.

Gel filtration of proteins (FPLC)

Dry hydrolysate powders were dissolved in 0.05 M Na-acetate buffer (pH=5.0), filtered (0.22μm) and separated on FPLC column (Superdex ® Peptide 10/300 GL). The flow rate was 0.5 ml/min, and the speed of recorder was 0.5 m/min. The standards used were Cytochrome c (M_w =12 384), Aprotinin (M_w =6 512), Vitamin B_{12} (M_w =1 356), Cysteine (M_w =121).

Amount of free amino acids composition by high pressure liquid chromatography (HPLC)

The content of free amino acids in the fish protein hydrolysates was determined according to Osnes and Mohr [29]. Protein was precipitated using sulfosalicylic acid, the supernatant was diluted using doubly distilled water, filtered through 0.2 μm pore size filters. Reversed phase HPLC by precolumn fluorescence derivatization with o-phthaldialdehyde (SIL-9A Auto Injector, LC-9A Liquid Chromatograph, RF-530 Fluorescence HPLC Monitor, Shimadzu,

Japan) was performed using a Nova-Pak C18 cartridge. Amino acid concentrations were determined once in each extract. Glycine/arginine and histidine/serine/glutamine were determined together, as their peaks merged.

Sensory analysis

Ranking test was used for evaluation of bitter taste in freeze dried FPH. The participants (12 persons) in the sensory panel were selected among the SINTEF Fisheries and Aquaculture (Norway) employees on the basis of their threshold level for bitter taste after training, using caffeine solutions (0%, 0.006%, 0.014% and 0.027% in water). Freeze dried fish protein hydrolysate (FPH) and stick water powders (soluble protein fraction before hydrolysis) were dissolved in water to concentration of 0.2 % and presented for panelists evaluation. A ranking test was used and the bitterness of 6 different samples was evaluated in range from 1 (the least bitter sample) up to 6 (the most bitter sample). The samples were served in a completely random order. Water was provided between samples to cleanse the palate.

Statistical analysis

Microsoft Excel was used for data processing and statistical analysis. Data were subjected to analysis of variance (ANOVA). Means were accepted as significantly different at 95% level ($p<0.05$).

Results and discussion

Yield, recovery and composition of FPH

Rest raw material used for hydrolysis (viscera, heads and frames) varied in chemical composition and all had lipids as the major component in the dry material (Table 1). High lipid content is a typical situation for fish rest raw materials especially when viscera make up a significant amount of the mixture [30]. Due to the high amount of lipids in the starting material, one of the hydrolysis was performed after separation of oil (V-oil ePaBr, Figure 1). An expected outcome of this technological solution would be better oil quality (Table 2) and increased productivity of the hydrolysis reactor due to lower hydrolysis volume. Oil quality evaluated by peroxide values (PV) and amount of free fatty acids (FFA), indicated that oil after hydrolysis of mixed raw material (VHF) had better quality compared to oil obtained after hydrolysis of viscera (V): Table 2. Viscera contain more active lipases compared to other fish rest raw materials [31] and this leads for higher amount of free fatty acids in oil obtained only from viscera (Table 2). Thermal treatment before hydrolysis (V PaBr) produced the oil with the lowest amount of FFA. This proves that lipase activity in the raw material was

greatly reduced by heating to 70 ^{o}C for 5 min. The oil separated before hydrolysis also contained low amount of FFA, which was significantly lower compared to the oil separated from the same raw material after hydrolysis (V-oil ePaBr).

Amount of primary oxidation products measured as peroxide value (PV) was also lower in oil after hydrolysis of mixed raw material (VHF) compared to oil obtained after hydrolysis of viscera (V). The oil separated before hydrolysis was significantly less oxidized compared to the oil separated from the same raw material after hydrolysis (V-oil ePaBr). These results suggest that in order to obtain high quality oil, one way could be to separate the oil before hydrolysis.

The kinetics of hydrolysis was influenced by both composition of raw material and hydrolysis time. The amount of separated oil after hydrolysis reflected the amount of oil in raw material (Table 1, Table 3). "Lean" raw material (VHF) and oil reduced viscera (V-oil ePaBr) gave significantly less oil compared to hydrolysis of viscera alone. Amount of liberated oil increased with hydrolysis time and was not influenced by the type of enzyme added (Table 3). Endogenous enzymes were less effective to separate oil compared to hydrolysis where endogenous enzymes were used together with Protamex and Papain/Bromelain mixture or only Papain/Bromelain mixture. Initial heating of raw material (70^{o}C, V PaBr) also gave less oil, probably due to formation of protein – lipid complexes which are more resistant to enzymatic hydrolysis and ended in the protein containing fractions (Table 5).

Approximately 0.5 g of dried material was found in the emulsion fraction which was slightly reduced with the time of hydrolysis. Hydrolysis of thermally treated viscera and hydrolysis after initial oil separation produced more emulsion compared to all other tested hydrolysis. This again can be due to formation of protein – lipid complexes during thermal treatment, which end in the mixed fraction between oil and hydrolysate.

The amount of sediments was reduced by increased time of hydrolysis. Mixture of viscera, heads and frames (VHF) yielded more sediments compared to hydrolysis of viscera alone, probably due to the bones which are not hydrolyzed. Endogenous enzymes were less effective to solubilize sediments compared to hydrolysis where endogenous enzymes were used together with Protamex and Papain/Bromelain mixture. Hydrolysis of heat denatured viscera (V PaBr) gave more sediments (less hydrolysis) compared to the same treatment on fresh and thermally untreated viscera.

High rate of hydrolysis and high yield of solubilized material are two main factors describing the economics of hydrolysis. Different factors influenced the yield and rate of the hydrolysis process (Table 3). Most striking results of the yield are found in the hydrolysis of viscera with heat denatured endogenous enzymes (70 ^{o}C) and subsequent proteolysis with added Papain/Bromelain (V PaBr). No hydrolysis seems to take place after adding the commercial enzymes as seen in the yield in Table 3 and Figure 2 and by the degree of hydrolysis given in Table 6. Summarizing all hydrolyses it was observed that the increase in FPH yields was faster during the first 60 min of hydrolysis at 52°C resulting in 30-60% increase in FPH compared to the subsequent 60 to 120 minutes of hydrolysis (15-35%). The exception was heat treated rest raw material (V PaBr) where no significant hydrolysis was observed and the increase in FPH amount was very low (Table 3) and hydrolysis of viscera with added Papain/Bromelain mixture (V ePaBr), which gave significant increase in amount of FPH (54%) during the last 60 minutes of hydrolysis.

Analysis of FPH data indicates that hydrolysis of viscera, head and frames mixture (VHF) gave more FPH compared to hydrolysis of viscera alone (Table 3). However, this data do not reveal the right picture of protein hydrolysis. As all raw materials contained high amounts of lipids (Table 1), which is inert material for protein hydrolysis, it can significantly influence calculation of yields. Recalculation, based of fat free raw material, gives a different picture of production of FPH (Figure 2): hydrolysis of both raw materials for 60 min gives very similar amounts independent on enzymes used. However, further hydrolysis for the next 60 min (120 min total hydrolysis time) gave significant more solubilized materials when only viscera was used as raw material. Added enzymes did not play any significant role for the yield of hydrolysate. In the viscera experiments, the concentration of endogenous enzymes were twice the amount compared to the VHF hydrolysis, which contained only 50% viscera. The protein concentration was lower in the viscera substrate than in VHF substrate (Table 1), but the VHF substrate contained more easily degradable muscle proteins than viscera, that contained slowly hydrolysable parts of connective tissue like the lining of the guts. As the amount of solubilized proteins is approximately 60 % higher with viscera as substrate, it is fair to assume that kinetics of solubilisation is determined by the endogenous enzyme concentration.

Hydrolysis of viscera with only endogenous enzymes (V e) gave similar yield compared to hydrolysis with added commercial enzymes. The same tendency is observed in a study when using Atlantic cod viscera as raw material [32]. The effect of adding commercial enzymes as Protamex, Papain/Bromelain to raw material containing endogenous enzymes is therefore

questionable. Viscera were collected from salmon that have been starved for minimum 7 days before slaughtering to empty their digestive system. The empty stomach contains proteases at comparable concentrations to feeding salmon [33]. This concentration of endogenous proteases could be sufficient or even more efficient compared to added commercial proteases. It can be concluded that endogenous enzymes can play the same or even more important role for hydrolysis and solubilisation compared to hydrolysis with added commercial enzymes. This is more evident in hydrolysis where only viscera was used as raw material as it is shown that endogenous proteolytic enzymes in viscera are more efficient than in other rest raw materials [34].

In order to control the hydrolysis process, especially when raw material like viscera contains high level of active endogenous enzymes, it is needed to heat the raw material to inactive these enzymes. However, this treatment drastically affects amount of solubilized proteins: amount from inactivated viscera hydrolyzed by mixture of Papain/Bromelain was less than half of the amount obtained with active endogenous enzymes (Table 3, Figure 2). This might be explained by either a lack of activity of the commercial enzymes on heat-denatured proteins or a need for co-operative effect of the endogenous and commercial enzymes. The co-operation theory is not supported by the other hydrolysis experiments on viscera, as the yield is the same for experiments with only endogenous enzymes and in experiments were also commercial enzymes are added. The lack of enzymatic hydrolyzing power on heat denatured proteins is also supported by other hydrolysis works [30, 35]. Protamex could be a better choice for hydrolysis of denatured rest raw materials as Protamex was more effective by hydrolyzing non heat treated rest raw materials (VHF ePr 120 vs VHF ePaBr 120 and V ePr 120 vs V ePaBr 120).

The high FPH yield at the starting point of the hydrolysis, after heating the raw material to 52^{o}C (time=0) is most probably due to the effect of the endogenous enzyme from the raw material. Endogenous enzymes are active during the heating process from 30 ^{o}C up to 52^{o}C [36]. In the experiment where endogenous enzymes were heated to 70^{o}C, the heating time was shorter compared to the time to reach 52 ^{o}C resulting in lower amount of FPH at time zero (Table 3).

Initial separation of oil did not influence the amount of FPH significantly (Figure 2). This indicates that heating of raw material up to 40°C to separate the oil do not denature proteins in a way that can influence the following hydrolysis. Initial oil separation also yields oil of better

quality (Table 2) compared to oil after enzymatic hydrolysis [37]. In addition, the productivity of the hydrolysis reactor will increase due to reduction of hydrolysis volume. The results show that it is possible to remove a substantial amount of high quality oil and still retain the activity of the endogenous enzymes.

The protein recovery (%) expressed as solubilized crude proteins in the hydrolysate fraction in percentage of available protein in the starting material, was lowest for hydrolysate with heat inactivated endogenous enzymes and denatured substrate: V PaBr (Table 4). No measurable increase in protein solubilisation was found during the hydrolysis process when commercial enzymes were added. However, a significant hydrolysis occurred during the heating process from 30 ^{o}C to 52 ^{o}C: samples before hydrolysis contained 41-56% of protein from raw materials in the hydrolysate fractions. This confirms that endogenous enzymes are very active and play a significant role for hydrolysis even at material preparation stage. The solubilisation of protein in the VHF hydrolysis is lower than solubilisation with viscera as sole substrate, most probably due to the higher concentration of endogenous enzymes in the viscera hydrolysis. There was no significant difference between the protein recovery in hydrolysis performed with intact and oil-depleted viscera. This again proves that efficient hydrolysis can be performed after removal of the oil. Crude protein recovery from thermally untreated viscera into FPH fraction varied from 65 to 76 % after 60 min, from 87 to 99 % after 120 min of hydrolysis and was proportionally reflected by FPH yield (Table 3). After 60 min of hydrolysis approximately 65 % of proteins can be recovered from both rest raw materials by technological solutions tested in this study independent on enzyme used. However, recovery after 120 min hydrolysis was higher for hydrolysis where only viscera were used as raw material: 92±7% compared to 75±9% recovery for the mixture of rest raw materials. Hydrolysis of thermally treated viscera (with inactivated endogenous enzymes) gave only 35% recovery and was not increased by the time of hydrolysis. This again proves the importance of endogenous enzymes for the progress of hydrolysis and proves results reported in earlier studies [35] that inactivation of endogenous enzymes leads to denaturation of substrate proteins which becomes more resistant to enzymatic degradation. Mixture of Papain/Bromelain was not effective for hydrolysis of denatured proteins.

Nutritional value of hydrolysates is determined by the amount of proteins and composition of amino acids. The composition of the hydrolysate varied with the extent of hydrolysis (Table 5). The crude protein concentration did not change significantly after the start of the hydrolysis at 52 ^{o}C, with exception of the hydrolysis of oil-reduced viscera (V-oil) and for

hydrolysis after inactivation of endogenous enzymes (V PaBr). V-oil hydrolysates contained higher amount of proteins due to lower amount of oil in hydrolysates, and furthermore, amount of proteins in V PaBr hydrolysates was lower due to higher amount of lipids, which increased due to formation of lipid-protein complexes during thermal inactivation. All hydrolysates from VHF showed lower fat content than hydrolysates from viscera. All hydrolysates had crude protein as the major component (65-80% of dry material) followed by lipids and ash (Table 5).

On contrary to proteins, lipids are not a desirable component in FPH. Even in low amount lipids can oxidize and negatively influence sensory properties in hydrolysates [6]. In general, the amount of lipids in FPH reflected the amount of lipids in starting raw materials: higher amount leads to more lipids in FPH (Table 5). Hydrolysis significantly reduced amount of lipids in FPH probably due to the breakage of bindings between proteins and lipids and releasing both components as pure fractions. As reported earlier [30, 35] thermal inactivation of enzymes before hydrolysis can influence formation of protein - lipid complexes which will lead to increased amount of lipids in the proteins containing fractions. FPH obtained after inactivation of endogenous enzymes contained significantly higher amount of lipids both before and after 60 min of hydrolysis. With the aim to reduce lipid content in the FPH, the initial separation of oil before hydrolysis seems to be a favorable technological option. The soluble protein fraction after separation of oil contained significantly less lipids compared with corresponding hydrolysis without initial oil separation (Table 5).

Extent of protein hydrolysis: degree of hydrolysis, amount of free amino acids and molecular weight distribution

Degree of hydrolysis (DH) of samples before addition of commercial enzymes (time = 0) was generally very high (Table 6). This can be explained by the nature of the raw materials containing high levels of active endogenous enzymes. It is obvious when only viscera were used as raw material and no commercial enzymes were added (V e). Another factor influencing the high measured DH might be shortcomings of the testing method. The method used in this study detects not only α-NH_2 groups from peptides, but also other nitrogen containing compounds and this can interfere with the final result. These N containing components can form a constant background level for all the measurements. Data from DH analysis confirms that thermal inactivation of endogenous enzymes stops the enzymatic activity: these samples (V PaBr) had the lowest DH among the all viscera samples at 0 point. However, initial separation of oil led to increase of DH at 0 point (V-oil ePaBr). This increase

is probably due to the endogenous enzymes which were active at elevated temperatures until the rest raw materials was warmed up for easier separation of oil. A temperature of 40^{o}C would be preferable for separation of oil as at this temperature minor denaturation of proteins occurs, while endogenous proteolytic enzymes has their highest or very closed to their highest activity at this temperature [36, 38]. In order to follow hydrolysis progress as well as the identification of differences among the samples the increase in DH was calculated (Table 6). Comparison of samples with only endogenous enzymes (V e) with the same raw material with added commercial enzymes (V ePr and V ePaBr) shows that increase of DH and hydrolysis rate was not significantly different. This once more confirms the importance of endogenous enzymes for hydrolysis progress and kinetics. Stable DH (without detected increase) for hydrolysis with inactivated endogenous enzymes (V PaBr) proves the assumption that denatured proteins seems to be resistant to hydrolysis by Papain/Bromelain.

High degree of hydrolysis was also found in **molecular weight distribution** data (Figure 3A, Figure 3B and Figure 3C). Most of the peptides in hydrolysates were smaller than approx. 1500 Da with the exception for hydrolysates obtained from raw material with inactivated endogenous enzymes (Figure 3B and Figure 3C). Thermal treatment of rest raw material, even warming of the material up to 70°C, leads to denaturation of proteins and formation of complexes which are very resistant for the following enzymatic hydrolysis (Figure 3B and Figure 3C). Activity and importance of endogenous enzymes is evident in the systems containing viscera in starting raw materials (Figure 3C). Heating to hydrolysis temperature (52^{o}C) plays a significant role for hydrolysis process as "0" point of hydrolysis with active endogenous enzymes have only a small peak representing big peptides while samples with inactivated endogenous enzymes have high peak showing that significant part of peptides in the samples are big peptides (Figure 3A). It is known that endogenous proteolytic viscera enzymes are active up to 65 ^{o}C with their maximum activity at 50 ^{o}C [31, 38] and this must be taken into account when handling fish rest raw materials.

Amount of free amino acids (FAA) in hydrolysates (Table 6) confirms earlier discussed DH and molecular weight distribution results. Already at "0" point of hydrolysis, the samples contains high amounts of free amino acids indicating that significant proteolysis took place before this point. Only FPH obtained from raw material with inactivated endogenous enzymes (V PaBr) contained significant lower amount of free amino acids. Amount of increased free amino acids as results of hydrolysis with added commercial enzymes was smaller compared to increase of free amino acid by the heating and preparation of rest raw materials for

hydrolysis (samples V ePr, V ePaB, V e and V-oil ePaBr compared to V PaBr, Table 6). This confirms that endogenous enzymes from viscera play an important role not only during hydrolysis processes, but also during samples preparation.

DH is a measure of hydrolysis and indication of the concentration of free amino acid is the final product of the same hydrolysis. It should then be expected a correlation between DH and concentration of free amino acids. Analysis of the test data indicates that correlation exists (Table 6). For all experiments, except hydrolysis with heat inactivated endogenous enzyme (V PaBr) the relationship is fairly constant. A small deviation is found in in hydrolysis with viscera where the oil was removed. This could be connected to the oil separation step where endogenous enzymes are active and most probably works more as exopeptidases while addition of commercial enzymes imply more endopeptidase activity. For the other hydrolysates the average ratio is 7.0 ±0.5 (SD), where the free amino acid concentration varies from 212 to 418 mg/g and DH is in the range from 31 to 52%. Total protein concentration is roughly 750 mg/g and concentration of FAA is around 300 mg/g, indicating that 40 % of the proteins are degraded all the way to FAA. With a DH of 45%, the theoretical average peptide length should be 2.5 amino acid per peptide, which corresponds to the above findings.

Bitterness of hydrolysates

The initial composition of raw materials is important for the taste of final product (Figure 4). Starting material composed of viscera, heads and frames had significantly lower median of bitterness compared to samples where only viscera was the starting raw material. The same tendency was also observed in the FPH after 60 min of hydrolysis. The importance of the composition of the fish raw material for the bitterness of the final hydrolyzed product is reported earlier [21]. Initial thermal inactivation of endogenous enzymes led to the bitterest samples both before hydrolysis and after 60 min of hydrolysis (Figure 4). Hydrolysis with only commercial enzymes (heat inactivated endogenous enzymes) gave more bitter taste compared to hydrolysates obtained with active endogenous enzymes combined with commercial enzymes. One of the explanations can be the amount of free leucine, which was at the lowest levels in the mentioned samples (19.2 mg free leucine/g crude protein) in FPH compared to 32 – 39 mg free leucine/g crude protein in FPH and other analyzed hydrolysates produced from viscera [39]. Leucine is hydrophobic and bitter amino acid [40], but is several times more bitter when is a part of peptide [41]. Percentage of free leucine in this sample is lower compared to other FPH from viscera and this indicates that more leucine are in the form

of peptides and could lead to a more bitter hydrolysate. The same tendency is observed with the amount of free bitter [40] amino acids: 9 mg free isoleucine/g crude protein in FPH compared to 20-23 mg free isoleucine/g crude protein in FPH; 9 mg free phenylalanine/g crude protein in FPH compared to 21-25 mg free phenylalanine/g crude protein in FPH; 9 mg free methionine/g crude protein in FPH compared to 17-20 mg free methionine/g crude protein in FPH; 19 mg free lysine/g crude protein in FPH compared to 33-39 mg free lysine/g crude protein in FPH; 12 mg free valine/g crude protein in FPH compared to 24-27 mg free valine/g crude protein in FPH. Parallel samples obtained on different days of the hydrolysis (V e0 and Ve0*) indicate the same level of bitterness and conforms the validity of the test even with the relatively wide distribution of evaluated values. As the bitterness of hydrolysate is one of the key factors for their application, a reduction of the bitterness may be obtained by hydrolysis with endogenous enzymes followed by addition of commercial enzymes. Separation of oil before hydrolysis did not influence bitterness of FPH obtained after 60 min of hydrolysis.

Conclusions

Composition together with freshness and quality of raw materials are the key factors for making ingredients from salmon rest raw material containing viscera. Endogenous enzymes are very active and intensively hydrolyze material even during preparation steps. Commercial enzymes can be used to improve the taste or release the oil during hydrolysis. Denatured proteins are difficult to hydrolyze and increase bitterness of hydrolysate. Bitterness is more affected by starting material than the enzymes and hydrolysis conditions.

Initial separation of oil before hydrolysis yields similar amount of FPH, separates significant amount of high quality oil and soluble protein fraction contains less lipids compared to corresponding treatment without oil separation.

Acknowledgement

The authors acknowledge Regional Research Found Midt-Norge (project number 217393) for the financial support in executing this research work. The authors want to thank Rakel Skjellegrind for great assistance during the hydrolysis experiment at SINTEF Sealab.

References

1. Olafsen, T., Richardsen, R., Nystøyl, R., Strandheim, G., and Kosmo, J.P. (2014). Analyse marint restråstoff, 2013: Analyse av tilgang og anvendelse for marint restråstoff i Norge. (Trondheim: SINTEF Fiskeri og havbruk AS).
2. Ngo, D.H., Vo, T.S., Ngo, D.N., Wijesekara, I., and Kim, S.K. (2012). Biological activities and potential health benefits of bioactive peptides derived from marine organisms. Int J Biol Macromol *51*, 378-383.
3. Rustad, T., Storro, I., and Slizyte, R. (2011). Possibilities for the utilisation of marine by-products. Int J Food Sci Tech *46*, 2001-2014.
4. RUBIN (2012). Varestrømanalyse for 2011.
5. Mackie, I.M. (1982). General review of fish protein hydrolysates. Animal Feed Science and Technology *7*, 113–124.
6. Kristinsson, H.G., and Rasco, B.A. (2000). Fish protein hydrolysates: Production, biochemical, and functional properties. Critical Reviews in Food Science and Nutrition *40*, 43-81.
7. Gildberg, A. (1993). Enzymatic Processing of Marine Raw-Materials. Process Biochemistry *28*, 1-15.
8. Mackie, I.M. (1974). Proteolytic enzymes in recovery of proteins from fish waste. Process biochemistry *9*, 12-14.
9. Dapkevicius, M.D.E., Batista, I., Nout, M.J.R., Rombouts, F.M., and Houben, J.H. (1998). Lipid and protein changes during the ensilage of blue whiting (Micromesistius poutassou Risso) by acid and biological methods. Food Chem *63*, 97-102.
10. Ramasubburayan, R., Iyapparaj, P., Subhashini, K.J., Chandran, M.N., Palavesam, A., and Immanuel, G. (2013). Characterization and Nutritional Quality of Formic Acid Silage Developed from Marine Fishery Waste and their Potential Utilization as Feed Stuff for Common Carp Cyprinus carpio Fingerlings. Turk J Fish Aquat Sc *13*, 281-289.
11. De Arruda, L.F., Borghesi, R., and Oetterer, M. (2007). Use of fish waste as silage - A review. Braz Arch Biol Techn *50*, 879-886.
12. Dapkevicius, M.L.N.E., Nout, M.J.R., Rombouts, F.M., Houben, J.H., and Wymenga, W. (2000). Biogenic amine formation and degradation by potential fish silage starter microorganisms. Int J Food Microbiol *57*, 107-114.

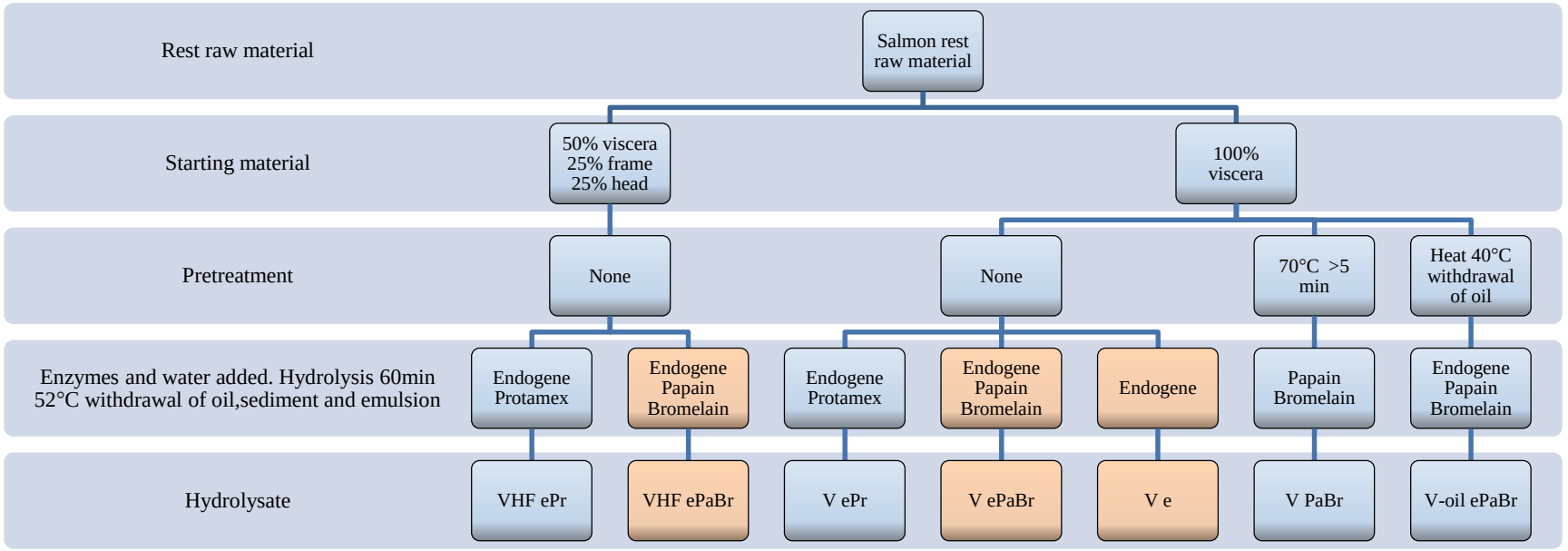
13. Goosen, N.J., de Wet, L.F., and Gorgens, J.F. (2014). The effects of protein hydrolysates on the immunity and growth of the abalone Haliotis midae. Aquaculture *428*, 243-248.
14. Roura, E., Baldwin, M.W., and Klasing, K.C. (2013). The avian taste system: Potential implications in poultry nutrition. Animal Feed Science and Technology *180*, 1-9.
15. Shi, P., and Zhang, J.Z. (2006). Contrasting modes of evolution between vertebrate sweet/umami receptor genes and bitter receptor genes. Mol Biol Evol *23*, 292-300.
16. Kudo, K., Shiraishi, J., Nishimura, S., Bungo, T., and Tabata, S. (2010). The number of taste buds is related to bitter taste sensitivity in layer and broiler chickens. Animal science journal = Nihon chikusan Gakkaiho *81*, 240-244.
17. Balog, J.M., and Millar, R.I. (1989). Influence of the Sense of Taste on Broiler Chick Feed Consumption. Poultry Sci *68*, 1519-1526.
18. Nelson, S.L., and Sanregret, J.D. (1997). Response of pigs to bitter-tasting compounds. Chem Senses *22*, 129-132.
19. Glaser, D., Wanner, M., Tinti, J.M., and Nofre, C. (2000). Gustatory responses of pigs to various natural and artificial compounds known to be sweet in man. Food Chem *68*, 375-385.
20. Hellekant, G., and Danilova, V. (1999). Taste in domestic pig, Sus scrofa. J Anim Physiol an N *82*, 8-24.
21. Dauksas, E., Slizyte, R., Rustad, T., and Storro, I. (2004). Bitterness in fish protein hydrolysates and methods for removal. Journal of Aquatic Food Product Technology *13*, 101-114.
22. Harnedy, P.A., and FitzGerald, R.J. (2012). Bioactive peptides from marine processing waste and shellfish: A review. Journal of Functional Foods *4*, 6-24.
23. Slizyte, R., Dauksas, E., Falch, E., Storro, I., and Rustad, T. (2005). Characteristics of protein fractions generated from hydrolysed cod (Gadus morhua) by-products. Process Biochemistry *40*, 2021-2033.
24. Shahidi, F., Han, X.Q., and Synowiecki, J. (1995). Production and characteristics of protein hydrolysates from capelin (Mallotus villosus). Food Chem *53*, 285-293.
25. Slizyte, R., Grimsmo, L., and Storrø, I. (2011). Prosessering av biråstoff fra sild til olje og proteinhydrolysat. Laboratorieforsøk med ulike proteaser og pilotforsøk med ultraferskt råstoff. Rapport Nr. 189. Volume Rapport Nr. 189. (RUBIN).

26. Bligh, E.G., and Dyer, W.J. (1959). A rapid method of total lipid extraction and purification. Canadian journal of biochemistry and physiology *37*, 911-917.
27. Bernardez, M., Pastoriza, L., Sampedro, G., Herrera, J.J.R., and Cabo, M.L. (2005). Modified method for the analysis of free fatty acids in fish. J Agr Food Chem *53*, 1903-1906.
28. Taylor, W.H. (1957). Formol titration: An evaluation of its various modifications. The Analyst *82*, 488-498.
29. Osnes, K.K., and Mohr, V. (1985). Peptide hydrolases of antarctic krill euphasa superbra. Comparative Biochemistry and Physiology *82B*, 599-606.
30. Slizyte, R., Rustad, T., and Storro, I. (2005). Enzymatic hydrolysis of cod (Gadus morhua) by-products - Optimization of yield and properties of lipid and protein fractions. Process Biochemistry *40*, 3680-3692.
31. Sovik, S.L., and Rustad, T. (2005). Effect of season and fishing ground on the activity of lipases in byproducts from cod (Gadus morhua). Lwt-Food Sci Technol *38*, 867-876.
32. Aspmo, S.I., Horn, S.J., and Eijsink, V.G.H. (2005). Enzymatic hydrolysis of Atlantic cod (Gadus morhua L.) viscera. Process Biochemistry *40*, 1957-1966.
33. Einarsson, S., Davies, P.S., and Talbot, C. (1996). The effect of feeding on the secretion of pepsin, trypsin and chymotrypsin in the Atlantic salmon, Salmo salar L. Fish Physiol Biochem *15*, 439-446.
34. Bower, C., Malemute, C., and Bechtel, P. (2011). Endogenous Protease Activity in by-Products of Pink Salmon (Oncorhynchus Gorbuscha). J Food Biochem *35*, 628-637.
35. Šližytė, R., Van Nguyen, J., Rustad, T., and Storrø, I. (2004). Hydrolysis of cod (Gadus morhua) by-products: influence of initial heat inactivaton, concentration and separation conditions. Journal of Aquatic Food Product Technology *13* 31-48.
36. Sovik, S.L., and Rustad, T. (2005). Proteolytic activity in byproducts from cod species caught at three different fishing grounds. J Agr Food Chem *53*, 452-458.
37. Carvajal, A.K., Šližytė, R., Storrø, I., and Aursand, M. (2015). Production of high quality fish oil by thermal treatment and enzymatic hydrolysis from fresh Norwegian spring spawning herring by-products. Journal of Aquatic Food Product Technology *in press*.
38. Sovik, S.L., and Rustad, T. (2004). Seasonal changes in Trypsin and Chymotrypsin activity in viscera from Cod species. Journal of Aquatic Food Product Technology *13*, 13-30.

39. Opheim, M., Slizyte, R., Sterten, H., Provan, F., Larssen , E., and Kjos, N.P. (2015). Hydrolysis of Atlantic salmon (Salmo salar) rest raw materials – Effect of raw material and processing on composition, nutritional value, and potential bioactive peptides in the hydrolysates. Process Biochemistry *50*, 1247-1257.

40. Lemieux, L., and Simard, R.E. (1992). Bitter Flavor in Dairy-Products .2. A Review of Bitter Peptides from Caseins - Their Formation, Isolation and Identification, Structure Masking and Inhibition. Lait *72*, 335-382.

41. Ishibashi, N., Arita, Y., Kanehisa, H., Kouge, K., Okai, H., and Fukui, S. (1987). Studies on Flavored Peptides .1. Bitterness of Leucine-Containing Peptides. Agr Biol Chem Tokyo *51*, 2389-2394.



591

592 **Figure 1.** Hydrolysis test design. The hydrolysis process was performed at 52°C for 120 minutes. The oil, possible emulsion, water

593 phase and sediment were separated by centrifugation after hydrolysis.

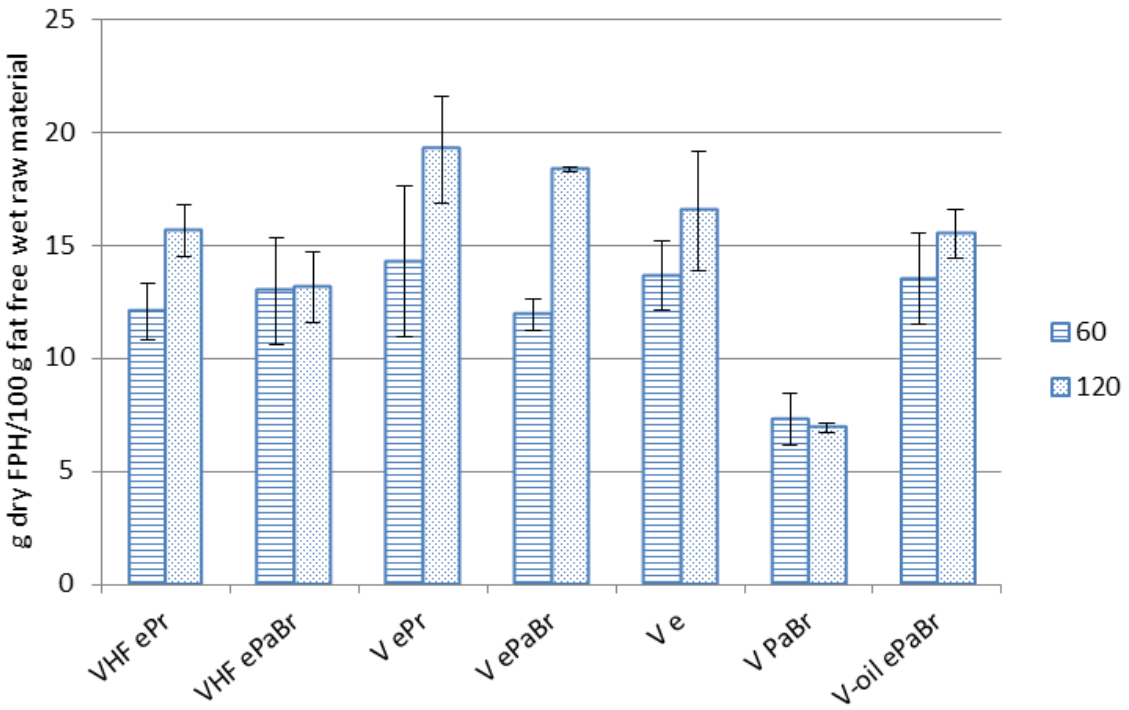
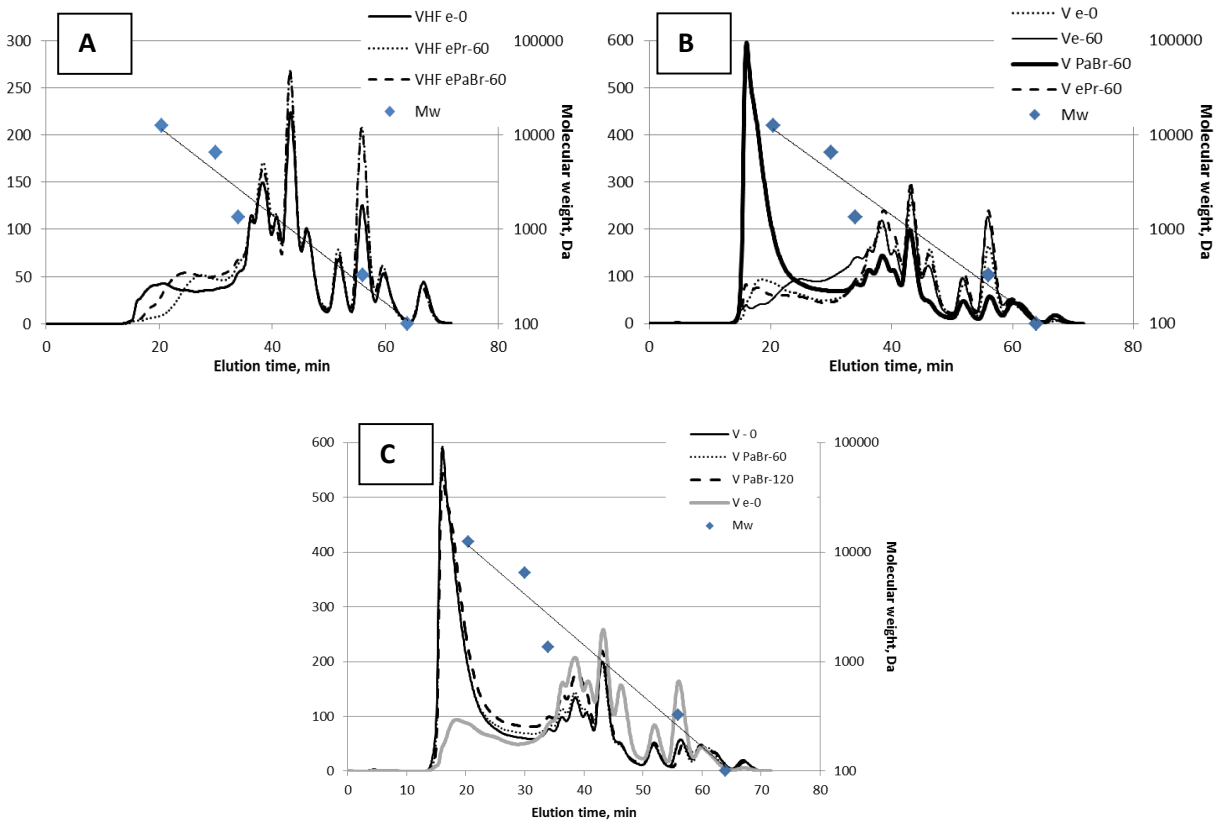
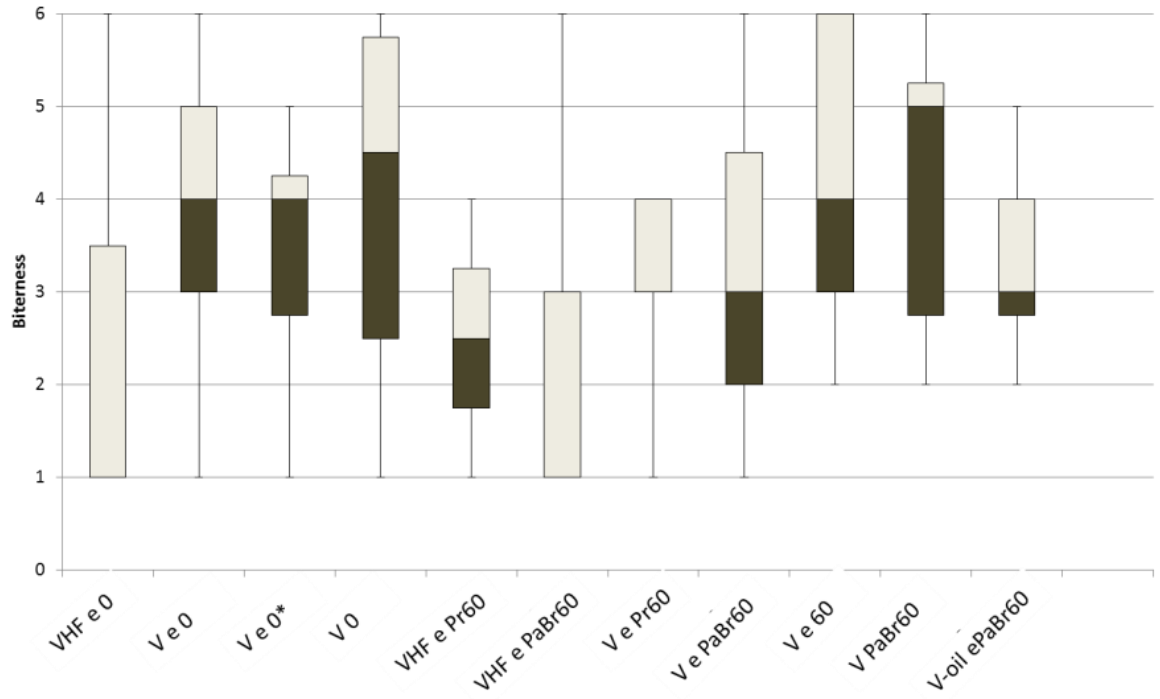


Figure 2. Amount of dry FPH obtained from 100 g wet fat free raw materials obtained after hydrolysis (60 and 120 min) and drying. Values are presented as average ± SD.



597 **Figure 3.** Molecular weight distribution Graph A: the hydrolysates obtained from mixture of
598 viscera, heads and frames (VHF) with active endogenous enzymes (e) hydrolyzed with
599 Protamex (Pr) or mixture of Papain/Bromelain (PaBr). 0 and 60 indicates time of hydrolysis;
600 Graph B: the hydrolysates obtained from hydrolysis of viscera hydrolyzed with endogenous
601 enzymes (e), Protamex (Pr) or mixture of Papain/Bromelain (PaBr). 0 and 60 indicates time of
602 hydrolysis; Graph C: the hydrolysates obtained from hydrolysis of viscera with inactivated
603 endogenous enzymes hydrolyzed with mixture of Papain/Bromelain (PaBr). V e represents
604 samples obtained from viscera with active endogenous enzymes (e) before hydrolysis started.
605 0, 60 and 120 indicates time of hydrolysis.



606

607 **Figure 4.** Relative bitterness of the hydrolysates and stick water (0.2% solution in water)
608 Scale of relative bitterness: 1 (the least bitter sample) up to 6 (the most bitter sample). Box
609 plot presents the median of the measurements, first and third quartiles as well as total
610 distribution of the measurements.

611 **Table 1.** Chemical composition of wet rest raw materials. Values are presented as average ±
612 SD.

Rest raw material	Dry material, %	Lipids, %	Ash, %	Protein, %	Mass balance, calculated (measured)
Frames (F)	42±2	27±1	4±1	15±1	46 (42)
Heads (H)	39±4	22±2	4±1	13±1	39 (39)
Viscera (V)	60±8	44±9	1±0	8±2	53 (60)
VHF (50:25:25)*	50.3	34.3	2.5	11	48 (50)

613 *Calculated based on composition of the three constituting parts.

614

615

616

617 **Table 2.** Quality of oil, obtained after 60 min of hydrolysis and after thermal separation
618 before hydrolysis. The quality markers are peroxide value (PV) and amount of free fatty
619 acids. The values and presented as average ± standard error. Vertical columns of means with
620 different superscripts differ significantly ($p<0.05$).

Treatments of obtained oil	PV (meq/kg)	FFA (%)
VHF ePr	15.0 ± 1.0^{a}	0.74 ± 0.01^{e}
VHF ePaBr	14.3 ± 0.9^{a}	0.74 ± 0.01^{e}
V ePr	16.8 ± 1.4^{ab}	0.92 ± 0.03^{f}
V ePaBr	17.8 ± 1.2^{ab}	0.86 ± 0.01^{f}
V e	18.4 ± 1.4^{b}	0.92 ± 0.01^{f}
V PaBr	17.0 ± 1.8^{ab}	0.24 ± 0.02^{g}
V-oil ePaBr	30.6 ± 2.0^{c}	1.33 ± 0.01^{h}
Oil thermal (V)	6.8 ± 1.4^{d}	0.29 ± 0.06^{g}

621

622 **Table 3.** Yield of oil, dried emulsion, dried FPH and dried sediments obtained from 100 g wet raw materials before hydrolysis (0) and
623 after 60 and 120 min hydrolysis and drying. Liberated oil is determined after separation by centrifugation. Values presented as average
624 ± SD

	Oil			Emulsion			FPH			Sediments		
Hydrolysis time	0	60	120	0	60	120	0	60	120	0	60	120
VHF ePr	22.9±2.6	30.3±1.3	33.9±4.2	0.6±0.3	0.5±0.2	0.4±0.1	5.7±1.2	8.2±0.9	10.6±0.7	15.6±0.6	7.9±0.0	5.0±0.9
VHF ePaBr	22.0±1.3	30.1±0.9	32.4±2.2	0.8±0.1	0.5±0.0	0.3±0.0	5.3±1.5	8.8±1.7	8.9±1.1	13.8±3.4	8.9±0.4	7.5±0.3
V ePr	45.6±6.7	46.7±5.2	45.1±1.8	0.6±0.2	0.4±0.1	0.3±0.1	4.8±0.9	8.0±1.3	10.8±0.6	7.0±2.3	4.7±0.5	3.8±0.1
V ePaBr	42.0±4.2	47.1±5.4	47.8±4.0	0.5±0.3	0.3±0.0	0.3±0.0	6.0±0.3	6.7±0.1	10.4±0.7	7.3±0.4	5.3±0.4	3.9±0.0
V e	39.1±0.2	40.4±2.6	41.9±2.5	0.6±0.0	0.2±0.1	0.4±0.0	5.1±0.8	7.5±0.5	9.0±1.0	8.1±0.2	8.2±1.1	6.7±2.9
V PaBr	35.3±4.8	33.0±2.5	45.9	0.9±0.2	1.1±0.1	1.1±0.1	3.6±0.1	4.0±0.4	3.8±0.3	13.4±2.6	11.3±0.4	6.6±1.7
V-oil ePaBr	10.3	9.0	12.8	0.9±0.2	2.3±0.0	1.2±07	6.0±1.9	7.2±1.4	8.8±0.8	8.7±0.5	7.0±2.2	7.1±2.5

Table 4. Protein recovery (%) for different substrates (VHF and V) and enzymes used (-e, e, Pr and PrBr) at different hydrolysis time. Recovery is calculated from protein in raw material (Table 1) and yield given in Table 3.

	VHF e		V e			V	V-oil e
	Pr	PaBr	Pr	PaBr	Endo	PaBr	PaBr
Before addition of enzymes	41-44		46-56			30	73
After hydrolysis: 60 min	62	69	76	65	73	36	75
After hydrolysis: 120 min	80	70	99	96	87	35	91

Table 5. Chemical composition of fish protein hydrolysates. Numbers given after hydrolysate description represents hydrolysis time in minutes.

Hydrolysate	**Ash,** average±9 % of value	**Lipids,** %	**Crude proteins,** %	**Moisture,** average±14 % of value	**Sum**
VHF e Pr0	10	3,8 ± 1,3	79 ± 1	7	100
VHF ePr60	8	4,2 ± 0,5	77 ± 1	7	97
VHF ePaBr 60	8	3,1 ± 0,4	80 ± 1	7	97
V e 0	10	9,0 ± 1,5	71 ± 1	6	96
V ePr 60	10	8,3 ± 0,5	71 ± 1	6	95
V ePaBr 60	10	7,0 ± 0,3	72 ± 1	6	96
V e 60	8	9,8 ± 1,3	72 ± 1	7	98
V PaBr 0	14	10,8 ± 0,1	63 ± 1	8	95
V PaBr 60	15	10,9 ± 0,2	65 ± 3	7	97
V-oil 0	9	6,4 ± 0,6	74 ± 1	6	96
V-oil ePaBr 60	7	5,7 ± 0,1	77 ± 3	7	97

635 **Table 6.** Correlation between amount of free amino acids (FAA, mg/g hydrolysate) and
636 degree of hydrolysis (DH, %) of the hydrolysates obtained from different raw materials,
637 different treatment and time of hydrolysis.

	FAA, mg/g hydrolysate			DH, %, average ±4% of value			FAA/DH			Average
Hydrolysis time	0	60	120	0	60	120	0	60	120	
VHF ePr	212	342	339	31	43	51	6.8	7.9	6.6	7.1
VHF ePBr	212	288	295	31	41	45	6.7	7.1	6.6	6.8
V ePr	299	344	372	44	50	52	6.8	6.9	7.1	6.9
V ePBr	299	418	421	44	49	52	6.8	8.6	8.1	7.9
V e	260	336	328	44	48	52	5.9	7.0	6.3	6.4
V PBr	136	101	156	37	35	36	3.7	2.9	4.3	3.6
V-oil ePBr	255	312	346	49	52	57	5.2	6.0	6.1	5.8

Paper II

Process Biochemistry 50 (2015) 1247–1257

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

Hydrolysis of Atlantic salmon (*Salmo salar*) rest raw materials—Effect of raw material and processing on composition, nutritional value, and potential bioactive peptides in the hydrolysates

Margareth Opheim[a,d,*], Rasa Šližytė[b], Hallgeir Sterten[a], Fiona Provan[c], Eivind Larssen[c], Nils Petter Kjos[d]

[a] Felleskjøpet Fôrutvikling, N-7018 Trondheim, Norway
[b] SINTEF, Fisheries and Aquaculture, N-7465 Trondheim, Norway
[c] International Research Institute of Stavanger (IRIS), N-4068 Stavanger, Norway
[d] Norwegian University of Life Sciences, N-1432 Ås, Norway

ARTICLE INFO

Article history:
Received 3 February 2015
Received in revised form 27 March 2015
Accepted 22 April 2015
Available online 4 May 2015

Keywords:
Enzymatic hydrolysis
Salmon
Viscera
Rest raw material
Nutritional value
Bioactive motifs

ABSTRACT

This study evaluates the composition, nutritional value, and potential bioactive peptides in hydrolysates obtained from different Atlantic salmon rest raw materials. The effect of enzymes (endogenous enzymes, Protamex, and papain plus bromelain), pretreatments, and industrial-compared to laboratory-scale production was evaluated. Proximal composition of the hydrolysates reflected the composition of the starting material. Removal of oil by slight warming (40 °C) before hydrolysis of viscera did not influence amino acid composition, nutritional value, or peptide analysis. However, excessive heating (70 °C) of viscera before hydrolysis reduced protein content, nutritional value, free amino acids, and the degree of hydrolysis (DH) compared to other viscera hydrolysates produced with papain plus bromelain with active endogenous enzymes. Hydrolysis of viscera with only endogenous enzymes was similar to hydrolysis with Protamex or papain plus bromelain concerning proximal and total amino acid composition, DH, nutritional value, and number of different bioactive motifs in the hydrolysates. However, the number of different peptides and the total number of bioactive motifs was higher when using papain plus bromelain compared to Protamex with viscera-containing starting material. Differences between laboratory- and industrial-scales of production of hydrolysates appear to be mainly due to lower efficiency in the hydrolysis, separating processes, and storage conditions.

1. Introduction

Aquaculture is a large and fast-growing industry in Norway, and the production of farmed Atlantic salmon (*Salmo salar*) reached 1.3 million tons in 2013, producing 336,000 t of rest raw material [1]. As viscera are a major constituent of rest raw material from aquaculture (salmon farming), it is of great importance to consider viscera in order to increase sustainability and to exploit valuable marine proteins and oils. In 2011, about 50% of the rest raw material from aquaculture in Norway was minced and acidified to reach a pH < 4 to produce a semisolid fluid called salmon–silage [2]. Most of the salmon–silage is used as a raw material in animal feed production [1], and is considered to be a valuable source of protein. It is, however, possible to increase the value of salmon rest raw material by more gentle processes than silage production, e.g., controlled enzymatic hydrolysis, thus allowing protein, oils, and minerals to be extracted to supply the market with higher value products with desirable features.

The enzymatic hydrolysis process is achieved by the activity of enzymes at certain temperatures for a defined time. Many factors influence the properties and constituents of the final hydrolysate, e.g., composition and variation of raw materials, specificity, activity and concentration of endogenous and added enzymes, pH, temperature, and time. During the hydrolyzing process, the proteins become more soluble due to cleavage of the peptide bonds between the amino acids, generating peptides with a smaller molecular size and with more ionizable amino and carboxyl groups, which probably contribute to the solubility [3]. After hydrolysis, by using phase separation, it is possible to obtain an oil fraction, possibly an emulsion phase, a protein-rich aqueous phase (hereinafter referred to as the hydrolysate), and an insoluble sludge fraction.

* Corresponding author at: Department of Animal and Aquacultural Sciences, Norwegian University of Life Sciences, P.O. Box 5003, N-1432 Ås, Norway. Tel.: +47 95871070.
E-mail address: margareth.opheim@fkf.no (M. Opheim).

http://dx.doi.org/10.1016/j.procbio.2015.04.017
1359-5113/

The amino acid composition in fish is well balanced, and provides both animals and humans with proteins of high nutritional value. In addition, many of the isolated protein fractions have shown promising effects on health by modulating blood pressure and the immune system, by inhibiting cancer development, and by exerting antioxidant and antibacterial effects, among others [4–6]. The knowledge of these effects has evoked interest from a wide range of scientists exploring the opportunity for producing high-value products by better utilization of the rest raw material [7–11].

Viscera are traditionally considered as a low-value raw material due to high enzymatic activity [12,13] and variable intestinal content and seasonal variations in wild-caught fish [14]. Many of these challenges can be solved or diminished in farmed Atlantic salmon that are slaughtered after fasting and under controlled and hygienic conditions. Together, this makes it possible to utilize fresh rest raw material directly after filleting, and to get the desired stability and composition needed for further controlled processing and for the final products.

Many of the earlier studies of salmon hydrolysates have used rest raw materials without viscera [15–18]. However, hydrolysates from viscera-containing rest raw material have been studied from other species, including pink salmon (*Oncorhynchus gorbuscha*) [14], cod (*Gadus morhua*) [19–21], yellowfin tuna (*Thunnus albacares*) [22], sturgeon (*Acipenser persicus*) [23], herring (*Clupea harrengus*) [24], and sardinella (*Sardinella aurita*) [25] with different aims for the final products.

The nutritional value of hydrolysates can be evaluated by protein content and amino acid composition-however, the molecular size of the peptides, content of free amino acids, and amount of bioactive motifs are important factors for defining the intended application of the hydrolysate. Generally, the goal is to obtain a hydrolysate that is rich in protein with a peptide size <10 kDa, is rich in essential amino acids, and at the same time has a low content of lipids and ash. The number and type of bioactive motifs, with expected bioactivity, may play a crucial role in some applications of protein hydrolysates and other important components, such as taurine and anserine, may be important.

The main aim of this study was to evaluate the composition, nutritional value, and potential bioactive peptides of different hydrolysates produced from viscera-containing salmon rest raw material that was processed with different enzymes and pretreatments. A further goal was to compare laboratory- and industrial-scale production of hydrolysates with respect to the above-mentioned parameters.

2. Materials and methods

2.1. Rest raw material for the laboratory hydrolysis

Fresh, farmed salmon was taken from the slaughtering line (Salmar, Norway) and packed in isophore boxes with ice. Salmon frames and heads from filleting lines (Salmar, Norway) were collected, and packed in isophore boxes with ice separately. Whole salmon, frames, and heads were transported to the SINTEF's SeaLab (Trondheim, Norway) and kept in a cold (4 °C) room until testing. The fish (weight 4.5–4.9 kg) were delivered every other day, and the raw material for hydrolysis was either 1 or 2 days old. Viscera (taken from the whole salmon), frames, and heads were minced in a Hobart mincer using 10-mm holes to produce a homogenous batch of each rest raw material. The minced raw materials were kept on ice (maximum 60 min) until further processing. Two different raw materials for hydrolysis were prepared. The first (VHF) was made of approx. 50% viscera, 25% minced heads, and 25% minced frames (calculated on a wet-weight basis), and the second was 100% viscera (V). This was done to ensure that the composition of the head and frames would be representative for later processing at a larger scale.

2.2. Enzymes and chemicals

Papain (powder on maltodextrin, 500 TU/mg) and bromelain (powder on maltodextrin, 400 GDU/g) (both from Enzybel Intl. SA, Belgium) and Protamex (Novozymes A/S, Bagsvaerd, Denmark) were used for the hydrolysis. Protamex was kindly donated by Novozymes, and complied with the recommend purity specifications for food-grade enzymes of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the Food Chemicals Codex (FCC) [26,27]. Methanol, chloroform, formaldehyde hexane, diethyl ether, and formic acid (Merck, Darmstad, Germany) were used for the chemical analysis. All chemicals used were of reagent grade.

2.3. Hydrolysis

The schematic hydrolysis process is outlined in Fig. 1.

The lab hydrolysis was performed in a 4-l closed glass vessel placed in a water bath (52 °C), and stirred with a marine impeller (approx. 80 rpm) in order to ensure the homogeneity of the mixture during the entire hydrolysis. The experiment was performed

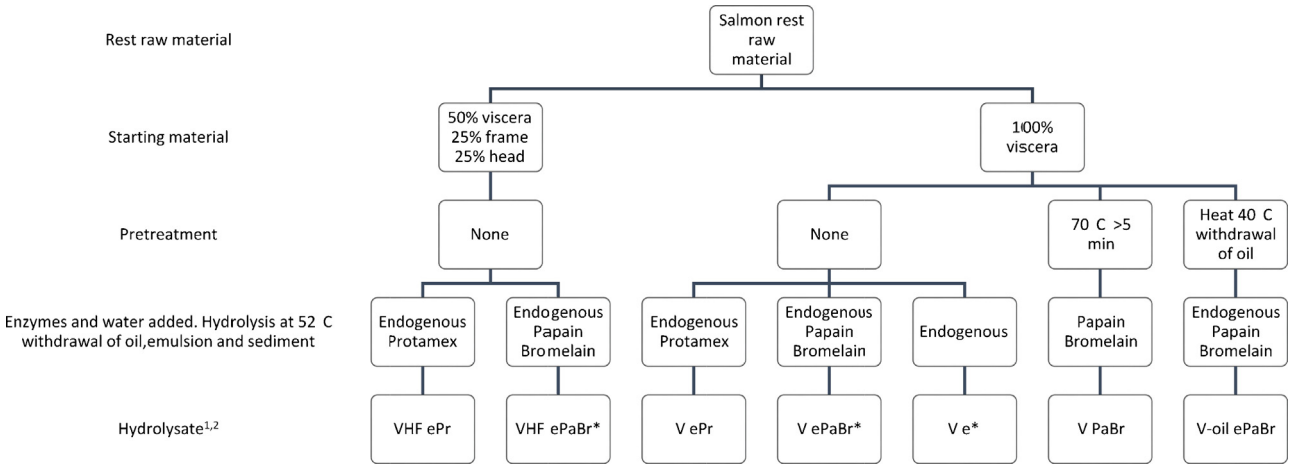


Fig. 1. Hydrolysis trial design. The hydrolysis process ran for 120 min and was sampled after 60 min. The oil, possible emulsion and sediment layers was separated by centrifugation after hydrolysis. * Hydrolysates also produced by an industrial hydrolyzing plant. 1 VHF: Viscera (50%), head (25%) and frame and trimmings (25%). V: viscera (100%). V-oil: viscera (100%) oil separated before hydrolysis. 2 e: endogenous enzymes active. Pr: Protamex (Novozymes A/S, Bagsvaerd, Denmark). Pa: papain (powder on maltodextrin, 500 TU/mg) and Br: bromelain (powder on maltodextrin, 400 GDU/g) (both from EnzybelIntl.s.a., Belgium).

by adding warm (approx. 50 °C) distilled water (1:1 of raw material mass). The enzymatic hydrolysis was started when the temperature of the mixture was 52 °C by adding either 0.1% Protamex (by wet weight of raw material) or 0.05% + 0.05% of papain plus bromelain, respectively (the first four hydrolyses in Fig. 1), and the hydrolysis reaction was incubated for 120 min. Samples were taken after 0, 60, and 120 min, followed by enzyme inactivation by microwave heating for 5 min at 90 °C. The mixtures were cooled to room temperature, then placed in 50-mL graduated centrifuge tubes and centrifuged at 6500 × *g* for 10 min. After centrifugation, the tubes were put upright in a freezer (−80 °C), and all fractions (oil, emulsion, fish protein hydrolysate, and sludge) were separated by cutting the frozen content of the tubes. The fish protein hydrolysate fractions were freeze-dried, and the 60-min samples were used for further analysis in this study.

Three additional hydrolyses were performed with 100% viscera as starting material. The first hydrolysis (V e) was performed with only active endogenous enzymes (without addition of commercial enzymes). Two other hydrolysis reactions were thermally treated before hydrolysis: the first (V PaBr) was heated to inactivate endogenous enzymes (70 °C for minimum 5 min), while the second (V-oil ePaBr) was warmed to 40 °C for 5 min to initiate better oil separation before hydrolysis. Oil separation in V-oil ePaBr was performed after warming before hydrolysis. The latter two hydrolyses were performed by adding 0.05% + 0.05% of papain plus bromelain (wet-weight basis of raw material). The whole hydrolysis process, as well as inactivation of enzymes and separation of different fractions, was performed as described above. All hydrolysis trials were performed twice.

2.4. Industrial-scale production

Three of the hydrolysates from the laboratory trial were selected for production by an industrial plant (Nutrimar A/S, Norway) based on raw material availability, expected nutritional value of the hydrolysate, and overall production costs. Endogenous enzymes were active during the industrial production of hydrolysate. The first production of industrial hydrolysate was from a viscera, head, and frame mixture as the starting material, and was processed with papain plus bromelain (IndVHF ePaBr). The second production used viscera as the raw material, which was hydrolyzed by use of papain plus bromelain together with active endogenous enzymes (IndV ePaBr). The third production was from viscera that were hydrolyzed without addition of commercial enzymes during hydrolysis (IndV e).

The industrial production mimicked the laboratory trials, and used the same type and concentration of enzymes, as well as the same processing parameters, such as time and temperature. For practical reasons, not all technological steps were reproduced at the industrial scale. Time from slaughter to hydrolysis was shorter at the industrial plant (within 120 min) compared to the laboratory, added water was not preheated, separation was conducted by a decanter, and no drying was performed on the hydrolysate. Industrially produced hydrolysates were acidified with formic acid to reach pH < 4.

2.5. Analyses

2.5.1. Protein

The total nitrogen (N) was determined by a CHN-S/N elemental analyzer 1106 (Costech Instruments ECS 4010 CHNSO Analysator), and crude protein (CP) was estimated by multiplying the total N by a factor of 6.25 in the laboratory hydrolysates. Measurements were performed in quadruplicate. For the industrially manufactured hydrolysates, the protein content was measured in duplicate by the Kjeldahl method according to ISO 5983-2 [28].

2.5.2. Water and ash

Content of water and ash in the samples was determined gravimetrically after drying for 24 h at 105 °C until constant weight of the samples was achieved. Ash content was determined according to Association of Analytical Communities [29]. The water and ash content in the industrially manufactured hydrolysates was performed according to ISO 6496 [30] and ISO 5984 [31].

2.5.3. Lipids

The method of Bligh and Dyer [32] was used for the extraction of lipids, and total lipid content was determined gravimetrically. In the laboratory hydrolysates, the extraction of lipids was performed on freeze-dried material while undried samples were used for industrial samples.

2.5.4. Water-soluble protein

Water-soluble protein from salmon protein hydrolysate was extracted with boiling water, filtered through a black ribbon filter paper, and the protein was determined by the Kjeldahl method.

2.5.5. Amino acids

Analysis of total and free amino acids was performed in duplicate after initial preparation from one sample, and as described in Cohen and Michaud [33] and Bidlingmeyer et al. [34].

2.5.6. Protein quality

The equation for the protein efficiency ratio (PER) was adapted from Alsmeyer et al. [35] and Lee et al. [36]. The protein digestibility corrected amino acid score (PDCAAS) was from the report from World Health Organization [37] with digestibility of CP set to 92 as given by the Videncenter for Svineproduktion (VSP) in Denmark [38]. The digestible indispensable amino acid score (DIAAS) was calculated as described in the Food and Agriculture Organization (FAO) report about dietary protein quality evaluation [39] using rooster coefficients from Folador et al. [40] (DIAASa), swine coefficients from VSP [38] (DIAASb), and mink coefficients from Kopenhagen Fur [41] (DIAASc), as ileal digestibility data from humans or newly weaned piglets is not available for salmon protein hydrolysates. The amino acid pattern for children (from 6 months to 3 years) was used for calculations of PDCAAS and DIAAS, as recommended by the FAO expert consultation report from 2013 [39].

2.5.7. Degree of hydrolysis

DH was evaluated as the proportion (%) of α-amino N with respect to the total N in the sample [42], and was measured in duplicate for laboratory samples. For the industrial samples, the *o*-phthaldialdehyde (OPA) method [43] was performed.

2.5.8. Molecular weight

The molecular weight was analyzed by high-performance liquid chromatography (HPLC) as described in Wang-Andresen and Haugsgjerd [44]. In short, Superdex peptide 10/300 GL columns (ID: 10 mm, length: 300 mm; GE Healthcare) were used with a flow-rate of 0.5 mL/min and with 214-nm UV detection. The eluent solutions were according to the manufacturer's recommendations.

2.5.9. Peptides

The peptide analysis was performed at the International Research Institute of Stavanger (IRIS), and was based on the method described by Pampanin et al. [45].

Samples were freeze-dried on arrival and stored at −20 °C. To prepare for analysis, samples were diluted 1:30 (w/v) in distilled water, and filtered with a 10-kDa cut-off filter. Samples were purified and concentrated using ZipTip pipette tips (C_{18}, Millipore, Merck KGaA), and were analyzed with LC–MS/MS. Analysis was performed using an UltiMate 3000 dual-pump nanoflow HPLC

system (Dionex, Sunnyvale, USA) connected to a linear ion trap-orbitrap mass spectrometer (LTQ-Orbitrap XL, Thermo Scientific). Two columns were used in series: a Nanoviper monolithic PS-DVB pre-column (PepSwift 200 μm × 5 mm; Thermo Scientific) and a monolithic PS-DVB PepSwift® analytical column (ID: 100 μm, length: 25-cm; Nano Viper Thermo Scientific). The sample-loading mobile phase was 0.1% formic acid. The separation mobile phase A was 2.5% ACN in 0.1% formic acid, and the separation mobile phase B was 80% ACN and 0.1% formic acid. Loading flow through the pre-column was 2 μL/min during injection, analytical flow was set at 300 nL/min, and the injection volume was 5 μL. The optimized gradient used was a multistep linear gradient between the A and B phases: 0–10 min, 100% A; 10–175 min, 100% to 70% A; 175–185 min, 70% to 0% A; 185–215 min, 0% to 0% A; 215–220 min, 0% to 100% A; and 220–250 min, 100% to 100% A. The MS method was data dependent, using dynamic exclusion-based MS/MS analysis on peptides with two or more charges.

The LC–MS/MS raw data files were analyzed using Proteome Discoverer 1.4 (Thermo Scientific) using the Sequest algorithm to search against the *Salmonidae* database (downloaded from Uniprot 02.01.13, Tax. Id 8015, containing 15,818 sequences). Peptide identification was performed by correlating acquired experimental MS/MS spectra with theoretical spectra predicted for each peptide contained in the protein sequence database. The best scoring peptide spectrum match was considered as the peptide identification and was subjected to statistical validation [46]. Raw data were searched against a decoy database with false-discovery rate strict: 0.01 and relaxed, 0.05 to remove false-positive peptide identifications. Precursor ion tolerance was set to 10 ppm, and fragment ion mass tolerance to 0.8 Da. Oxidation (M) was set as a dynamic modification. The peptide confidence filters were set with the following combinations of X correlation and charge (z): high significance, 1.9 ($z = 2$), 2.3 ($z = 3$), and 2.6 ($z \geq 4$); medium significance, 0.8 ($z = 2$), 1 ($z = 3$), and 1.2 ($z \geq 4$).

The identified peptides were exported to a text file and compared to the sequences in an in-house bioactive database using an algorithm for motif searching. The database was generated through literature searches for bioactive motifs, and thereafter sorted for their different physiological properties (cardiosystem, antioxidant, immunomodulation, and antimicrobial).

2.5.10. Statistical analysis

Data handling and statistics were performed using Microsoft Excel 2010 and JMP 11.1.1 from SAS (SAS Institute Inc., Cary, NC). The evaluation of data was performed by relative standard deviation when comparing groups and one-way analysis of variance (ANOVA). Tukey adjustments were used in the multiple comparison of means when a significant F-test was detected. p-Values <0.05 were considered significant.

3. Results and discussion

3.1. Proximate composition

The proximate composition of different rest raw materials from Atlantic salmon varied significantly, depending on which fraction was selected, and is shown in Table 1.

The CP content in the hydrolysates varied from 69.9% to 86.0% on a dry-matter basis (Table 2), with the lowest content in hydrolysate from viscera that was initially heated (70 °C) before hydrolysis (V PaBr). The highest content of protein was obtained in the hydrolysate from the mixed starting material obtained with added papain plus bromelain in the laboratory test (VHF ePaBr).

Generally, hydrolysates from viscera contained less CP (orthogonal contrast, $p = 0.0004$, HFV vs. V) and more lipids (orthogonal contrast, $p < 0.0001$, HFV vs. V) than hydrolysates from mixed raw material. This reflects the composition of the starting material before hydrolysis, and was also confirmed in the hydrolysate of viscera where the oil was removed by slight warming (40 °C) before hydrolysis (V-oil ePaBr). Šližytė et al. [20] found an inverse linear correlation between lipids in cod rest raw material and protein content in the hydrolysate by use of Neutrase and Flavourzyme. This tendency was also detected for the hydrolysis of salmon viscera-containing rest raw material by use of Protamex and papain plus bromelain. Hence, oil separation before hydrolysis makes it possible to increase the protein content in hydrolysates.

Table 1
Proximate composition of Atlantic salmon (*Salmo salar)* rest raw material. (Values given as % of wet material. Mean values ± SD. $n = 2$).

Raw material	Viscera (V)	Head (H)	Frame (F)	VHF[1]
Dry material	60 ± 8	39 ± 4	42 ± 2	50 ± 6
Crude protein	8 ± 2	13 ± 1	15 ± 1	11 ± 2
Ash	1 ± 0	4 ± 1	4 ± 1	3 ± 1
Lipids	44 ± 9	22 ± 2	27 ± 1	34 ± 5

[1] VHF values are calculated from a 50% plus 25% plus 25% mixture of viscera (V), head (H) and frame (F), respectively, on wet weight basis.

Addition of Protamex or papain plus bromelain compared to only endogenous hydrolysis of viscera (V e) did not increase the protein content in hydrolysates, but significantly decreased the lipid content from 10.5% to 7.4–8.8%. It is well known that proteins and peptides are good emulsifiers and the observed decrease in lipid content in hydrolysates when adding Protamex or papin plus bromelain during hydrolysis of viscera, might be caused by reduced emulsifying properties in these hydrolysates. This might have been influenced by higher total enzymatic activity in these hydrolysates (V ePr and V ePaBr) as endogenous enzymes, in addition to the commercial ones, were active during these hydrolysis. Lipid content was significantly higher in the hydrolysates obtained by use of Protamex compared to hydrolysates obtained by use of papain and bromelain (orthogonal contrast, $p < 0.001$ VHF ePr and V ePr vs. VHF ePaBr and V ePaBr). This might be a significant factor, as quality and stability of hydrolysate depend largely on lipid content and the risk of lipid oxidation. Despite a numerical increase in the protein content in hydrolysates obtained by the use of papain plus bromelain compared to Protamex, the choice of enzyme did not significantly influence the content of CP and ash produced with the same process parameters and starting material. Others have found differences in proximate composition of hydrolysates that are dependent on the enzyme used, and often Alcalase revealed the highest content of protein in the hydrolysates compared to other commercial enzymes [21,47,48]. The proximate composition of hydrolysate is, however, very much dependent on the raw material composition, enzymes used, enzyme activity, and concentration and process conditions. Choice of enzyme is usually a cost/benefit-dependent consideration [49], and depends on the process conditions and the final uses of the hydrolysates.

Inactivation of endogenous enzymes by heat (70 °C) prior to hydrolysis changed the composition of the hydrolysate and significantly reduced the CP (orthogonal contrast $p = 0.01$ V ePaBr vs. V PaBr) and increased the lipid content (orthogonal contrast $p < 0.001$ V ePaBr vs. V PaBr) in the hydrolysates. The reduced efficiency of hydrolysis after excessive heat pretreatment has also been described by others [21,50,51], and is hypothesized to be due to conformational changes in the protein structure [52,53] or formation of lipid–protein complexes in the hydrolysate [21] making it resistant to enzymatic breakdown, in addition to reduced endogenous proteolytic activity.

The industrial hydrolysates contained less CP and ash but more lipids compared to the hydrolysates produced in the laboratory from the same raw material. This might be the result of a less

Table 2
Proximate composition of laboratory and industrial hydrolysates from Atlantic salmon (*Salmo salar*) rest raw material (g per 100 g DM hydrolysate. Statistical testing for laboratory hydrolysates. Laboratory: $n = 2$, Industrial $n = 1$).

Raw material[1]	VHF	VHF	V	V	V	V	V-oil		ANOVA	Ind VHF	Ind V	Ind V
Enzyme[2]	ePr	ePaBr	ePr	ePaBr	e	PaBr	ePaBr	s.e.m. [3]	*p*-Value	ePaBr	ePaBr	e
Crude protein	82.8^{ab}	86.0^{a}	75.5^{bc}	76.6^{bc}	77.4^{bc}	69.9^{c}	82.8^{ab}	1.4	<0.001	78.8	71.9	73.0
Ash	8.6^{c}	8.6^{c}	10.6^{b}	10.6^{b}	8.6^{c}	16.1^{a}	7.5^{c}	0.3	<0.001	6.6	7.1	7.3
Lipids	4.5^{d}	3.3^{d}	8.8^{b}	7.4^{c}	10.5^{a}	11.7^{a}	6.1^{c}	0.2	<0.001	12.9	13.9	16.1

^{a,b,c}: Significant ($p < 0.05$) differences, within rows, between laboratory hydrolysates using Tukey adjustments for multiple comparison.
[1] VHF: viscera (50%), head (25%) and frame and trimmings (25%). V: viscera (100%). V-oil: viscera (100%) oil separated before hydrolysis. Ind VHF: industrial production with VHF. Ind V: industrial production with V.
[2] e: endogenous enzymes active. Pr: Protamex (Novozymes A/S, Bagsvaerd, Denmark). Pa: papain (powder on maltodextrin, 500 TU/mg) and Br: bromelain (powder on maltodextrin, 400 GDU/g) (both from EnzybelIntl.s.a., Belgium).
[3] s.e.m.: pooled standard error of the mean.

efficient separating process and different processing parameters despite the effort to standardize the process between the laboratory and the industrial plant.

3.2. Total amino acids

The total sum of amino acids varied between 73.0% and 85.1% of analyzed CP (Table 3). This was lower than expected, and might be explained by the multiplication factor 6.25 used in the Kjeldahl method. The factor of 6.25 is based on the average content of N in proteins, and might give estimates of CP that are too high in these hydrolysates as smaller peptides and free amino acids have relatively higher water content compared to intact proteins, hence decreasing the gram N in CP. Furthermore, non-protein N in the hydrolysates will be analyzed as CP by the Kjeldahl method.

Different raw materials have different amino acid compositions, and this was reflected in the hydrolysates from different raw materials. This is in accordance with previous work on capelin (*Mallotus villosus*) [54]. The content of glycine and hydroxyproline, which are the main constituents of collagen, was about 30% higher in the hydrolysates from the mixture of raw material compared to the hydrolysates produced from viscera alone. On the other hand, the content of tryptophan, cysteine, and tyrosine was higher in the hydrolysates from viscera alone. Oil removal before hydrolysis did not affect the amino acid composition in the hydrolysate from viscera. The exception to this pattern was the hydrolysate that was initially heated to 70 °C before hydrolysis with papain plus bromelain (V PaBr). This hydrolysate contained a lower amount of amino acids per gram of CP compared to the other hydrolysates. The reason for this observation is unknown-however, a possible explanation might be that strong bonds in the denatured proteins in this hydrolysate may inhibit hydrolysis during amino acid analysis. In particular, indispensable and hydrophobic amino acids, such as isoleucine, leucine, phenylalanine, tryptophan, and valine, were lower in this hydrolysate (V PaBr) compared to the other hydrolysates. Additionally, the content of tyrosine was lower than the other hydrolysates from viscera. During excessive heating, unfolding of the proteins takes place, and the hydrophobic interiors of proteins are exposed. This facilitates packing of the hydrophobic residues inside the molecule, and hence reduces the solubility. The locking of hydrophobic amino acids after excessive heat before hydrolysis was also described by Liu and Zhao [51], and a higher proportion of hydrophobic indispensable amino acids in the insoluble fraction was shown by Šližytė et al. [20] and Liaset and Espe [55].

No major differences were revealed in the total content of amino acids per gram of CP in the hydrolysates produced by Protamex or papain plus bromelain. However, the tendency was that the choice of enzyme seemed to have more impact on the amino acid composition in the mixed starting material than the viscera alone. Hydrolysates produced by Protamex increased the content of the majority of amino acids from the mixed starting material compared to hydrolysates obtained by the use of papain plus bromelain.

The industrial hydrolysates produced a lower amount of amino acids per gram of CP compared to laboratory hydrolysates (806 vs. 838–860 mg amino acid per gram of CP for mixed material, and 767–778 vs. 811–839 mg amino acid per gram of CP for visceral material). This lower content was especially evident for lysine, threonine, alanine, glycine, hydroxyproline, and proline. Glycine, hydroxyproline, and proline are abundant in collagen, which forms a stable triple helix of α-chains that is more resistant to different proteases compared to other proteins of the viscera, head, or frame. Interestingly, industrial hydrolysates contained more tyrosine compared to the laboratory hydrolysate on the same rest raw material.

3.3. Protein quality

Different protein quality calculations (Table 3), such as content of aromatic and branched-chain amino acids, PER values, and PDCAA and DIAAS, almost all showed higher values for the hydrolysates produced from viscera compared to hydrolysates produced from mixed raw material. An exception to this observation was the hydrolysate that was initially heated before hydrolysis (V PaBr), which produced lower values for these parameters compared to the other hydrolysates. From a nutritional point of view, it is important that supply of amino acids fulfills the requirements for maintenance and production in the animal. Feed formulations are designed to supply sufficient amounts of the limiting essential amino acids, which might result in excess supply of other amino acids, thus challenging the amino acid metabolism. The high values of aromatic and branched-chain amino acids, PER, PDCAA, and DIAAS in the hydrolysates implies that hydrolysates from viscera-containing rest raw material is well balanced according to the FAO-recommended amino acid pattern for a preschool child (from 6 months to 3 years) [39]. All hydrolysates, except the hydrolysate from viscera after initial heating (V PaBr), had DIAAS values exceeding 78, with the highest value of 92 in the viscera hydrolysate produced at the industrial plant without addition of commercial enzymes (IndV e). In comparison, the DIAAS values for wheat, peas, and whole milk powder are calculated to be 40, 64, and 122, respectively [39].

3.4. Free amino acids

Initial heating of the raw material up to 70 °C before hydrolysis (V PaBr) markedly affected the amount and composition of free amino acids (Table 4). This was especially evident for the content of histidine, tryptophan, and the small molecule anserine compared to the other hydrolysates from viscera produced in the laboratory. This supports the hypothesis that conformational changes of proteins during excessive heating makes them more resistant to

Table 3
Total amino acid composition and protein quality evaluations of hydrolysates from Atlantic salmon (*Salmo salar*) rest raw material (amino acids given as mg per gram crude protein. Presented values are average ± SD of duplicate measurements where SD is given).

Raw material[1]	VHF	VHF	V	V	V	V	V-oil	Ind VHF	Ind V	Ind V
Enzyme[2]	ePr	ePaBr	ePr	ePaBr	e	PaBr	ePaBr	ePaBr	ePaBr	e
IAA[3]										
Arginine	56.2 ± 0.3	55.0 ± 0.6	50.8 ± 0.7	50.5 ± 0.2	49.6 ± 1.0	48.4 ± 0.2	48.7 ± 0.0	54.3 ± 1.3	49.4 ± 0.1	49.7 ± 0.1
Histidine	26.3 ± 0.2	25.8 ± 0.3	23.2 ± 0.7	23.4 ± 0.3	23.5 ± 0.5	18.2 ± 0.3	23.4 ± 0.1	27.9 ± 0.1	25.0 ± 0.3	25.4 ± 0.4
Isoleucine	36.0 ± 0.2	33.9 ± 0.5	37.4 ± 0.0	36.7 ± 0.1	36.0 ± 0.2	25.0 ± 0.1	36.2 ± 0.1	34.1 ± 0.5	35.9 ± 0.2	36.8 ± 0.3
Leucin	57.9 ± 0.4	55.6 ± 0.6	62.6 ± 0.3	61.2 ± 0.5	60.3 ± 0.4	45.5 ± 0.1	59.9 ± 0.0	58.1 ± 0.1	60.8	63.1 ± 0.6
Lysine	71.2 ± 0.6	69.7 ± 1.2	69.4 ± 0.4	72.3 ± 1.9	68.7 ± 1.9	64.5 ± 0.6	68.0 ± 2.2	64.9 ± 1.3	62.9 ± 1.5	63.8 ± 0.5
Methionine	24.3 ± 0.1	23.4 ± 0.2	24.4 ± 0.8	24.3 ± 0.4	23.7 ± 0.3	18.5 ± 0.0	24.1 ± 0.1	23.8 ± 0.8	22.7 ± 0.0	22.9 ± 0.3
Phenylalanine	31.2 ± 0.2	29.6 ± 0.3	33.9 ± 0.3	33.3 ± 0.2	32.6 ± 0.0	22.3 ± 0.0	33.2 ± 0.0	31.4 ± 0.3	33.3 ± 0.1	34.7 ± 0.4
Threonine	39.3 ± 0.1	37.9 ± 0.4	42.4 ± 0.3	41.9 ± 0.7	41.9 ± 1.0	36.4 ± 0.4	40.5 ± 0.3	32.3 ± 0.4	34.9 ± 0.6	34.4 ± 0.1
Tryptophane	8.2	7.8	10.4	10.1	10.7	6.8	10.6	8.7	10.2	10.3
Valine	42.6 ± 0.2	40.5 ± 0.5	45.6 ± 0.1	44.9 ± 0.5	44.2 ± 0.5	33.0 ± 0.1	44.3 ± 0.0	40.9 ± 0.7	43.6 ± 0.2	44.6 ± 0.2
DAA[4]										
Alanine	57.0 ± 0.0	55.4 ± 0.6	53.4 ± 2.0	52.4 ± 0.4	52.1 ± 0.9	52.3 ± 0.3	50.7 ± 0.3	50.2 ± 1.5	45.5 ± 0.0	45.1 ± 0.1
Aspartic acid	77.8 ± 0.8	75.6 ± 0.6	77.0 ± 0.1	76.1 ± 0.3	73.6 ± 1.9	59.6 ± 0.1	73.5 ± 0.5	70.5 ± 1.0	68.2 ± 1.5	69.6 ± 0.6
Cysteine	9.4	9.5	12.4	11.9	11.9	11.1	12.3	10.8	11.9	12.1
Glutamic acid	113.1 ± 1.3	111.6 ± 1.2	109.6 ± 1.9	109.8 ± 0.1	107.1 ± 2.6	107.6 ± 0.4	104.2 ± 0.6	103.3 ± 2.1	97.9 ± 1.7	100.6 ± 1.1
Glycine	85.8 ± 1.4	85.1 ± 0.9	66.5 ± 0.0	65.7 ± 0.9	63.3 ± 1.7	70.9 ± 0.1	64.4 ± 0.1	78.6 ± 1.6	57.4 ± 0.8	57.3 ± 0.5
OH-proline	16.9 ± 0.3	17.1 ± 0.1	9.1 ± 0.0	9.1 ± 0.5	8.5 ± 0.1	11.8 ± 0.1	9.4 ± 0.0	14.2 ± 0.2	7.0 ± 0.0	6.9 ± 0.8
Proline	49.2 ± 0.5	48.5 ± 0.4	47.0 ± 0.3	46.2 ± 1.9	45.9 ± 0.4	48.6 ± 0.4	46.5 ± 0.4	43.2 ± 1.7	38.7 ± 0.8	38.1 ± 1.1
Serine	39.2 ± 0.2	38.4 ± 0.7	41.2 ± 0.9	41.2 ± 0.3	40.4 ± 1.8	35.1 ± 0.2	39.1 ± 0.1	37.5 ± 0.4	36.9 ± 0.2	38.1 ± 0.1
Tyrosine	18.9 ± 0.5	17.7 ± 0.0	22.2 ± 0.2	21.3 ± 0.4	21.5 ± 1.0	14.3 ± 0.0	21.3 ± 0.6	21.3 ± 0.5	24.5 ± 0.0	25.1 ± 0.1
$\sum$AA	860.5	838.1	838.5	832.3	815.7	729.8	810.5	806.2	766.7	778.5
$\sum$IAA	393.1	379.2	400.2	398.4	391.2	318.6	389.0	376.5	378.7	385.5
$\sum$DAA	467.3	458.9	438.3	433.8	424.5	411.2	421.5	429.7	388.1	392.9
Ratio $\sum$IAA:$\sum$DAA	0.8	0.8	0.9	0.9	0.9	0.8	0.9	0.9	1.0	1.0
$\sum$Aromatic AA[5]	84.6	80.8	89.7	88.0	88.3	61.6	88.5	89.4	92.9	95.5
$\sum$Branched Chain AA [6]	136.5	130.0	145.6	142.8	140.5	103.5	140.5	133.1	154.4	144.4
PERa[7]	1.94	1.85	2.12	2.06	2.02	1.43	2.01	1.92	2.01	2.11
PERb[8]	2.53	2.41	2.53	2.50	2.38	1.57	2.40	2.32	2.13	2.27
PERc[9]	2.34	2.24	2.44	2.43	2.38	1.87	2.37	2.20	2.27	2.32
PERd[10]	2.40	2.31	2.45	2.43	2.39	1.91	2.37	2.31	2.33	2.38
PDCAAS[11]	80.7	77.5	87.3	85.3	84.1	63.4	83.5	80.9	84.7	87.9
DIAASa[12]	84.2	80.9	91.1	89.1	87.8	66.2	87.2	84.4	88.4	91.7
DIAASb[12]	83.1	79.9	89.9	87.9	86.6	65.3	86.1	83.4	87.2	90.5
DIAASc[12]	81.6	78.4	88.2	86.3	85.0	64.1	84.5	81.8	85.6	88.7

[1] VHF: viscera (50%), head (25%) and frame and trimmings (25%). V: viscera (100%). V-oil: viscera (100%) oil separated before hydrolysis. IndVHF: industrial production with VHF. IndV: industrial production with V.
[2] e: endogenous enzymes active. Pr: Protamex (Novozymes A/S, Bagsvaerd, Denmark). Pa: papain (powder on maltodextrin, 500 TU/mg) and Br: bromelain (powder on maltodextrin, 400 GDU/g) (both from EnzybellIntl.s.a., Belgium).
[3] IAA: indispensable amino acid.
[4] DAA: dispensable amino acid.
[5] Aromatic AA: His + trp + phe + tyr.
[6] Branched chain AA: Leu + iso + val.
[7] PERa: protein efficiency ratio: −0.468 + 0.45[leu]−0.105[tyr].
[8] PERb: protein efficiency ratio −1.816 + 0.435[met] + 0.780[leu] + 0.211[his]−0.944[tyr].
[9] PERc: protein efficiency ratio 0.08084[$\sum$AA7]−0.1094 where $\sum$AA7 = threonine + valine + methionine + isoleucine + leucine + phenylalanine + lysine.
[10] PERd: protein efficiency ratio 0.06320[$\sum$AA10]−0.1539 where $\sum$AA10 = $\sum$AA7 + histidin + arginin + tyrosin.
[11] PDCAAS: protein digestibility corrected amino acid score: based on WHO/FAO/UNU report 2007 [37]. Reference pattern for a preschool child from FAO [39]. Fecal digestibility of crude protein set to 92% in accordance with Videncenter for Svineproduction (VSP) [38].
[12] DIAAS: digestible indispensable amino acid. Based on the FAO report from 2013 [39]. a: Coefficients from Folador et al. [40] in roosters. b: Coefficients from Videncenter for Svineproduction (VSP) [38]. c: Coefficients from Kopenhagen Fur [41].

degradation by proteases. The exception was the taurine content, which was much higher in this hydrolysate (V PaBr) compared to the other hydrolysates (67.8 vs. 14.0–33.1 mg/g of CP). This might be of importance as taurine has antioxidant and anti-inflammatory properties, and is involved in several important physiological functions, such as osmoregulation, organ development, and vascular, cardiac, muscular, and retinal regulation [56]. The beneficial effect of taurine in human nutrition during low availability is also shown in, e.g., preterm babies and parenteral nutrition, as reviewed by Stapleton et al. [57] and Redmond et al. [58]. Taurine is a small water-soluble amine and sulfur-containing acid, which is not incorporated into proteins or metabolized in the body [59], and the higher concentration of taurine in this hydrolysate (V PaBr) might be due to lower hydrolysate yield in this hydrolysate compared to the others.

Hydrolysates from viscera (except for V PaBr) had a higher content of free amino acids (389–426 mg free amino acid per gram of protein) compared to hydrolysates from mixed starting material (292–305 mg free amino acid per gram of protein). This was most evident from the higher content of free isoleucine, tryptophan, valine, proline, and serine, together with taurine. The only exception was the content of anserine, which was higher in hydrolysates from mixed starting material compared to hydrolysates from viscera. Anserine (*b*-alanyl-1-methyl-histidine) is more abundant in skeletal muscles compared to heart and brain in vertebrates [60], and is also found in salmon frame hydrolysates [16]. The higher content of anserine in hydrolysate from mixed starting material compared to hydrolysates from viscera alone might be of importance in some applications as anserine has antioxidant [61], buffering [62], and osmotic [60] properties. Different starting

Table 4
Free amino acid composition and degree of hydrolysis of hydrolysates from Atlantic salmon (*Salmo salar*) rest raw material (amino acids given as mg per gram crude protein. Presented values are average ± SD of duplicate measurements).

Raw material[1]	VHF	VHF	V	V	V	V	V-oil	Ind VHF	Ind V	Ind V
Enzyme[2]	ePr	ePaBr	ePr	ePaBr	e	PaBr	ePaBr	ePaBr	ePaBr	e
IAA[3]										
Arginine	26.1 ± 1.0	29.0 ± 0.4	29.2 ± 1.4	32.0 ± 0.6	30.7 ± 1.3	16.6 ± 1.2	33.3 ± 0.5	23.4 ± 0.2	31.0 ± 0.3	29.4 ± 0.9
Histidine	8.0 ± 0.1	7.8 ± 0.1	10.1 ± 0.2	10.5 ± 0.2	9.8 ± 0.1	3.4 ± 0.2	10.5 ± 0.5	4.9 ± 0.0	7.5 ± 0.1	7.4 ± 0.4
Isoleucine	15.9 ± 0.8	13.8 ± 0.0	20.2 ± 0.3	19.6 ± 0.3	20.2 ± 0.7	8.8 ± 0.3	23.0 ± 0.7	9.3 ± 0.0	16.0 ± 0.0	16.1 ± 0.1
Leucine	32.7 ± 1.1	30.3 ± 0.1	39.6 ± 1.2	39.5 ± 0.7	39.8 ± 0.9	18.7 ± 0.8	44.0 ± 0.1	23.2 ± 0.1	34.7 ± 0.3	34.7 ± 0.3
Lysine	26.7 ± 0.5	29.9 ± 0.7	35.6 ± 1.4	39.3 ± 0.5	32.8 ± 2.2	19.2 ± 1.1	36.2 ± 2.4	24.1 ± 0.2	34.8 ± 0.1	31.7 ± 0.7
Methionine	14.5 ± 0.6	13.9 ± 0.0	17.1 ± 0.4	17.6 ± 0.3	18.0 ± 0.5	8.5 ± 0.2	20.3 ± 0.3	9.9 ± 0.0	13.3 ± 0.6	15.2 ± 1.4
Phenylalanine	16.8 ± 1.1	16.1 ± 0.2	20.6 ± 0.0	20.7 ± 0.5	20.9 ± 0.6	9.0 ± 0.2	24.7 ± 1.6	11.5 ± 0.0	17.9 ± 0.2	17.9 ± 0.4
Threonine	11.7 ± 0.1	13.4 ± 2.6	17.3 ± 0.2	18.4 ± 0.2	17.7 ± 1.1	10.1 ± 0.4	19.7 ± 0.1	4.3 ± 0.2	6.6 ± 0.1	6.6 ± 0.3
Tryptophane	4.8 ± 0.4	4.5 ± 0.2	6.9 ± 0.0	6.8 ± 0.1	7.2 ± 0.5	2.5 ± 0.1	8.7 ± 0.7	2.0 ± 0.0	3.9 ± 0.0	3.8 ± 0.2
Valine	17.4 ± 0.5	15.9 ± 0.2	23.6 ± 0.4	23.7 ± 0.4	24.4 ± 0.6	11.8 ± 0.5	27.1 ± 0.3	11.8 ± 0.1	19.1 ± 0.5	19.3 ± 0.2
DAA[4]										
Alanine	15.9 ± 1.0	17.7 ± 0.5	20.3 ± 1.4	23.1 ± 0.5	22.5 ± 2.1	16.5 ± 0.6	23.6 ± 0.1	13.7 ± 0.1	16.9 ± 0.3	18.5 ± 0.5
Asparagine	1.8 ± 0.1	1.1 ± 0.0	1.6 ± 0.0	1.6 ± 0.3	1.0	0.9 ± 0.1	1.9 ± 0.2	0.4 ± 0.1	1.2 ± 0.1	1.0 ± 0.1
Aspartic acid	9.9 ± 2.3	14.2 ± 1.4	13.8 ± 2.4	19.3 ± 0.4	14.3 ± 2.8	8.3 ± 1.2	14.8 ± 2.4	8.5 ± 0.1	15.1 ± 0.2	15.4 ± 0.3
Cysteine/cystin	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.1	<0.1	<0.1
Glutamine	24.3 ± 1.1	23.8 ± 0.4	36.8 ± 2.1	38.1 ± 1.1	35.0 ± 1.8	19.5 ± 2.1	35.3 ± 2.3	1.3 ± 0.0	1.1 ± 0.2	1.0 ± 0.3
Glutamic acid	15.6 ± 2.2	20.1 ± 1.0	20.7 ± 2.4	26.4 ± 0.5	21.7 ± 3.0	21.0 ± 2.6	22.0 ± 2.3	13.0 ± 0.1	19.4 ± 0.0	20.1 ± 0.6
Glycine	7.4 ± 0.3	7.4 ± 0.1	10.2 ± 0.1	10.7 ± 0.2	10.8 ± 0.6	8.0 ± 0.5	12.1 ± 0.8	7.1 ± 0.1	8.7 ± 0.5	8.7 ± 0.1
OH-proline	0.7 ± 0.0	0.9 ± 0.0	1.0 ± 0.0	1.2 ± 0.0	0.8 ± 0.0	1.5 ± 0.2	0.8 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.2 ± 0.1
Proline	3.1 ± 0.1	3.2 ± 0.0	5.8 ± 0.1	6.0 ± 0.1	6.6 ± 0.4	4.3 ± 0.2	8.8 ± 0.9	2.9 ± 0.1	4.8 ± 0.2	5.1 ± 0.0
Serine	11.1 ± 0.9	11.9 ± 0.3	17.3 ± 1.0	19.5 ± 0.4	17.5 ± 1.5	10.0 ± 0.8	19.1 ± 1.6	7.4 ± 0.1	12.3 ± 0.3	12.4 ± 0.1
Tyrosine	9.6 ± 0.6	10.1 ± 0.1	10.9 ± 0.1	10.6 ± 0.3	11.0 ± 0.5	8.6 ± 0.3	11.1 ± 0.8	10.5 ± 0.0	10.3 ± 0.0	12.5 ± 0.3
Anserine	4.5 ± 0.2	3.8 ± 0.3	1.1 ± 0.0	1.1 ± 0.2	1.1	0.2 ± 0.1	1.3 ± 0.1	6.6 ± 0.1	1.7 ± 0.4	1.4 ± 0.0
Taurine	14.0 ± 0.7	16.3 ± 0.4	29.4 ± 0.0	30.9 ± 0.6	32.7 ± 0.8	67.8 ± 3.6	28.4 ± 1.2	20.2 ± 0.5	28.5 ± 1.0	33.1 ± 0.0
$\sum$Free AA +anserine + taurine	292.5	305.0	389.2	416.8	396.4	275.1	426.4	216.2	305.2	311.5
Ratio $\sum$FreeAA:$\sum$AA	0.34	0.36	0.46	0.50	0.49	0.38	0.53	0.27	0.39	0.40
Degree of hydrolysis	43.1	40.6	50.0	48.6	48.0	35.0	52.2	27.0	40.8	37.3

[1] VHF: viscera (50%), head (25%) and frame and trimmings (25%). V: viscera (100%). V-oil: viscera (100%) oil separated before hydrolysis. IndVHF: industrial production with VHF. IndV: industrial production with V.
[2] e: endogenous enzymes active. Pr: Protamex (Novozymes A/S, Bagsvaerd, Denmark). Pa: papain (powder on maltodextrin, 500 TU/mg) and Br: bromelain (powder on maltodextrin, 400 GDU/g) (both from EnzybelIntl.s.a., Belgium).
[3] IAA: indispensable amino acid.
[4] DAA: dispensable amino acid.

materials and a more extensive hydrolysis in the viscera hydrolysates compared to the hydrolysates from mixed starting material probably caused these differences in the amount and composition of free amino acids.

Different enzyme treatments revealed minor differences in the composition of free amino acids. However, there was a tendency towards a higher content of free amino acids, such as arginine, lysine, aspartic acid, and glutamic acid, in hydrolysates treated with papain plus bromelain compared to Protamex, which was not dependent on the raw material used. Different enzymes have different binding-site preferences and theoretically result in hydrolysates with different compositions of free amino acids. The minor differences seen in this study is probably a result of high proteolytic activity in the raw material itself, as it contained at least 50% viscera and may have dominated the proteolytic activity during hydrolysis.

Industrially produced hydrolysate contained a lower concentration of free amino acids compared to the laboratory hydrolysates. In particular, the content of histidine, isoleucine, threonine, tryptophan, glutamine, hydroxyproline, and serine was lower in the industrial hydrolysates with a decrease between 22% (isoleucine) and 97% (glutamine) compared to the laboratory-scale hydrolysates. This was also the case for asparagine in the mixed raw material. Glutamine is a labile amino acid in solution and is degraded to ammonia depending on the storage conditions, such as temperature, pH, and time [63,64]. The industrial hydrolysates were preserved by addition of formic acid, and it is likely that the observed low content of glutamine in the industrial hydrolysates was due to degradation during storage and not due to the hydrolyzing process itself. Interestingly, the content of anserine and taurine was higher in the industrial hydrolysates from the mixed raw material compared to the corresponding hydrolysates in the laboratory. This was most probably due to the lower amount of protein in industrial samples giving a higher concentration of small free molecules, such as anserine and taurine, when calculated on a CP basis.

3.5. Degree of hydrolysis

Two different analysis methods were used to evaluate DH for laboratory and industrial hydrolysates, which made direct comparison difficult despite there being a strong positive correlation between these two analytical methods in hydrolysates from whey proteins [65].

Hydrolysates from viscera had higher DH (Table 4) compared to the hydrolysates from mixed starting material (48.0–52.2% vs. 40.6–43.1%), with the exception of the hydrolysate obtained after initial heating before hydrolysis, which reached 35.0% DH after 60 min of hydrolysis. Oil separation before hydrolysis (V-oil ePaBr) appeared to enhance DH in viscera hydrolysates, which was probably due to the slight warming to 40 °C before hydrolysis, during which the endogenous enzymes are still active [13] and facilitate endogenous hydrolysis during warming and oil-separation steps.

Hydrolysis by use of papain plus bromelain only slightly enhanced DH compared to the viscera hydrolysate produced with only endogenous enzymes (V e) in both laboratory (48.6 vs. 48.0) and industrial (40.8 vs. 37.3) hydrolysates. The high proteolytic activity in viscera found in this study are supported by the work of Pastoriza et al. [66] who found that endogenous enzymes increased DH compared to commercial enzymes (papain and pepsin) during

hydrolysis of rayfish (*Raja clavata*) residues. A number of other studies have also reported high proteolytic activity in different fish viscera [13,14,19]. Protamex seems to produce hydrolysates with higher DH in both visceral and mixed starting materials compared to papain plus bromelain. Interestingly, Protamex seems to produce a slightly lower concentration of free amino acids compared to papain plus bromelain in the same starting materials. Despite this, there was a significant and strong positive correlation between DH and the content of free amino acids ($r = 0.95$, $p < 0.0001$). A similar finding was also described by Šližytė et al. [20] during hydrolysis of cod by-products, and by Silvestre et al. [65] in whey protein hydrolysates, although this was not significant.

In general, DH in industrial hydrolysates was lower than that in the laboratory hydrolysates. The endogenous enzymes are active during storage and pre-heating of raw material before addition of commercial enzymes, which is often defined as the start of the hydrolysis. A different degree of endogenous hydrolysis due to storage, heating capacity, and time used to reach 52 °C might therefore influence DH in the hydrolysates. Despite the effort to replicate the laboratory trials, the observed differences in DH are probably partly due to the shorter time from slaughter to hydrolysis and the higher heating capacity in the industrial plant. It is expected that the most resistant proteins would be hydrolyzed later in the process.

3.6. Molecular weight

As hydrolysates from VHF had lower DH, it was expected that they would contain larger peptides (>1 kDa) compared to visceral hydrolysates. This was proved by the molecular weight distribution analysis in which VHF hydrolysates contained 16.9% and 24.7% of peptides >1 kDa compared to 10.9% and 14.7% in the corresponding V hydrolysates (Table 5).

Initial heating of viscera to 70 °C before hydrolysis had a great impact on the molecular weight distribution of the hydrolysate, as 7% of the components still had a molecular weight of >10 kDa after hydrolysis compared to <2% in the other hydrolysates from viscera. Only 51% of the peptides had a molecular weight below 1 kDa in the initially heated viscera hydrolysate (V PaBr), compared to >81% in the other hydrolysates from viscera. These findings are in contrast to those of Adjonu et al. [67], who did not find significant differences in molecular weight distribution between initially heated and non-heated raw material when hydrolyzing whey protein isolate with chymotrypsin and pepsin. On the other hand, our findings are in accordance with those of Liu and Zhao [51] and Duan et al. [50] who found lower efficiency of hydrolysis and larger peptides after excessive heating before hydrolysis of blue mackerel (*Decapterus maruadsi*) and silver carp (*Hypophthalmichthys molitrix*), respectively.

Hydrolysates produced with Protamex tended to result in smaller peptides (<1 kDa) compared to papain plus bromelain from both V and VHF. This explains the higher DH in Protamex hydrolysates, as the content of peptides >10 kDa from Protamex compared to papain plus bromelain hydrolysates was 1.1% vs. 1.7% from viscera raw material, and 0.7% vs. 1.4% from the mixture of raw material, respectively.

The molecular weight distribution in the industrial hydrolysates revealed differences, as the content of peptides between 1 and 10 kDa was higher in the industrial hydrolysates compared to those produced in the laboratory. The content of peptides between 1 and 10 kDa was 30.8% in industrial hydrolysates from mixed starting material (IndVHF ePaBr) and 17.3–17.4% in industrial hydrolysates from viscera (IndV e and IndV ePaBr). For laboratory hydrolysates, the percentages were 16.2–23.3% for the mixed starting material and 9.8–14.2% for the viscera hydrolysates, respectively.

The molecular weight distribution of proteins in hydrolysates has a great impact on the use of hydrolysates. Peptides between 3 and 20 amino acid residues are regarded as the most promising in the search for biologically active peptides as the chance of crossing the intestinal membrane and exerting a biological effect is higher for the lower molecular weight peptides [68], and published bioactive peptides usually contain between 3 and 20 amino acid residues [69].

3.7. Bioactive peptides

Hydrolysates from different raw materials were expected to give rise to different peptides. This was, however, difficult to detect, as the difference between the two enzymes used during hydrolysis were substantial in the presented peptide analysis. The number of different peptides was lower in hydrolysates produced with Protamex compared to those produced with papain plus bromelain (Table 6). In hydrolysates obtained by use of Protamex, the number of different peptides was 157 (VHF ePr) from mixed starting materials and 147 (V ePr) from viscera as the starting material, compared to 355 (VHF ePaBr) and 298 (V ePaBr) in hydrolysates obtained by the use of papain plus bromelain from mixed starting material and viscera as the starting material, respectively. However, the number of different bioactive motifs was approximately equal among the hydrolysates VHF ePr, V ePr, VHF ePaBr, and V ePaBr, although the total number of bioactive motifs was lower in hydrolysates obtained by using Protamex compared to papain plus bromelain. The reason for these findings are unknown, as other analyses performed in this research, such as DH, and content of CP, free amino acids, and amino acids, did not reveal substantial differences between the enzymes used during hydrolysis. The

Table 5
Molecular weight distribution of hydrolysates from Atlantic salmon (*Salmo salar*) rest raw material (molecular weight class given as Da and content as % of water-soluble protein).

Raw material[1]	VHF	VHF	V	V	V	V	V-oil	Ind VHF	Ind V	Ind V
Enzyme[2]	ePr	ePaBr	ePr	ePaBr	e	PaBr	ePaBr	ePaBr	ePaBr	e
>20,000	0.3	0.7	0.6	1.1	0.8	2.9	0.3	0.1	0.2	0.2
20,000–15,000	0.1	0.2	0.1	0.1	0.1	0.9	0.1	0.1	0.1	0.1
15,000–10,000	0.3	0.5	0.4	0.5	0.5	3.2	0.4	0.5	0.4	0.5
10,000–8000	0.3	0.4	0.4	0.5	0.4	3.5	0.3	0.6	0.4	0.5
8000–6000	0.6	0.9	0.6	0.8	0.8	5.9	0.5	1.3	0.7	0.8
6000–4000	1.2	2.1	1.0	1.4	1.5	8.8	0.9	3.3	1.6	1.7
4000–2000	4.3	7.3	2.6	3.6	4.4	13.7	2.8	10.7	5.4	5.4
2000–1000	9.8	12.6	5.2	6.7	7.1	10.2	5.7	14.9	9.2	9.0
1000–500	13.2	12.8	8.6	8.7	8.4	7.0	8.4	13.9	11.1	10.5
<500	69.9	62.6	80.5	76.6	76.0	44.0	80.7	54.6	71.0	71.3

[1] VHF: viscera (50%), head (25%) and frame and trimmings (25%). V: viscera (100%). V-oil: viscera (100%) oil separated before hydrolysis. IndVHF: industrial production with VHF. IndV: industrial production with V.
[2] e: endogenous enzymes active. Pr: Protamex (Novozymes A/S, Bagsvaerd, Denmark). Pa: papain (powder on maltodextrin, 500 TU/mg) and Br: bromelain (powder on maltodextrin, 400 GDU/g) (both from EnzybelIntl.s.a., Belgium).

Table 6
Number of recognized peptides in Atlantic salmon (*Salmo salar*) rest raw material hydrolysates. (Values given as number of different recognized peptides except in total number of bioactive motifs).

Raw material[1]	VHF	VHF	V	V	V	V	V-oil	Ind VHF	Ind V	Ind V
Enzyme[2]	ePr	ePaBr	ePr	ePaBr	e	PaBr	ePaBr	ePaBr	ePaBr	e
No. of peptides detected[3]	157	355	147	298	314	168	282	48	274	326
No. of bioactive motifs detected[4]	40	48	45	44	45	49	44	25	52	47
No. of cardiovascular	27	35	34	35	35	41	34	20	38	40
No. of antioxidant	10	12	8	7	8	6	8	4	12	6
No. of pep[5]	1	0	1	1	1	1	1	1	1	1
No. of immunomodulation	2	1	2	1	1	1	1	0	0	0
No. of antimicrobial	0	0	0	0	0	0	0	0	1	0
No. of total number of bioactive motifs[6]	184	453	232	402	423	252	385	60	380	455

[1] VHF: viscera (50%), head (25%) and frame and trimmings (25%). V: viscera (100%). V-oil: viscera (100%) oil separated before hydrolysis. IndVHF: industrial production with VHF. IndV: industrial production with V.
[2] e: endogenous enzymes active. Pr: Protamex (Novozymes A/S, Bagsvaerd, Denmark). Pa: papain (powder on maltodextrin, 500 TU/mg) and Br: bromelain (powder on maltodextrin, 400 GDU/g) (both from EnzybelIntl.s.a., Belgium).
[3] Number of different peptides detected by described method based on Pampanin et al. [45].
[4] Number of different bioactive motifs detected.
[5] pep: prolyl endopeptidase.
[6] Sum of all bioactive motifs recognized.

hydrolysate from viscera produced with only endogenous enzymes (V e) appears to have a high number of different peptides, and high total number of bioactive motifs compared to hydrolysate from viscera using papain plus bromelain in both the laboratory and industrial production of the hydrolysates.

The effect of initial inactivation of the endogenous enzymes (V PaBr) affected the number of different peptides, but not the number of different bioactive motifs. In addition, this hydrolysate (V PaBr), compared to all the other hydrolysates, contained six unique bioactive motifs. These unique and potentially bioactive peptides were found in small numbers (from one to five detected peptides in the hydrolysate) and might be caused by conformational changes uncovering different binding sites for the enzymes during hydrolysis.

Based on the total number of different peptides, the hydrolysate from the mixed material with papain plus bromelain (VHF ePaBr) appears to be the most efficiently hydrolyzed with 355 different peptides detected, whereas the hydrolysis process was least efficient in the industrial production of the same hydrolysate (IndVHF ePaBr) with only 48 detected different peptides. Theoretically, these two hydrolysates should contain the same number of different peptides, but as described earlier, DH was lower in the industrial hydrolysates and the number of different peptides was expected to be lower from the industrially processed hydrolysates. However, this tendency was not observed in the hydrolysis of viscera between industrial- and laboratory-scales of production of the hydrolysates.

The identified bioactive motifs are located within larger peptides (usually 8–10 amino acids in length) and from the current analysis it cannot be determined whether or not the peptides are bioactive. Further in vivo and in vitro testing is recommended to verify the proposed bioactive properties presented here. As expected, the majority of recognized bioactive peptides were associated with the cardiosystem, upon which most of the published data are based on.

4. Conclusion

Hydrolysates from farmed Atlantic salmon rest raw material contain high levels of proteins, with high nutritional value, and a number of bioactive motifs. The present work shows that hydrolysate composition may be designed by selection of starting material, and by choosing the correct pretreatments before hydrolysis and commercial enzymes in order to obtain the desired final products. Composition of starting material, V and VHF, was reflected in the proximal composition in the hydrolysate, as the high lipid content in raw material tended to increase the lipid content in hydrolysate at the expense of CP content. Composition of amino acids in the hydrolysates was also influenced by composition of the raw material. Removal of oil before hydrolysis reduced lipids and increased CP content in the hydrolysate, but had little effect on amino acid composition, and slightly changed the molecular weight distribution and enhanced the content of free amino acids together with DH.

Small differences were seen between hydrolysates obtained by the use of Protamex and papain plus bromelain in the current work on viscera-containing rest raw material, except for the number of different peptides detected and total amount of bioactive motifs, which was higher after papain plus bromelain treatment compared to Protamex. However, the number of different bioactive motifs was at the same level for all the laboratory hydrolysates, despite different viscera-containing raw materials, enzymes, or pretreatments. Compared to the other laboratory viscera hydrolysates, the hydrolysate without commercial enzymes added (V e), gave the highest amount of detected bioactive motifs, which was nearly equal to that treated with papain plus bromelain (V ePaBr), which yielded the next best results.

Heat inactivation of endogenous enzymes had a great impact on the hydrolysate composition, as this hydrolysate contained less protein and amino acids (both free and total), larger peptides, and a smaller number of recognizable peptides compared to initially non-heated hydrolysates. Up-scaling is not a direct repetition of the technology from the small scale, as laboratory findings are not necessarily easy to mimic at an industrial scale. Possible challenges are the time from fish slaughter to hydrolysis of the rest raw material, storage conditions, separating processes, and heating capacity in industrial production.

However, the present work shows that the hydrolyzing process increases the possibilities for refinement and further usage of viscera-containing rest raw material from Atlantic salmon. It is possible, to some extent, to design the size of peptides formed, amino acid composition, and potential bioactive peptides by combining different raw material, enzymes, and pretreatments.

Acknowledgements

The authors want to thank Rakel Skjellegrind for great assistance during the hydrolysis experiment at Sintef Sealab and Kjell Birger Øysæd at IRIS for analytical contribution of peptide analysis. The authors also acknowledge the financial support from Regional

Research Found Mid-Norway (ES486682/217393) and the National Research Council of Norway (221364/O30).

References

[1] Olafsen T, Richardsen R, Nystøyl R, Strandheim G, Kosmo JP. Analyse marint restråstoff, 2013. SINTEF Fisheries and Aquaculture; 2014.
[2] RUBIN. Varestrømanalyse for 2011. In: Årsrapport for RUBIN 2011. RUBIN; 2012.
[3] Panyam D, Kilara A. Enhancing the functionality of food proteins by enzymatic modification. Trends Food Sci Technol 1996;7:120–5.
[4] Thorkelsson G, Sigurgisladottir S, Geirsdottir M, Johannsson R, Guerard F, Chabeaud A, et al. Mild processing techniques and development of functional marine protein and peptide ingredients. In: Borresen T, editor. Improving seafood products for the consumer. Cambridge, England: Woodhead Publishing Limited; 2008. p. 363–98.
[5] Ewart HS, Dennis D, Potvin M, Tiller C, Fang LH, Zhang R, et al. Development of a salmon protein hydrolysate that lowers blood pressure. Eur Food Res Technol 2009;229:561–9.
[6] Harnedy PA, FitzGerald RJ. Bioactive peptides from marine processing waste and shellfish: a review. J Funct Foods 2012;4:6–24.
[7] Slizyte R, Mozuraityte R, Martinez-Alvarez O, Falch E, Fouchereau-Peron M, Rustad T. Functional, bioactive and antioxidative properties of hydrolysates obtained from cod (*Gadus morhua*) backbones. Process Biochem 2009;44:668–77.
[8] Kim SK, Wijesekara I. Development and biological activities of marine-derived bioactive perptides: a review. J Funct Foods 2010;2:1–9.
[9] Rustad T, Storro I, Slizyte R. Possibilities for the utilisation of marine by-products. Int J Food Sci Technol 2011;46:2001–14.
[10] Chalamaiah M, Kumar BD, Hemalatha R, Jyothirmayi T. Fish protein hydrolysates: proximate composition, amino acid composition, antioxidant activities and applications: a review. Food Chem 2012;135:3020–38.
[11] Ngo DH, Vo TS, Ngo DN, Wijesekara I, Kim SK. Biological activities and potential health benefits of bioactive peptides derived from marine organisms. Int J Biol Macromol 2012;51:378–83.
[12] Kristinsson HG, Rasco BA. Kinetics of the hydrolysis of Atlantic salmon (*Salmo salar*) muscle proteins by alkaline proteases and a visceral serine protease mixture. Process Biochem 2000;36:131–9.
[13] Sovik SL, Rustad T. Proteolytic activity in byproducts from cod species caught at three different fishing grounds. J Agric Food Chem 2005;53:452–8.
[14] Bower C, Malemute C, Bechtel P. Endogenous protease activity in by-products of pink salmon (*Oncorhynchus gorbuscha*). J Food Biochem 2011;35: 628–37.
[15] Kristinsson HG, Rasco BA. Hydrolysis of salmon muscle proteins by an enzyme mixture extracted from Atlantic salmon (*Salmo salar*) pyloric caeca. J Food Biochem 2000;24:177–87.
[16] Liaset B, Julshamn K, Espe M. Chemical composition and theoretical nutritional evaluation of the produced fractions from enzymic hydrolysis of salmon frames with Protamex (TM). Process Biochem 2003;38:1747–59.
[17] Aksnes A, Hope B, Hostmark O, Albrektsen S. Inclusion of size fractionated fish hydrolysate in high plant protein diets for Atlantic cod, *Gadus morhua*. Aquaculture 2006;261:1102–10.
[18] He S, Franco C, Zhang W. Process optimisation and physicochemical characterisation of enzymatic hydrolysates of proteins from co-products of Atlantic Salmon (*Salmo salar*) and Yellowtail Kingfish (*Seriola lalandi*). Int J Food Sci Technol 2012;47:2397–404.
[19] Aspmo SI, Horn SJ, H. Eijsink VG. Enzymatic hydrolysis of Atlantic cod (*Gadus morhua* L.) viscera. Process Biochem 2005;40:1957–66.
[20] Šližytė R, Daukšas E, Falch E, Storrø I, Rustad T. Characteristics of protein fractions generated from hydrolysed cod (*Gadus morhua*) by-products. Process Biochem 2005;40:2021–33.
[21] Šližytė R, Rustad T, Storrø I. Enzymatic hydrolysis of cod (*Gadus morhua*) by-products: optimization of yield and properties of lipid and protein fractions. Process Biochem 2005;40:3680–92.
[22] Ovissipour M, Kenari AA, Motamedzadegan A, Nazari RM. Optimization of enzymatic hydrolysis of visceral waste proteins of yellowfin tuna (*Thunnus albacares*). Food Bioprocess Technol 2012;5:696–705.
[23] Ovissipour M, Safari R, Motamedzadegan A, Shabanpour B. Chemical and biochemical hydrolysis of Persian sturgeon (*Acipenser persicus*) visceral protein. Food Bioprocess Technol 2012;5:460–5.
[24] Šližytė R, Carvajal AK, Mozuraityte R, Aursand M, Storrø I. Nutritionally rich marine proteins from fresh herring by-products for human consumption. Process Biochem 2014;49:1205–15.
[25] Souissi N, Bougatef A, Triki-Ellouz Y, Nasri M. Biochemical and functional properties of sardinella (*Sardinella aurita*) by-product hydrolysates. Food Technol Biotechnol 2007;45:187–94.
[26] NovoNordisk. Novo Nordisk. Protamex TM. Product sheet. B 716d-GB; 1998.
[27] NovozymesA/S. Novozymes, A/S. Fish processing-fish fillet. Fish flavour and fishmeal production. In: Application sheet. No. 2003-32152-02; 2003.
[28] ISO. ISO 5983-2 Animal feeding stuffs – determination of nitrogen content and calculation of crude protein content – Part 2: Block digestion/steam distillation method. International Standards Organisation; 2009.
[29] AOAC. Official methods of analysis. 15th ed. Washington, DC, USA: Assosiation of Official Analytic Chemists; 1990.
[30] ISO. ISO 6496 Animal feeding stuffs—determination of moisture and other volatile matter content. In: ISO standard 6496. International Standards Organization; 1999.
[31] ISO. ISO 5984:2002 Animal feeding stuffs—determination of crude ash. In: ISO standard 5984:2002. International Standards Organization; 2002.
[32] Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. Can J Biochem Physiol 1959;37:911–7.
[33] Cohen SA, Michaud DP. Synthesis of a fluorescent derivatizing reagent, 6-aminoquinolyl-*n*-hydroxysuccinimidyl carbamate, and its application for the analysis of hydrolysate amino acids via high-performance liquid chromatography. Anal Biochem 1993;211:279–87.
[34] Bidlingmeyer BA, Cohen SA, Tarvin TL, Frost B. A new, rapid, high sensitivity analysis of amino acids in food type samples. J Assoc Off Anal Chem 1987;70:241–7.
[35] Alsmeyer RH, Cunningham AE, Happich ML. Equations predict PER from amino acid analysis. Food Technol 1974;28:34–40.
[36] Lee YB, Elliott JG, Rickansrud DA, Hagberg EYC. Predicting protein efficiency ratio by the chemical determination of connective tissue content in meat. J Food Sci 1978;43:1359–62.
[37] WHO/FAO/UNU. Protein and amino acid requirements in human nutrition: report of a joint WHO/FAO/UNU expert consultation. WHO technical report series, vol. 935. WHO/FAO/UNU; 2007.
[38] Videncenter for Svineproduktion. Videncenter for Svineproduktion—Råvaretabellen; 2014.
[39] FAO. Dietary protein quality evaluation in human nutrition. In: Report of an FAO Expert Consultation. 31 March–2 April, 2011. Auckland, New Zealand: Food and Agriculture Organization of the United Nations; 2013.
[40] Folador JF, Karr-Lilienthal LK, Parsons CM, Bauer LL, Utterback PL, Schasteen CS, et al. Fish meals, fish components, and fish protein hydrolysates as potential ingredients in pet foods. J Anim Sci 2006;84:2752–65.
[41] Fiskeensilage. Kopenhagen Fur Rapport for fordøyelighetsforsøg med mink (Neovison vison). In: Fiskeensilage FishPro (1124F); 2013.
[42] Taylor WH. Formol titration: an evaluation of its various modifications. Analyst 1957;82:488–98.
[43] Nielsen PM, Petersen D, Dambmann C. Improved method for determining food protein degree of hydrolysis. Food Chem Toxicol 2001;66:642–6.
[44] Wang-Andersen J, Haugsgjerd BO. Forbedret analyse-metodikk for peptidstørrelsesfordeling i marine proteinhydrolysater. Tromsø, Norway: Nofima A/S; 2011.
[45] Pampanin DM, Larssen E, Provan F, Sivertsvik M, Ruoff P, Sydnes MO. Detection of small bioactive peptides from Atlantic herring (*Clupea harengus* L.). Peptides 2012;34:423–6.
[46] Nesvizhskii AI. A survey of computational methods and error rate estimation procedures for peptide and protein identification in shotgun proteomics. J Proteomics 2010;73:2092–123.
[47] Kristinsson HG, Rasco BA. Biochemical and functional properties of Atlantic salmon (*Salmo salar*) muscle proteins hydrolyzed with various alkaline proteases. J Agric Food Chem 2000;48:657–66.
[48] Ovissipour M, Benjakul S, Safari R, Motamedzadegan A. Fish protein hydrolysates production from yellowfin tuna *Thunnus albacares* head using Alcalase and Protamex. Int Aquat Res 2010;2:87–95.
[49] Lahl WJ, Braun SD. Enzymatic production of protein hydrolysates for food use. Food Technol 1994;48:68–71.
[50] Duan ZH, Wang JL, Yi MH, Yin AQ. Recovery of proteins from silver carp by-products with enzymatic hydrolysis and reduction of bitterness in hydrolysate. J Food Process Eng 2010;33:962–78.
[51] Liu TX, Zhao MM. Thermal pretreatment and chemical modifications as a means to alter hydrolytic characteristics and prevent bitterness in hydrolysates of fishery by-catch (*Decapterus maruadsi*) protein. Int J Food Sci Technol 2010;45:1852–61.
[52] Mutilangi WAM, Panyam D, Kilara A. Functional properties of hydrolysates from proteolysis of heat-denatured whey protein isolate. J Food Sci 1996;61: 270–5.
[53] Branden C, Tooze J. Introduction to protein structure. New York, NY: Garland Publishing; 1999.
[54] Shahidi F, Han X-Q, Synowiecki J. Production and characteristics of protein hydrolysates from capelin (*Mallotus villosus*). Food Chem 1995;53: 285–93.
[55] Liaset B, Espe M. Nutritional composition of soluble and insoluble fractions obtained by enzymatic hydrolysis of fish-raw materials. Process Biochem 2008;43:42–8.
[56] Wu GY. Amino acids: metabolism, functions, and nutrition. Amino Acids 2009;37:1–17.
[57] Stapleton PP, Charles RP, Redmond HP, BouchierHayes DJ. Taurine and human nutrition. Clin Nutr 1997;16:103–8.
[58] Redmond HP, Stapleton PP, Neary P, Bouchier-Hayes D. Immunonutrition the role of taurine. Nutrition 1998;14:599–604.
[59] Han X, Patters AB, Jones DP, Zelikovic I, Chesney RW. The taurine transporter: mechanisms of regulation. Acta Physiol (Oxford, England) 2006;187: 61–73.
[60] Baslow MH. The existence of molecular water pumps in the nervous system: a review of the evidence. Neurochem Int 1999;34:77–90.
[61] Kang JH, Kim KS, Choi SY, Kwon HY, Won MH, Kang T-C. Protective effects of carnosine, homocarnosine and anserine against peroxyl radical-mediated Cu,Zn-superoxide dismutase modification. Biochim Biophys Acta, Gen Subj 2002;1570:89–96.

[62] Abe H. Role of histidine-related compounds as intracellular proton buffering constituents in vertebrate muscle. Biochemistry (Moscow) 2000;65:757–65.
[63] Khan K, Elia M. Factors affecting the stability of L-glutamine in solution. Clin Nutr 1991;10:186–92.
[64] Snowden MK, Baxter JH, Mamula Bergana M, Reyzer I, Pound V. Stability of *N*-acetylglutamine and glutamine in aqueous solution and in a liquid nutritional product by an improved HPLC method. J Food Sci 2002;67:384–9.
[65] Silvestre MPC, Morais HA, Silva VDM, Silva MR. Degree of hydrolysis and peptide profile of whey proteins using pancreatin. Rev Soc Bras Alim Nutr 2013;38:278–90.
[66] Pastoriza L, Sampedro G, Cabo ML, Herrera JJR, Bernárdez M. Solubilisation of proteins from rayfish residues by endogenous and commercial enzymes. J Sci Food Agric 2004;84:83–8.
[67] Adjonu R, Doran G, Torley P, Agboola S. Screening of whey protein isolate hydrolysates for their dual functionality: influence of heat pre-treatment and enzyme specificity. Food Chem 2013;136:1435–43.
[68] Roberts PR, Burney JD, Black KW, Zaloga GP. Effect of chain length on absorption of biologically active peptides from the gastrointestinal tract. Digestion 1999;60:332–7.
[69] Pihlanto-Leppälä A. Bioactive peptides derived from bovine whey proteins: opioid and ace-inhibitory peptides. Trends Food Sci Technol 2000;11:347–56.

Paper III

Atlantic salmon (*Salmo salar*) protein hydrolysate – Effect on growth performance and intestinal morphometry in broiler chickens

M. Opheim[a,b,*], H. Sterten[a], M. Øverland[b], N. P. Kjos[b]

[a]*Felleskjøpet Fôrutvikling, Nedre Ila 20, N-7018 Trondheim, Norway*

[b]*Department of Animal and Aquacultural Sciences, Norwegian University of Life Sciences, P. O. Box 5003, N-1432 Aas, Norway*

Corresponding author: Margareth Opheim. E-mail: margareth.opheim@fkf.no

Abstract

An experiment was conducted to evaluate the effect of different salmon protein hydrolysates on growth performance and intestinal morphometry in broiler chickens. Two different Atlantic salmon protein hydrolysates (SPH) from viscera were produced, one without the addition of exogenous enzymes and the other with added papain+bromelain during hydrolysis. The hydrolysates were included with 5% and 10% in both the starter and grower diets for broiler chickens. Additionally, one fish meal diet (FM) and a plant protein-based (PP) diet were included as controls in each period. Immediately after hatching, 5282 Ross308 broiler chickens were allotted to 66 pens and fed the starter diet from days 0–9 and the grower diet from days 10–28. Growth performance data were recorded for the starter period, early grower period (days 10–20) and late grower period (days 21–28), and duodenum, jejunum and ileum were sampled at day 10 for morphometric analysis. Inclusion of 10% SPH in the diet led to increased average daily gain (ADG) ($P < 0.05$) in the broiler chickens compared with the broiler chickens fed the PP, FM and 5% SPH diets. Using contrast statistical analysis, the gain to feed ratio (G:F) was significantly higher in the groups fed the 10% SPH diets compared with the groups fed the PP and FM diets in the starter period. Also, ADG values were higher for the 5% SPH diet groups compared with the groups fed the FM (FM vs. 5% SPH, $P < 0.001$) and PP (PP vs. 5% SPH, $P = 0.015$) diets in the starter period. In the grower period, the impact of SPH inclusion on ADG diminished compared with the FM diet group; however, broiler chickens fed the PP diet did not reach the same final live-weight as broiler

chickens fed the marine protein diets. There was a tendency towards longer villi in the duodenum and ileum, as well as larger villi absorption area in the ileum, of broiler chickens fed marine proteins compared with those fed the PP diet. No adverse effect on mortality or litter quality was detected when salmon protein hydrolysates were included in the broiler chicken diet. In conclusion, addition of salmon protein hydrolysates to broiler chicken starter diets improved the growth performance compared with the PP and FM diets. Additionally, starter diets with inclusion of marine proteins tended to improve the small intestinal development in broiler chickens compared to a plant protein-based diet.

Keywords: broiler chicken, hydrolysate, intestinal morphometry, salmon viscera, growth performance

1. Introduction

Broiler chickens have a number of critical periods when they are prone to delayed or retarded growth and to developing diseases. The first critical period is during hatching, at which time there is extensive intestinal development (Uni et al., 1998; Batal and Parsons, 2002) and the chickens are sorted by sex, moved to a new environment where they experience new physiological and microbiological challenges, and are introduced to solid feed. Another critical period is one to two weeks after hatching when the concentration of plasma IgG (IgY) is at its lowest due to the short half-life of maternal IgG (IgY) and low endogenous production post-hatching (Hamal et al., 2006). A highly digestible and palatable feed is often needed to stimulate feed intake and to support a healthy growth of the intestine and broiler chickens during these periods. The importance of a healthy intestine for productivity and disease resistance is well known and plays a role in animal welfare and economic considerations in commercial broiler chicken production (Choct, 2009).

Fish meal is often used as a feed ingredient in young animals and during periods where high protein digestibility and a balanced amino acid composition are needed to support the growth potential without challenging the protein metabolism. The positive effect of fish meal compared with traditional plant proteins on growth performance is well known. However, due to increasing prices of traditional fish meal, the feed industry has been searching for protein sources with the same – or better – qualities as fish meal. Novel marine protein sources have the potential to serve as an alternative to traditional fish meal. The annual Norwegian production of Atlantic salmon is 1.3 million tons and after filleting and withdrawal of the

primary products (e.g. filet, eviscerated salmon), about 336,000 tons of rest raw material are available for further processing into oils, silage, hydrolysates and fish meal (Olafsen et al., 2014). Viscera accounts for about 40% (wet weight basis) of the rest raw material (Olafsen et al., 2014) and it is of great importance to utilise this fraction to increase sustainability in salmon aquaculture. Viscera are often considered as a minor value product due to high enzymatic activity and immediate loss of integrity post-mortem. The high enzymatic activity can, however, be exploited in downstream applications, as the addition of water and slight heating for a defined time facilitates hydrolysis. After phase separation, it is possible to obtain oil, as well as an emulsion phase, a protein-rich aqueous phase (hereinafter referred to as salmon protein hydrolysate, **SPH**), and a sediment phase. Although not needed for hydrolysis of Atlantic salmon viscera, the addition of exogenous enzymes may produce other peptide fractions that are potentially rich in bioactive peptides with different properties (Opheim et al., 2015).

The high levels of smaller peptides and free amino acids may increase the digestibility and absorption rate of SPH. Additionally, as reviewed by Ngo et al. (2012), many marine peptides are reported to possess biological activity, including anti-oxidative, anti-hypertensive, anti-cancer and cholesterol-lowering effects with many potential applications in functional foods and pharmaceuticals.

A positive effect on growth rates from diets containing small proteins and peptides from marine sources has been reported in Atlantic salmon (*Salmo salar*) (Refstie et al., 2004; Hevroy et al., 2005; Kousoulaki et al., 2009), Japanese flounder (*Paralichthys olivaceus)* (Zheng et al., 2012) and in broiler chickens (Wagner and Bregendahl, 2007). However, no or little effect on growth performance was reported in piglets fed diets that included fish protein hydrolysate (Tucker et al., 2011; Nørgaard et al., 2012) or in broiler chickens fed diets with up to 8% shrimp hydrolysate inclusion (Mahata et al., 2008). Additionally, reduced growth performance was reported in Atlantic salmon fed diets with high inclusion levels of SPH (Refstie et al., 2004; Hevroy et al., 2005), and in broiler chickens fed diets with high levels of a fish silage (Santana-Delgado et al., 2008) and shrimp hydrolysate (Mahata et al., 2008).

The main aim of this study was to investigate the effects of two inclusion levels of SPH in broiler chicken diets on growth performance and intestinal morphometry. A further goal was to examine the effects of using two different types of SPH from Atlantic salmon viscera with

endogenous enzymes only or endogenous enzymes in addition to papain and bromelain during hydrolysis.

2. Material and methods

2.1. Animals, feeding and weighing

All chickens were cared for according to the laws and regulations controlling experiments with live animals in Norway (the Animal Welfare Act of December 28, 2009, and the Animal Protection Ordinance concerning experiments with animals of January 15, 1996).

Immediately after hatching, 5282 Ross308 broiler chickens were sorted by sex and allocated into 66 pens in four rooms. One room contained 30 pens, while three rooms contained 12 pens each. Each pen (5.3–5.9 m^2) consisted of 40 females and 40 males, except one pen that consisted of 82 broiler chickens (41 females and 41 males). The lighting regime was 23 hours of light and 1 hour in darkness for the first 2 days, and 16 hours of light and 8 hours of darkness from day 3 onwards.

Feed intake was recorded for days 0–9, 10–20 and 21–28 post-hatch and average daily feed intake (ADFI) was calculated within these periods. Broiler chickens were weighed in each pen at days 0, 10, 21 and 28, and the ADG was calculated for each period and used for further analysis. The feed efficiency ratio (G:F) was calculated as g weight gain/g feed intake. Apparent metabolic energy content in the experimental diets was calculated by use of analysed values of the diets and the equation from the (European Commission, 2009) with the modification that the value 2.2 was used as fixed factor for sugar. Energy utilisation was calculated as g weight gain/ME intake. Daily water intake was recorded in each pen in one of the rooms consisting of 12 pens. Litter quality was recorded weekly on a 0–5 scale (0 = dry and 5 = watery). There was no exchange or addition of litter bedding during the experiment. Dead birds were recorded each day and the mortality was calculated for each period. Growth performance data was adjusted for mortality.

2.2. Hydrolysates

Viscera from Atlantic salmon (*Salmo salar*) were used as the starting material for the hydrolysing process at an industrial hydrolysing plant (Nutrimar A/S, Frøya, Norway). The hydrolysis process was performed within 2 hours after slaughter and ran for 1 hour at 52°C before separation of the hydrolysate using a decanter. After heat-inactivation at 90°C for 10

minutes, formic acid was added to the hydrolysates until the pH was <4. One hydrolysis was performed with only the endogenous enzymes (Ve), and a second hydrolysis was performed after the addition of the commercial enzymes papain plus bromelain (VePaBr). Both papain (powder on maltodextrin, 500 TU/mg) and bromelain (powder on maltodextrin, 400 GDU/g), both from Enzybel Intl. SA, (Belgium), were added to 0.05% on a wet weight basis. Hydrolysate composition is outlined in Table 1 and further details of these hydrolysates are given in Opheim et al. (2015).

2.3. Experimental diets

Six experimental diets were produced for the starter period (days 0-10) and six diets for the grower period (days 11-28), all diets were based on standards for commercial starter and grower feed formulations for broiler chickens. One negative control diet with protein from only plant sources (PP) and one positive control diet with 4% fish meal (FM). Two diets were produced with 10% inclusion of SPH (10% SPH diets, both Ve and VePaBr) replacing 100% of the fish meal protein and two diets with 5% inclusion of SPH (5% SPH diets, both Ve and VePaBr) replacing 50% of the fish meal protein. Nutrient digestibility values from CVB (2005) were used for all ingredients, except for SPH where the protein and amino acids digestibility values were set to 95%. The experimental diets in each period were formulated to be isoenergetic, equal in digestible lysine per energy unit, and equal for digestible methionine+cysteine, threonine, tryptophan and arginine per lysine unit, together with an equal concentration of sodium, calcium and sum EPA+DHA (Table 2). All diets were formulated to meet or exceed the recommendations from the National Research Council (NRC, 1994). Diets were allocated randomly to pens within each room, and chickens were fed *ad libitum*. All diets were well tolerated and the voluntary feed intake was satisfactory during the experiment. Diet formulation, calculations and chemical compositions are outlined in Table 2. Due to the high water content in the 10% SPH diets, these diets were dried after production for better preservation. On a dry matter basis, the calculated and analysed values of the experimental diets were as expected.

2.4. Sampling and morphometry of the small intestine

At day 10 post-hatch, one bird from each pen (in total, 11 birds from each diet group) was euthanised by cervical dislocation after sedation with a head blow before sampling. Weight and sex were recorded. Between 3 and 4 cm of the distal segments of the duodenum (denoted

from the gizzard to the pancreatic and gallbladder ducts), jejunum (from the pancreatic ducts to Meckel's diverticulum) and ileum (Meckel's diverticulum to the ileo-cecal junction) were dissected and rinsed carefully with 10% formaldehyde before being submersed in 10% formaldehyde. After 4 days' fixation, the middle part of each intestinal segment sample was cut, dehydrated in alcohol and embedded in paraffin by standard procedures. Two sections, at least 25 µm apart, were mounted on glass slides and stained with hematoxylin and eosin following standard protocols. All processed slides were examined on a Jenoptic ProgRes® CapturePro 2.8 camera (Jena, Germany) mounted on a Leica DM2000 microscope. The handling of the software was as recommended by the manufacturer and a calibration slide was used as a control for each day of reading to ensure calibrated measurements. From each of the intestinal sections, the 10 longest and most intact villi were measured. Villi height was considered as the distance from the top of the villi to the villi–crypt junction. Villi width was measured in two places (upper and lower thirds of the villi), and the mean value was used in further calculations. Crypt depth was from the villi–crypt junction to the base of the crypt. Villi absorption area was calculated by mean villi width x villi height on each measured villi. The mean value of 10 measurements from each intestinal site was considered as the value from one broiler chicken and was used for further analysis.

2.5. Chemical analysis

In the hydrolysates, CP was measured in duplicate by the Kjeldahl method according to ISO 5983-2 (ISO, 2009) and water and ash content according to ISO 6496 (ISO, 1999) and ISO 5984 (ISO, 2002). The method of Bligh and Dyer (1959) was used for the extraction of lipids. Analysis of amino acids was performed in duplicate after initial preparation from one sample, and as described according to Cohen and Michaud (1993). The degree of hydrolysis was determined by the o-phthaldialdehyde (OPA) method (Nielsen et al., 2001). The molecular weight distribution was determined as described by Wang-Andersen and Haugsgjerd (2011) using high-performance liquid chromatography **(**HPLC). In brief, the molecular weight distribution was analysed by use of Superdex peptide 10/300 GL columns (ID: 10 mm, length: 300 mm; GE Healthcare) with a flow-rate of 0.5 mL/min and with 214-nm UV detection. The eluent solutions were according to the manufacturer's recommendations. Analyses of crude protein, fat, water and ash in the diets were performed with methods adapted from the EU directive 76/371 EC regulation EF nr. 152/2009 (European Commission, 2009).

186 *2.6. Statistical analysis*

187 The experimental unit was pen for the growth performance analysis, whereas broiler chicken
188 was the unit for the intestinal analysis. Statistical analyses were performed using the PROC
189 MIXED procedure in the SAS 9.2 statistical package (SAS Institute Inc, Cary, USA). Tukey
190 adjustments were used in the multiple comparisons when a significant F-test was detected.
191 Data are presented as the least-squares mean with a pooled standard error of the mean
192 (s.e.m.). P-values <0.05 were considered significant and P<0.10 as a tendency.

193 For the growth performance parameters, the model consisted of the diet as the fixed effect and
194 the room as a random effect. The interaction term diet x room was tested and, when
195 significant, was included in the model as a random effect, but otherwise was excluded.

196 In the morphometry analysis, the model consisted of diet and sex as fixed effects. The
197 interaction term diet x sex was included in the model as a random variable when it was found
198 to be significant. Weight did not have a strong correlation with any of the morphometric
199 parameters and hence was excluded from the model.

200 Contrast analysis were performed by the contrast option in SAS.

201 **3. Results**

202 *3.1. Starter period*

203 In the starter period, the ADG was significantly higher in broiler chickens fed the 10% SPH
204 diets compared with broiler chickens fed the PP, FM and 5% SPH diets (Table 3). The largest
205 difference was detected between the 10% Ve and the FM diet group, where the 10% Ve diet
206 group showed 11.6% higher ADG than the group fed the FM diet. Additionally, the 5% SPH
207 diet groups showed increased growth compared with the PP and FM diet groups, although it
208 was not significant for the 5% Ve diet group. No significant differences were found in the
209 ADFI (P = 0.565) or ME intake (P = 0.750) between the diet groups in the starter period. No
210 significant differences in the G:F ratio, which varied between 0.89 and 1.01, were detected
211 between the diet groups. However, by contrast analysis, broiler chickens fed the 10% SPH
212 diets showed a higher G:F ratio than the PP (PP vs. 10% SPH, P = 0.036), FM (FM vs. 10%
213 SPH, P = 0.009) and 5% SPH (5% SPH vs. 10% SPH, P = 0.002) diet groups in the starter
214 period. No significant differences (P = 0.172) in energy utilisation were detected between the
215 diet groups. However, by contrast analysis, broiler chickens fed the 10% SPH diets showed

significant higher energy utilisation than the broiler chickens fed the 5% SPH diets (P = 0.012) and a tendency towards higher energy utilisation than the broiler chickens fed the FM diet (P = 0.067). No statistical differences were found between the PP and FM diet groups for ADG, ADFI, G:F ratio, ME intake or energy utilisation during the starter period.

3.2. Grower period

In the early grower period (days 10–20), ADG was significantly higher in groups fed the 5% Ve, 10% Ve and 10% VePaBr diets compared with the PP diet group (Table 3). Additionally, three of the hydrolysate diet groups (5% Ve, 10% Ve and 5% VePaBr) showed significantly higher ADFI than the PP diet group. Although a significant statistical model (P = 0.019), the G:F ratio showed only small numerical variations and the post-hoc test did not detect any significant differences between the diet groups on an as-fed basis. However, by contrast analysis, broiler chickens fed the PP and FM diets showed significantly higher G:F ratio compared with the 5% SPH diet groups during days 10–20 (PP vs. 5% SPH, P = 0.003 and FM vs. 5% SPH, P = 0.043). The PP diet group showed significant higher energy utilisation compared with the 5% Ve and 10% Ve diet groups during days 10–20 post-hatch. In the second phase of the grower period (days 21–28), the broiler chickens fed 5% SPH and FM diets showed the highest ADG, which were significantly higher than the PP diet. No significant differences in ADFI were detected between any of the diet groups and the G:F ratio was significantly higher in the FM diet group compared with the PP diet group. Energy utilisation was significantly higher in the group fed the FM diet compared with the PP diet, and both 10% SPH diets.

The ADG during the whole grower period (days 10–28) was significantly higher in the broiler chickens fed marine protein sources compared with the PP diet. However, the marine protein groups showed higher ADFI compared with the PP diet group during the overall grower period, although this was non-significant for the 10% VePaBr diet group. Despite only small numerical differences in the G:F ratio, significant differences were found between the groups fed the 10% VePaBr and the FM diets compared with the group fed the 5% VePaBr diet, which showed the lowest G:F ratio.

3.3. Whole period

Broiler chickens fed the PP diet showed significant lower ADG and ME intake values than those of the FM and SPH diet groups. In addition, broiler chickens fed the PP diet showed

significantly lower ADFI than broiler chickens fed the FM diet, both of the Ve diets, and the 5% VePaBr diet. The ME intake was significantly higher in the groups fed the Ve diets compared with the groups fed the PP and FM diets. The overall G:F ratios were highest in the groups fed the 10% SPH diets, which were higher than the PP (PP vs. 10% SPH, $P = 0.002$), FM (FM vs. 10% SPH, $P = 0.017$), and 5% SPH (5% SPH vs. 10% SPH, $P < 0.001$) diet groups. No major differences between the diet groups Ve and VePaBr were detected for ADG, ADFI or G:F ratio when the inclusion level was equal, except for ADG in the period 10-20 days, in which the 5% Ve diet group showed significantly higher ADG compared with the 5% VePaBr diet group.

No differences in litter quality were detected until day 28. At day 28, the litter in the PP diet group was more moist (score 3.37) compared with the diet groups containing marine proteins (score 3.18–3.23), both numerically and by contrast (PP vs. SPH, $P = 0.003$ and PP vs. FM, $P = 0.007$). No difference in water intake was detected between any of the diet groups at any time during the experiment (data not shown). Similarly, no differences between the diet groups were found for mortality rate, which varied from 1.9% to 3.2% for the whole period ($P = 0.660$).

3.4. Morphometry

The duodenal villi height was significantly longer in the broiler chickens fed marine protein diets compared with the broiler chickens fed the PP diet (PP vs. SPH, $P = 0.019$ and PP vs. FM, $P = 0.013$) at day 10 post-hatch (Table 4). Furthermore, the ileal villi tended to be longer (PP vs. SPH, $P = 0.09$ and PP vs. FM, $P = 0.09$) and to have a larger absorption area (PP vs. SPH, $P = 0.06$ and PP vs. FM, $P = 0.04$) in the marine protein-fed groups compared with the group fed the PP diet.

4. Discussion

The present study showed a significant increase in ADG in broiler chickens fed starter diets (days 0–9) with 10% inclusion of SPH compared with PP, FM and 5% SPH diets. A similar 12% increase in ADG was reported by Wagner and Bregendahl (2007) when 11.5% condensed salmon protein concentrate was added to a broiler chicken diet compared with a plant protein-based diet. The same authors concluded that the increased growth rate was due to a higher feed intake in the salmon protein concentrate diet. However, we did not observe any significant differences in ADFI or ME intake between the diet groups in the starter period

278 in the current study. An increase in ADG without affecting feed intake or the feed:gain ratio
279 during the first 7 days by use of a porcine mucosa hydrolysate in the broiler chicken diet is
280 also reported by Mateos et al. (2014). Increased ADG together with no increase in ADFI
281 normally improves the G:F ratio. Our study did not detect differences in the G:F ratio despite
282 a statistically significant model. However, by contrast analysis, the effect of increased ADG
283 was associated with improved G:F when comparing the 10% SPH diets with the PP and FM
284 diets.

285 The observed increase in ADG might be associated with increased absorption rates of amino
286 acids from peptides by use of SPH in the diets compared with FM and PP diets. Absorption of
287 amino acids into the portal blood was shown to be more efficient from peptides compared
288 with free amino acids and intact protein in rats (Kodera et al., 2006). Gilbert et al. (2010)
289 reported higher mRNA expression of several peptide and amino acid transporters when a
290 hydrolysed whey protein source was given to chickens compared with both intact whey
291 protein and free amino acids. Additionally, although the broiler chicken intestine develops
292 extensively during the first 2 weeks and appears immature at hatching (Uni et al., 1998; Iji et
293 al., 2001; Batal and Parsons, 2002), there is a high capacity for absorption of glucose, amino
294 acids and easily digestible proteins immediately after hatching (Batal and Parsons, 2002).

295 The marine protein diet groups (FM and SPH diets) showed higher ADG during the grower
296 period (days 10–28) compared with the group fed the PP diet. This difference between the
297 marine protein and PP diets might be caused by factors in the marine protein diets that
298 enhance growth or factors in the PP diet that impair intestinal development and growth, or
299 combinations thereof. The PP diet contained more soya compared with the other diets and the
300 anti-nutritional factors – such as lectins, oligosaccharides, saponins and trypsin inhibitors,
301 which are known to be present in soya – might have had a negative impact on growth
302 performance. Marine protein sources contain nitrogenous components, such as taurine and
303 hydroxyproline, which are not detected in soy protein isolate (Liaset et al., 2003). Both
304 taurine and hydroxyproline are reported to be positively correlated with growth parameters in
305 Atlantic salmon (Kousoulaki et al., 2009). Surprisingly, the inclusion of taurine in broiler
306 chicken diets resulted in impaired intestinal mucosal development and enhanced feed
307 utilisation for the first 7 days, without affecting the ADG or ADFI (Huang et al., 2014).
308 Despite several possible factors, no obvious candidates were detected in our study and further

investigation is needed to fully understand the underlying relationships between the observed differences between the PP and marine protein diets.

Withholding feed from broiler chickens for 36 hours after hatching leads to retarded growth of the intestines for up to 9 days (Uni et al., 1998), and this impaired intestinal growth is difficult to remedy later in the production (Juul-Madsen et al., 2004). Luminal stimulation and adequate feed intake, therefore, seem to be crucial for intestinal development in broiler chickens. In our experiment, inclusion of fish meal and SPH in the broiler chicken starter diets tended to increase the villi height in the duodenum and ileum, in addition to the ileal absorption area, compared with the PP diet group. The improved development of the small intestine during the first days of life may favour growth performance at older ages (Wijtten et al., 2010) as nutrient absorption is thought to be facilitated with increased villi height associated with increased luminal absorptive area. In the present study, broiler chickens fed the FM diet showed higher ADG than broiler chickens fed the PP diet during the late grower period (days 21–28) despite similar ADG during the starter period. The delayed increase in ADG in the groups fed the FM diet compared with the SPH diets corresponds well with the morphometric findings in the broiler chicken intestine at day 10 as no morphometric differences were detected between the FM and SPH diets at this stage.

In the late grower period, the effect of SPH compared with FM diminished and no significant differences were detected in ADG, ADFI or G:F ratio. At this stage, the broiler chicken intestines are more mature and compensatory regulation of amino acid absorption and metabolism might conceal the effects of SPH on growth performance. Maturity of the intestines, in addition to the differences in hydrolysate composition and species, may explain some of the conflicting results reported on the effect of hydrolysates compared with intact proteins in growth performance. The efficiency of protein absorption seems to be highly regulated. In a dog study, Zhao et al. (1997), it was observed a dose-dependent slowing of the intestinal transit time and reported that intact protein reduces the transit time more effectively than the hydrolysed form. An almost constant protein absorption efficiency was reported when using different concentrations of hydrolysate and intact protein, although the amount of protein absorbed in the intestinal proximal parts was significantly higher when protein was given in the hydrolysed form compared with the intact form (Zhao et al., 1997).

As different enzymes were used during hydrolysis, this may have influenced the peptide composition of the two different hydrolysates from Atlantic salmon viscera that were used in

the present study (Opheim et al., 2015). However, no differences were detected on growth performance between the hydrolysates (Ve and VePaBr) at the same inclusion level. Hence, no additional effects on growth performance or intestinal morphometry in broiler chickens were detected by the addition of papain and bromelain when hydrolysing Atlantic salmon viscera for inclusion in broiler chicken diets. Endogenous enzymes in viscera, therefore, seem sufficient for hydrolysis of viscera in order to obtain the reported effects detected in this study. High levels of fish protein hydrolysates in the diets of Atlantic salmon were reported to have inhibitory effects on growth performance (Refstie et al., 2004; Hevroy et al., 2005), and two inclusion levels of 5% and 10% SPH were used in this study in order to detect a dose-response effect. The largest effect of inclusion level was seen during the starter period, as the 10% SPH diet groups showed higher ADG than the 5% SPH diet groups. However, for the overall period, broiler chickens fed the 10% SPH diets showed similar ADG and lower ADFI, and hence higher G:F ratio, compared with the broiler chickens fed the 5% SPH diets.

High initial growth rates are associated with metabolic disorders and leg problems in broiler chickens (Bessei, 2006), and therefore, high initial ADG is not necessarily an ultimate goal. Despite significantly different growth rates in the starter period, no differences in mortality between any of the diet groups were detected at any time in our experiment. Conflicting results were also described by Wijtten et al. (2010) who reported lower mortality due to leg problems in Ross308 broiler chickens with high early weight gain compared with the lower early weight gain group. The same researchers did not find any relationship between weight development in the small intestines and leg problems. The observed increase in ADG in the groups fed the SPH diets during the starter period might be due to increased visceral weight, although this was not examined in the present study. The litter score indicates that there was more moisture in the groups fed the PP diet than the groups fed the marine protein diets at day 28. This might be an important result, both related to growth performance and animal welfare, as litter high in moisture predisposes towards higher microbial activity and an increase in temperature and ammonia in the environment. Such an environment increases the incidence of contact dermatitis and respiratory diseases in the chickens (Bessei, 2006) and hence should be avoided.

5. Conclusion

The present study shows that hydrolysed Atlantic salmon viscera at 5% and 10% inclusion levels increase broiler chicken growth performance when included in starter diets compared

with either a plant protein-based or a fish meal diet. In the grower period, the effect between hydrolysate and fish meal diets diminished – however, the plant protein-based diet group achieved lower final live-weight than the marine protein diet groups. A tendency towards longer villi in the proximal and distal parts of the small intestine, which might facilitate increased nutrient absorption and higher live-weight later in the production, was detected in the marine protein diet groups compared with the group fed the plant protein-based diet. No adverse effects on mortality or litter quality were detected when salmon hydrolysate was included in the broiler chicken diet. Salmon protein hydrolysate might be a novel protein source for broiler chickens.

Acknowledgements

The authors thank Benny Berg at the Scandinavian Poultry Research Center for management and ethical handling of the chickens during the feeding experiment. We are grateful to Tore Remman at Nutrimar A/S for production and delivery of specified hydrolysates, and Hanne R. Skjelstad and Ketil Skår at the National Veterinary Institute Trondheim for lending microscopic facilities and assistance in the morphometric analysis. The authors also acknowledge the financial support from Regional Research Found Mid-Norway (ES486682/217393) and the National Research Council of Norway (221364/O30).

References

Batal, A.B., Parsons, C.M., 2002. Effects of age on nutrient digestibility in chicks fed different diets. Poult. Sci. 81, 400-407.

Bessei, W., 2006. Welfare of broilers: a review. World's Poultry Science Journal 62, 455-466.

Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37, 911-917.

Choct, M., 2009. Managing gut health through nutrition. Br. Poult. Sci. 50, 9-15.

Cohen, S.A., Michaud, D.P., 1993. Synthesis of a fluorescent derivatizing reagent, 6-aminoquinolyl-n-hydroxysuccinimidyl carbamate, and its application for the analysis of hydrolysate amino acids via high-performance liquid chromatography. Anal. Biochem. 211, 279-287.

CVB, 2005. Veevoedertabel [Feedstuff table, nutritional value of feed ingredients]. Central Veevoederbureau, Lelystad, The Netherlands.

404 European Commission, 2009. Commission Regulation 2009/152/EC of 27 January 2009
405 laying down the methods of sampling and analysis for the official control of feed.
406 Official Journal of European Communities L 54 (2009), 1-130.
407 Gilbert, E.R., Li, H., Emmerson, D.A., Webb, K.E., Wong, E.A., 2010. Dietary protein
408 composition influences abundance of peptide and amino acid transporter messenger
409 ribonucleic acid in the small intestine of 2 lines of broiler chicks. Poult. Sci. 89, 1663-
410 1676.
411 Hamal, K.R., Burgess, S.C., Pevzner, I.Y., Erf, G.F., 2006. Maternal antibody transfer from
412 dams to their egg yolks, egg whites, and chicks in meat lines of chickens. Poult. Sci. 85,
413 1364-1372.
414 Hevroy, E.M., Espe, M., Waagbo, R., Sandnes, K., Ruud, M., Hemre, G.I., 2005. Nutrient
415 utilization in Atlantic salmon (Salmo salar L.) fed increased levels of fish protein
416 hydrolysate during a period of fast growth. Aquacult. Nutr. 11, 301-313.
417 Huang, C.X., Guo, Y.M., Yuan, J.M., 2014. Dietary taurine impairs intestinal growth and
418 mucosal structure of broiler chickens by increasing toxic bile acid concentrations in the
419 intestine. Poult. Sci. 93, 1475-1483.
420 Iji, P.A., Saki, A., Tivey, D.R., 2001. Body and intestinal growth of broiler chicks on a
421 commercial starter diet. 1. Intestinal weight and mucosal development. Br. Poult. Sci.
422 42, 505-513.
423 ISO, 1999. ISO 6496 Animal feeding stuffs — Determination of moisture and other volatile
424 matter content. ISO standard 6496. International Standards Organization.
425 ISO, 2002. ISO 5984:2002 Animal feeding stuffs — Determination of crude ash. ISO
426 standard 5984:2002. International Standards Organization.
427 ISO, 2009. ISO 5983-2 Animal feeding stuffs — Determination of nitrogen content and
428 calculation of crude protein content — Part 2: Block digestion/steam distillation
429 method. International Standards Organisation.
430 Juul-Madsen, H.R., Su, G., Sorensen, P., 2004. Influence of early or late start of first feeding
431 on growth and immune phenotype of broilers. Br. Poult. Sci. 45, 210-222.
432 Kodera, T., Hara, H., Nishimori, Y., Nio, N., 2006. Amino acid absorption in portal blood
433 after duodenal infusions of a soy protein hydrolysate prepared by a novel soybean
434 protease D3. J. Food Sci. 71, 517-525.

435 Kousoulaki, K., Albrektsen, S., Langmyhr, E., Olsen, H.J., Campbell, P., Aksnes, A., 2009.
436 The water soluble fraction in fish meal (stickwater) stimulates growth in Atlantic
437 salmon (Salmo salar L.) given high plant protein diets. Aquaculture 289, 74-83.
438 Liaset, B., Julshamn, K., Espe, M., 2003. Chemical composition and theoretical nutritional
439 evaluation of the produced fractions from enzymic hydrolysis of salmon frames with
440 Protamex (TM). Process Biochem. 38, 1747-1759.
441 Mahata, M.E., Dhama, A., Ryanto, H.I., Rizal, Y., 2008. Effect of substituting shrimp waste
442 hydrolysate of Penaeus merguensis for fish meal in broiler performance. Pak. J. Nutr. 7,
443 806-810.
444 Mateos, G.G., Mohiti-Asli, M., Borda, E., Mirzaie, S., Frikha, M., 2014. Effect of inclusion of
445 porcine mucosa hydrolysate in diets varying in lysine content on growth performance
446 and ileal histomorphology of broilers. Anim. Feed Sci. Technol. 187, 53-60.
447 Ngo, D.H., Vo, T.S., Ngo, D.N., Wijesekara, I., Kim, S.K., 2012. Biological activities and
448 potential health benefits of bioactive peptides derived from marine organisms. Int. J.
449 Biol. Macrormolec. 51, 378-383.
450 Nielsen, P.M., Petersen, D., Dambmann, C., 2001. Improved method for determining food
451 protein degree of hydrolysis. Food Chem. Toxicol. 66, 642-646.
452 NRC, 1994. Nutrient Requirements of Poultry: Ninth Revised Edition, 1994. The National
453 Academies Press.
454 Nørgaard, J.V., Blaabjerg, K., Poulsen, H.D., 2012. Salmon protein hydrolysate as a protein
455 source in feed for young pigs. Anim. Feed Sci. Technol. 177, 124-129.
456 Olafsen, T., Richardsen, R., Nystøyl, R., Strandheim, G., Kosmo, J.P., 2014. Analyse marint
457 restråstoff, 2013. SINTEF Fisheries and Aquaculture.
458 Opheim, M., Slizyte, R., Sterten, H., Provan, F., Larssen, E., Kjos, N.P., 2015. Hydrolysis of
459 Atlantic salmon (Salmo salar) rest raw materials–Effect of raw material and processing
460 on composition, nutritional value, and potential bioactive peptides in the hydrolysates.
461 Process Biochem. 50, 1247-1257.
462 Refstie, S., Olli, J.J., Standal, H., 2004. Feed intake, growth, and protein utilisation by post-
463 smolt Atlantic salmon (Salmo salar) in response to graded levels of fish protein
464 hydrolysate in the diet. Aquaculture 239, 331-349.
465 Santana-Delgado, H., Avila, E., Sotelo, A., 2008. Preparation of silage from Spanish mackerel
466 (Scomberomorus maculatus) and its evaluation in broiler diets. Anim. Feed. Sci.
467 Technol. 141, 129-140.

468 Tucker, J.L., Naranjo, V.D., Bidner, T.D., Southern, L.L., 2011. Effect of salmon protein
469 hydrolysate and spray-dried plasma protein on growth performance of weanling pigs. J.
470 Anim. Sci. 89, 1466-1473.
471 Uni, Z., Ganot, S., Sklan, D., 1998. Posthatch development of mucosal function in the broiler
472 small intestine. Poult. Sci. 77, 75-82.
473 Wagner, C., Bregendahl, K., 2007. Effect of dietary salmon protein concentrate on growth
474 performance of broiler chickens. Animal Industry Report: AS 653,ASL R2220. Iowa
475 State University, http://lib.dr.iastate.edu/ans_air/vol653/iss1/46.
476 Wang-Andersen, J., Haugsgjerd, B.O., 2011. Forbedret analyse-metodikk for
477 peptidstørrelsesfordeling i marine proteinhydrolysater. Nofima, Tromsø, Norway.
478 Wijtten, P.J., Hangoor, E., Sparla, J.K., Verstegen, M.W., 2010. Dietary amino acid levels
479 and feed restriction affect small intestinal development, mortality, and weight gain of
480 male broilers. Poult. Sci. 89, 1424-1439.
481 Zhao, X.-T., McCamish, M.A., Miller, R.H., Wang, L., Lin, H.C., 1997. Intestinal transit and
482 absorption of soy protein in dogs depend on load and degree of protein hydrolysis. J.
483 Nutr. 127, 2350-2356.
484 Zheng, K., Liang, M., Yao, H., Wang, J., Chang, Q., 2012. Effect of dietary fish protein
485 hydrolysate on growth, feed utilization and IGF-I levels of Japanese flounder
486 (Paralichthys olivaceus). Aquacult. Nutr. 18, 297-303.

487 **Table 1**

488 Composition of Atlantic salmon viscera hydrolysates (As is basis).

	Salmon protein hydrolysate (SPH)	
Hydrolysate	Ve[a]	VePaBr[b]
Proximal content (%)		
Dry matter	38.5	39.6
Crude protein	28.1	28.5
Lipids	6.2	5.5
Ash	2.8	2.8
Indispensible amino acids (%)		
Arginine	1.40	1.41
Histidine	0.71	0.71
Isoleucine	1.03	1.02
Leucine	1.77	2.13
Lysine	1.79	1.79
Methionine	0.64	0.65
Phenylalanine	0.97	0.95
Threonine	0.97	0.70
Tryptophan	0.29	0.29
Valine	1.25	1.24
Dispensible amino acids (%)		
Alanine	1.27	1.30
Aspartic acid	1.96	1.94
Cysteine	0.34	0.34
Glutamic acid	2.83	2.79
Glycine	1.61	1.63
OH-proline	0.19	0.20
Proline	1.07	1.10
Serine	1.07	1.05
Tyrosine	0.70	0.70
Molecular weight distribution (Dalton) (% of soluble protein)		
>20 000	0.2	0.2
20 000-15 000	0.1	0.1
15 000-10 000	0.5	0.4
10 000-8 000	0.5	0.4
8 000-6 000	0.8	0.7
6 000-4 000	1.7	1.6
4 000-2 000	5.4	5.4
2 000-1 000	9.0	9.2
1 000-500	10.5	11.1
<500	71.3	71.0
Degree of hydrolysis	37.3	40.8

489 [a] Ve: SPH from viscera obtained with endogenous enzymes.

490 [b] VePaBr: SPH from viscera obtained by addition of papain and bromelain.

491 **Table 2**

492 Diet formulation, calculated and analysed composition of experimental diets in broiler chickens (As is basis).

	Starter diets						Grower diets					
			Salmon protein hydrolysate (SPH)						Salmon protein hydrolysate (SPH)			
			Ve		VePaBr				Ve		VePaBr	
	PP	FM	5%	10%	5%	10%	PP	FM	5%	10%	5%	10%
Ingredient g/kg												
LT Fishmeal[a]	-	4.00	2.00	-	2.00	-	-	4.00	2.00	-	2.00	-
SPH – Ve[b]	-	-	5.00	10.00	-	-	-	-	5.00	10.00	-	-
SPH – VePaBr[c]	-	-	-	-	5.00	10.00	-	-	-	-	5.00	10.00
Wheat	36.16	39.54	36.39	33.22	36.38	33.22	40.81	43.83	41.04	37.87	41.03	37.87
Soya HI-PRO	26.08	20.70	21.47	22.25	21.46	22.21	22.05	17.00	17.44	18.22	17.42	18.18
Corn	14.00	14.00	14.00	14.00	14.00	14.00	14.00	14.00	14.00	14.00	14.00	14.00
Corn gluten	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00
Oats	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Soy oil	4.22	4.17	4.84	5.40	4.61	5.06	4.14	4.13	4.75	5.31	4.53	4.97
Vitamin - mineral premix[d]	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00
Salmon oil	1.27	-	-	0.11	0.23	0.46	1.27	-	-	0.11	0.23	0.46
Limestone	0.74	0.64	0.73	0.82	0.73	0.82	0.43	0.32	0.41	0.51	0.41	0.50
Mono calcium phosphate	0.44	0.20	0.25	0.31	0.26	0.31	0.24	-	0.05	0.10	0.06	0.11
Sodium bicarbonate	0.30	0.23	0.28	0.33	0.28	0.33	0.31	0.23	0.28	0.33	0.28	0.33
L-Lysine	0.40	0.32	0.32	0.32	0.32	0.32	0.40	0.30	0.32	0.32	0.32	0.32
DL-Methionine	0.21	0.16	0.17	0.19	0.17	0.19	0.19	0.14	0.16	0.17	0.15	0.17
L-Threonine	0.07	0.05	0.05	0.05	0.06	0.08	0.08	0.05	0.05	0.05	0.07	0.08
NaCl	0.10	-	-	-	-	-	0.10	-	-	-	-	-
Water evaporation[e]	-	-	-1.50	-3.00	-1.50	-3.00	-	-	-1.50	-3.00	-1.50	-3.00
Calculated value												
Metabolisable energy,	11.7	11.7	12.0	12.2	11.9	12.2	11.9	11.9	12.0	12.4	11.6	12.3

ME (MJ/kg)[f]												
Analysed composition (%)												
Dry matter	89.4	88.8	88.1	91.4	87.6	88.9	89.0	88.4	86.9	88.9	86.3	89.2
Crude protein	24.0	24.0	24.1	25.7	23.9	24.9	22.6	23.4	24.5	22.8	22.4	23.0
Crude fat	7.4	6.6	7.1	8.0	7.0	8.1	7.5	6.5	7.3	7.9	7.3	8.0
Starch	31.1	32.7	33.1	31.3	32.7	31.4	33.0	34.3	32.3	35.2	32.2	34.3
Crude ash	5.6	5.6	5.5	6.1	5.5	5.7	5.1	5.0	4.9	5.5	4.9	5.3
Calcium	0.89	0.89	0.88	1.01	0.92	0.87	0.73	0.72	0.77	0.80	0.71	0.80
Phosphorous	0.62	0.63	0.61	0.68	0.60	0.61	0.54	0.55	0.54	0.62	0.53	0.59

493 PP = Plant protein-based diet; FM = Fishmeal diet; Ve = SPH from viscera obtained with endogenous enzymes; VePaBr = SPH from
494 viscera obtained by addition of papain and bromelain

495 [a] LT Fishmeal: Norse-LT 94 Fishmeal (http://www.norsildmel.no).
496 [b] SPH-Ve: salmon protein hydrolysate from viscera obtained by use of endogenous enzymes.
497 [c] SPH-VePaBr: salmon protein hydrolysate from viscera obtained by use of papain and bromelain mixture.
498 [d] Premix: Vitamins, minerals, enzymes and coccidiostatics (Narasin).
499 [e] Water evaporation: Estimated water evaporation during feed production.
500 [f] Calculated ME value by equation from the European commission 2009 with the modification 2.2 used for sugar, based on diet
501 analysis.

Table 3

Effect of two different Atlantic salmon protein hydrolysates, with two inclusion levels, on growth performance in broiler chickens in the starter and grower period (n=11 pens).

			Salmon protein hydrolysate (SPH)						Contrast analysis			
			Ve		VePaBr				PP vs. SPH[a]		FM vs. SPH[b]	
	PP	FM	5%	10%	5%	10%	s.e.m.[c]	*P*-value[d]	5%	10%	5%	10%
Daily gain (g/day)												
Day 0-9	28.0 [c]	27.7 [c]	28.4 [bc]	30.9 [a]	29.0 [b]	30.0 [a]	0.5	<0.001	0.015	<0.001	<0.001	<0.001
Days 10-20	71.7 [c]	72.5 [bc]	74.4 [a]	73.6 [ab]	72.6 [bc]	73.6 [ab]	0.5	<0.001	<0.001	<0.001	0.031	0.015
Days 21-28	103.3 [b]	110.2 [a]	108.7 [a]	106.4 [ab]	108.0 [a]	106.8 [ab]	1.0	<0.001	<0.001	0.011	0.148	0.006
Grower period 10-28	84.1 [b]	87.2 [a]	87.9 [a]	86.5 [a]	86.5 [a]	86.7 [a]	0.5	<0.001	<0.001	<0.001	0.927	0.301
Whole period 0-28	64.1 [b]	66.0 [a]	66.6 [a]	66.6 [a]	65.9 [a]	66.4 [a]	0.4	<0.001	<0.001	<0.001	0.489	0.214
Daily feed intake (g/day)												
Days 0-9	31.0	32.4	31.9	30.5	33.2	30.6	2.4	0.565	0.300	0.790	0.913	0.237
Days 10-20	88.8 [c]	91.4 [bc]	96.7 [a]	94.0 [ab]	94.3 [ab]	91.3 [bc]	1.2	<0.001	<0.001	0.006	0.004	0.335
Days 21-28	146.7	147.8	148.9	147.7	150.6	147.0	2.1	0.355	0.073	0.723	0.267	0.767
Grower period 10-28	111.8 [d]	113.7 [bcd]	117.6 [a]	115.5 [abc]	116.8 [ab]	113.3 [cd]	1.1	<0.001	<0.001	0.005	<0.001	0.427
Whole period 0-28	83.0 [d]	85.2 [bc]	86.8 [ab]	85.0 [c]	87.1 [a]	83.7 [cd]	0.9	<0.001	<0.001	0.012	0.002	0.116
Gain to feed ratio (gain/intake)												
Days 0-9	0.92 [a]	0.89 [a]	0.90 [a]	1.01 [a]	0.89 [a]	0.98 [a]	0.05	0.031	0.513	0.036	0.965	0.009
Days 10-20	0.81 [a]	0.79 [a]	0.77 [a]	0.78 [a]	0.77 [a]	0.81 [a]	0.01	0.019	0.003	0.262	0.043	0.940
Days 21-28	0.71 [b]	0.75 [a]	0.73 [ab]	0.72 [ab]	0.72 [ab]	0.73 [ab]	0.01	0.049	0.085	0.109	0.053	0.041
Grower period 10-28	0.75 [ab]	0.77 [a]	0.75 [ab]	0.75 [ab]	0.74 [b]	0.76 [a]	0.01	0.007	0.147	0.642	0.001	0.114
Whole period 0-28	0.77 [bc]	0.77 [b]	0.77 [bc]	0.78 [ab]	0.76 [c]	0.79 [a]	0.01	<0.001	0.061	0.002	0.015	0.017
ME intake (MJ)												
Days 0-9	3.64	3.80	3.82	3.74	3.94	3.72	0.28	0.750	0.184	0.616	0.672	0.659
Days 10-20	11.61 [c]	11.94 [bc]	12.75 [a]	12.80 [a]	12.08 [bc]	12.33 [ab]	0.16	<0.001	<0.001	<0.001	0.010	<0.001

Days 21-28	12.20 [b]	12.29 [ab]	12.49 [ab]	12.79 [a]	12.28 [b]	12.62 [ab]	0.18	0.005	0.206	<0.001	0.531	0.007
Grower period 10-28	23.91 [d]	24.31 [cd]	25.37 [ab]	25.73 [a]	24.49 [cd]	25.02 [bc]	0.25	<0.001	<0.001	<0.001	0.005	<0.001
Whole period 0-28	27.57 [d]	28.29 [c]	29.16 [ab]	29.42 [a]	28.46 [c]	28.71 [bc]	0.28	<0.001	<0.001	<0.001	0.016	<0.001
Energy utilisation (g gain/ME intake)												
Days 0-9	78.00	75.77	74.74	82.38	74.77	80.90	4.26	0.172	0.292	0.240	0.738	0.067
Days 10-20	68.00 [a]	66.87 [ab]	64.15 [bc]	63.24 [c]	66.23 [ab]	65.70 [ab]	0.96	0.001	0.006	<0.001	0.093	0.018
Days 21-28	59.40 [bc]	62.94 [a]	61.10 [abc]	58.29 [c]	61.71 [ab]	59.37 [bc]	0.93	<0.001	0.039	0.551	0.125	<0.001
Grower period 10-28	63.34 [ab]	64.68 [a]	62.30 [bc]	60.44 [c]	63.56 [ab]	62.31 [bc]	0.59	<0.001	0.469	<0.001	0.004	<0.001
Whole period 0-28	65.02 [a]	65.32 [a]	63.90 [ab]	63.34 [b]	64.82 [ab]	64.71 [ab]	0.46	0.004	0.159	0.035	0.048	0.008

505 PP = Plant protein-based diet; FM = Fishmeal diet; Ve = SPH from viscera obtained with endogenous enzymes; VePaBr = SPH from
506 viscera obtained by addition of papain and bromelain; ME = metabolisable energy

507 [a] PP diet group vs. SPH diet groups, both Ve and VePaBr, and 5% and 10% levels of SPH.
508 [b] FM diet group vs. SPH diet groups, both Ve and VePaBr, and 5% and 10% levels of SPH.
509 [c] Pooled standard error of mean.
510 [d] *P*-value, all groups included.
511 [a,b,c,d] Values within a row with different superscripts differ significantly at $P<0.05$.

Table 4

Intestinal morphometric data in broiler chickens at day 10 fed experimental diets during the starter period (days 0-9 post-hatch). Diets contained two different hydrolysates with two different inclusion-levels in addition to one plant protein-based and one fish meal diet (n=11).

			Salmon protein hydrolysate (SPH)						Contrast analysis (P-values	
			Ve		VePaBr					
	PP	FM	5%	10%	5%	10%	s.e.m.[a]	*P*-value[b]	PP vs. SPH[c]	PP vs. FM[d]
Duodenum										
Villi height (µm)	1498	1660	1605	1598	1589	1680	45	0.082	0.019	0.013
Vabs ($\mu m^2 \times 10^{-3}$)	291	317	294	279	305	331	17	0.308	0.561	0.288
Crypt depth (µm)	105	111	105	107	98	120	12	0.836	0.830	0.735
Villi:Crypt ratio	14.4	15.3	15.2	15.6	16.3	15.5	0.9	0.747	0.187	0.472
Jejunum										
Villi height (µm)	617	612	570	607	619	610	27	0.813	0.604	0.896
Vabs ($\mu m^2 \times 10^{-3}$)	104	105	90	111	109	112	8	0.338	0.859	0.934
Crypt depth (µm)	87	90	77	83	80	82	6	0.675	0.316	0.758
Villi:Crypt ratio	7.6	7.2	7.5	7.5	8.0	7.5	0.5	0.925	0.935	0.567
Ileum										
Villi height (µm)	388	456	460	446	416	443	28	0.409	0.086	0.085
Vabs ($\mu m^2 \times 10^{-3}$)	55	71	66	70	63	69	5	0.284	0.056	0.038
Crypt depth (µm)	67	79	71	76	70	70	4	0.339	0.307	0.046
Villi:Crypt ratio	5.9	5.9	6.6	5.9	6.0	6.3	0.4	0.674	0.511	1.000

PP = Plant protein-based diet; FM = Fishmeal diet; Ve = SPH from viscera obtained with endogenous enzymes; VePaBr = SPH from viscera obtained by addition of papain and bromelain; Vabs = villi absorption area

[a] Pooled standard error of mean.

[b] *P*-value, all groups included.

[c] PP diet group vs. all SPH diet groups.

[d] PP diet group vs. FM diet group.

Paper IV

Atlantic salmon (*Salmo salar*) protein hydrolysate in diets for weaning piglets – Effect on growth performance, intestinal morphometry and microbiota composition

Margareth Opheim[a,c]*, Mikael Lenz Strube[b], Hallgeir Sterten[a], Margareth Øverland[c], Nils Petter Kjos[c]

[a]*Felleskjøpet Fôrutvikling AS, N-7018 Trondheim, Norway*

[b]*National Veterinary Institute, Technical University of Denmark, DK-1870 Frederiksberg C, Denmark*

[c]*Department of Animal and Aquacultural Sciences, Norwegian University of Life Sciences, N-1432 Aas, Norway*

*Corresponding author. Margareth Opheim. Email: margareth.opheim@fkf.no

Abstract

Salmon protein hydrolysates (SPH) from two different rest raw materials were evaluated in diets for weaning piglets. Four experimental diets were included in the study: one diet based on plant protein (PP), one diet based on fishmeal (FM), and two diets in which different SPHs replaced fishmeal. The experimental diets were fed to piglets from the day of weaning until 32 days post-weaning. In addition to live-weight and feed intake, intestinal sampling for morphometry and 16S rRNA gene sequencing were performed at day 11 on a subset of the animals. The duodenal villi absorption area was significantly larger in the SPH diet groups compared with the PP diet group ($p < 0.05$) and there was a tendency towards an increased duodenal villi height-to-crypt-depth ratio in the SPH diet groups compared to both PP (PP vs. SPH, $p = 0.13$) and FM (FM vs. SPH, $p = 0.13$) diet groups. A significant positive correlation between duodenal villi height and ADG during the first 11 days post-weaning was detected. Only small differences in intestinal microbiota community and no differences in growth performance were detected between the experimental diets. An inverse correlation was shown between *Turicibacter* and *Lactobacillus*, which together comprised most of the bacteria found in the ileal content. To conclude, SPH seem to be an interesting novel protein source with possible effects on intestinal health in weanling piglets.

Keywords: weaning piglet; hydrolysate; intestinal morphometry; growth performance; microbiota

1 Introduction

At weaning, the relationship between sow and offspring is abruptly disconnected and the piglets are forced to adapt immediately to a grain-based diet. In addition, piglets are often moved to nurseries and mixed with unfamiliar piglets, and hence are exposed to a new macro- and microenvironment and to psychological disruption. This period may induce intestinal barrier dysfunction, digestive disorders and reduced performance (Peace et al. 2011; Kim et al. 2012), which increases the piglet susceptibility to anorexia and post-weaning diarrhoea. In addition, the pancreatic and brush border proteolytic enzyme systems are still undergoing development at weaning (Pluske et al. 2003). Normally, post-weaning feed is highly digestible and the protein content and amino acid composition is fine-tuned to minimize the amount of protein reaching the large intestine, as undigested protein serves as a nutrient source for potential pathogens. It is therefore hypothesised that by using highly digestible feed ingredients and taking intestinal development into account, the feed utilisation and intestinal integrity will be improved, as a healthy intestine is important for high utilisation of feed, improved growth and disease resistance (Wu 2014). By feeding a mixture of peptides to rats, the availability and absorption of peptides, especially in the proximal part of the digestive system, was enhanced and stimulated (Kodera et al. 2006). Additionally, as reviewed by Harnedy and FitzGerald (2012) and Ngo et al. (2012), many marine peptides are proposed to exert a biological activity, including anti-oxidative, anti-hypertensive, anti-cancer and cholesterol-lowering effects.

It is shown that marine protein hydrolysates have a positive effect on growth performance when included in diets for salmon (Espe et al. 1999; Refstie et al. 2004; Hevroy et al. 2005). However, conflicting results are reported regarding the use of hydrolysates in other species as none or minor effects on growth performance were detected in piglets fed diets with salmon protein hydrolysate (Tucker et al. 2011; Nørgaard et al. 2012) or plant-based hydrolysates (Vente-Spreeuwenberg et al. 2004). Furthermore, reduced growth performance are reported in salmon fed diets with high levels of salmon protein hydrolysates (SPH) (Hevroy et al. 2005) and in broiler chickens fed diets with high levels of shrimp hydrolysate (Mahata et al. 2008).

The main aim of this study was to 1) investigate possible effects of SPH compared to non-hydrolysed fish protein and plant protein on growth performance, intestinal morphometry

and intestinal microbiota composition in piglets during the post-weaning period, and 2) compare two SPHs produced from different compositions of Atlantic salmon rest raw materials, namely viscera alone and a mixture of viscera, head and frame using the same processing conditions, in diets for piglets on growth performance, intestinal morphometry and ileal microbiota.

2 Material and methods

2.1 Animals, feeding and weighing

All piglets were cared for according to the laws and regulations controlling experiments with live animals in Norway (the Animal Welfare Act of 28 December, 2009, and the Animal Protection Ordinance concerning experiments with animals of 15 January, 1996).

The experiment was conducted at the Animal Production Experimental Centre at the Norwegian University of Life Sciences (Aas, Norway) in a randomised block design. At weaning, 96 piglets (age 34 ± 4 days, mean weight 12.5 ± 3.5 kg) of the breed (Norwegian Landrace x Yorkshire) x (Norwegian Landrace x Duroc) were blocked by sex, litter and live weight, and randomly allocated to four dietary treatments with four replicate pens per treatment. Each pen consisted of six piglets. At day 11 post-weaning, two piglets from each pen were sacrificed. Individual piglet weight was recorded at days 0, 11, and 32 post-weaning, and average daily gain (ADG) was calculated for each pen for the period 0–11 and 0–32 days. Accumulated feed intake was recorded for each pen on the same day as weighing, and average daily feed intake (ADFI) was calculated for the periods 0–11 and 0–32 days. Metabolisable energy was calculated from chemical analysis of the diets and according to Central Veevoederbureau (CVB 2005) before being included in calculation of energy intake. Feed efficiency is given as g gain/g feed (G:F) and energy utilisation as g gain/energy intake. Feed was given *ad libitum* from automatic feeders.

2.2 Hydrolysates

Two different rest raw materials from farmed Atlantic salmon (*Salmo salar*) were used as starting material for hydrolysis at the Nutrimar A/S industrial plant (Frøya, Norway), and hydrolysates were produced within two hours after slaughter. One starting material was 100% viscera (Ve), and the other consisted of a mixture of viscera, head and frame/trimmings (VHFe); 50:25:25% wet weight, respectively. No exogenous enzymes were added and hence only endogenous enzymes were active during hydrolysis. The hydrolysis process ran for one hour at

a temperature of 52°C before separation of the protein-rich aqueous phase (hereinafter referred to as salmon protein hydrolysate, **SPH)** using a decanter. After heat-inactivation at 90°C for 10 min, formic acid was added to the hydrolysates until pH <4. Chemical composition and molecular weight distribution of the hydrolysates are displayed in Table 1.

2.3 Experimental diets

Four experimental diets were included in the experiment: one diet based on plant protein (PP), one diet based on fishmeal (FM), and two diets with 10% inclusion of each of the hydrolysates (Ve and VHFe) replacing the fishmeal protein. The experimental diets were optimised to be isoenergic, equal in digestible lysine, methionine+cysteine, threonine, tryptophan, and valine per energy unit, together with an equal amount of EPA+DHA. All diets were formulated to meet or exceed the recommendations from the National Research Council (NRC 1998). Diet formulation, calculations and chemical compositions are displayed in Table 2. The two hydrolysate-containing diets were dried after feed production, due to high moisture content, to avoid spoilage during storage. On a dry matter basis, the calculated and analysed chemical values of the diets were equivalent.

2.4 Sampling

Two piglets (one male and one female) with average weaning weight from each pen were sampled at day 11. The 32 piglets were sedated and anesthetised with azaperone (Stresnil 40 mg/mL) and ketamine (Ketalar 50 mg/mL) before being euthanised with pentobarbital (Euthasol 400 mg/mL). Immediately after cardiac arrest, the abdominal wall was opened and the small intestine was dissected. The small intestinal length was recorded and intestinal samples of 4–5 cm in length were taken from 15–20 cm distal from the pylorus (duodenum), 300–305 cm (jejunum) and 20–25 cm (ileum) proximal to the ileocecal junction. Approximately 40–50 µL of the terminal ileal content was suspended in 1.5 mL RNAlaterTM (Ambion, Inc., Austin, Texas) and refrigerated at 4°C for 24 hours before being frozen at -80°C until further analysis. The small intestines were gently emptied of intestinal content and weighed after sampling. The middle part of each intestinal segment sample was cut, dehydrated in alcohol and embedded in paraffin using standard procedures after four days of fixation in 10% formaldehyde. Two sections, at least 25 µm apart, were mounted on glass slides and stained with haematoxylin and eosin following standard protocols. The slides were examined on a Jenoptic ProgRes® CapturePro 2.8 camera (Jena, Germany) mounted on a Leica DM2000

microscope (Wetzlar, Germany). The handling of the software was as recommended by the manufacturer. From each of the intestinal segments, the 10 longest, well-oriented and most intact villi were measured. Villi height was considered as the distance from the top of the villi top to the villi-crypt junction. Villi width was measured in two places (upper- and lower-thirds of the villi), and the mean value was used in further calculations. Crypt depth was measured as the distance from the villi-crypt junction to the base of the crypt. Villi absorption area was calculated by mean villi width x villi height of each measured villi. The mean value of 10 measurements from each intestinal site was considered as the value from one piglet and was used for further analysis.

2.5 Chemical analysis

The experimental diets were analysed with methods adapted from the EU directive 76/371 EC regulation EF nr. 152/2009 (European Commission 2009). Nitrogen was measured using a Kjeltec Auto System, and was multiplied by 6.25 to obtain CP. Fat was extracted after acid hydrolysis and weighed, and water and ash weighed after drying and combustion, respectively. Chemical analyses of hydrolysates were performed as described in Opheim et al. (2015) with the exception that fat was extracted using the ethyl acetate method NS 9402 (Standards Norway 1994).

2.6 Microbiota analysis

2.6.1 DNA extraction

Material from the terminal ileum was stored in RNAlater at -80˚C until DNA purification, which was done with the Maxwell® LEV Blood DNA Purification Kit (Promega Corporation, Madison, WI, USA). Briefly, 200 mg of sample was incubated for 30 min at 37°C in 200 µL of lysozyme mixture (20 mM Tris-HCL (pH 8), 2 mM EDTA, 1.2% Triton X and 25 mg/mL lysozyme). Afterwards, the samples were mixed with 350 µL lysis buffer, and one 5-mm stainless steel bead (Qiagen GmbH, Hilden, Germany) was added to the samples followed by shaking on a Qiagen TissueLyser II (Qiagen GmbH, Hilden, Germany) for 2 min at 20 Hz. Samples were then incubated for 1 hour at 56°C with 30 µL proteinase K. The DNA was then extracted on a Maxwell®16 Research Instrument System (Promega Corporation, Wisconsin, USA) according to the manufacturer's instructions. The concentration of the DNA was then quantified on a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies,

Wilmington, DE, USA).

2.6.2 16S rRNA gene PCR

The 16S rRNA gene was amplified through PCR using the universal primers V1-forward (5'-AGAGTTTGATCCTGGCTCAG-3') and V2-reverse (5'-CTGCTGCCTYCCGTA-3 ') (Strube et al. 2015) (Sigma-Aldrich, Broendby, Denmark) that target the V1/V2 region of the gene. Both primers were 5'-barcode tagged with unique hexameric barcodes for each sample. The reaction was carried out in 50-µL volumes containing 5 µL of 5x Gold *Taq* buffer (Applied Biosystems, Branchburg, NJ, USA), 0.5 µL AmpliTaq Gold® polymerase (Applied Biosystems, Branchburg, NJ, USA), 34.5 µL nuclease-free H_2O and 2 µL DNA template (10 ng/µL), 1 µL of each primer (20 µM), 2 µL of 10 mM dNTP and 4 µL of 25 mM $MgCl_2$. Reaction conditions were 94°C for 6 min, 30 cycles of 94°C for 45 s, 57°C for 45 s and 72°C for 90 s, followed by 72°C for 10 min. The PCR products were then checked for quality and concentration on an Agilent 2100 Bioanalyzer using the Agilent DNA 1000 kit (Agilent Technologies, Waldbronn, Germany) and then pooled in equimolar ratios (50 ng per barcoded sample). Following this, the pooled DNA samples were then purified for detergents and primers using a Qiagen MinElute® PCR purification kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions.

2.6.3 Illumina MiSeq sequencing

The DNA was sequenced at the National High-Throughput DNA Sequencing Centre at University of Copenhagen, Denmark, on the Illumina MiSeqTM 250PE platform. The BION-meta software was then used for further analysis (for more information about BION-meta and for acquisition of software, see http://box.com/bion). Sequences were demultiplexed into original samples according to their barcodes, followed by joining of forward and reverse sequences, allowing no gaps, a maximum mismatch percentage of 80, and a minimum overlap length of 35 bp. Next, the sequences were cleaned at both ends by removal of bases of quality less than 98%, equivalent to a Phred score of 17. Sequences with 100% identity were further de-replicated into consensus sequences for downstream analysis. Following removal of chimeric sequences, the consensus sequences were taxonomically classified against the Ribosomal Database Project II (RDP-II) SSU database (RDP-II; http://rdp.cme.msu.edu/index.jsp) using a word length of 8 and a match minimum of 90%. The top 1% of the obtained similarities from the RDP-II database was used for taxonomical

classification of the consensus sequences, and the counts of each operational taxonomic unit (OTU) were re-replicated to the original counts. The resulting counts in each barcoded sample were normalised to 100,000 in order to enable direct statistical comparisons of relative abundance in each sample.

2.7 Statistical analysis

For the growth performance and morphometric data, the PROC MIXED procedure in the SAS 9.2 statistical package (SAS institute Inc., Cary, North Carolina, USA) was used, in which group-wise comparisons were made by Tukey's test when a significant F-test was detected. Pen and piglet were the experimental unit in growth performance and intestinal morphometric data respectively, and diet was the fixed variable in the statistical model. Contrast statistical analyses were performed by the contrast option in SAS. The significance level was set at $p < 0.05$, and $p > 0.05$ to < 0.15 was considered a tendency. Correlations and linear regression were performed by use of JMP 11.1.1 (SAS institute Inc. Cary, North Carolina, USA).

The software R, version 3. 1. 0, (R Core Team, Vienna, Austria. URL http://www.R-project.org/.) was used for statistical analysis of the microbiota data. Sequencing data was analysed at a given taxonomic level by analysis of variance (ANOVA) on log-transformed data corrected for multiple comparisons by the Šidák correction (Šidák 1967). Given a significant ANOVA, this was followed by Tukey's post-hoc test. A multivariate analysis of the microbial community was performed on species-level data by canonical analysis of principal coordinates (CAP) with treatment diets as constraints and using the Bray-Curtis dissimilarity index, followed by analysis of similarities (ANOSIM), as well as k-means clustering. Beta-diversity was calculated as Shannon indices.

3 Results

3.1 Health

All diets were well tolerated, and voluntary feed intake was satisfactory during the experiment. From days 12–13 post-weaning, there was an outbreak of weaning diarrhoea and the two most affected pens (one from the FM diet and one from the VHFe diet) were excluded from the study from day 11. In addition, one piglet was removed from the study due to leg problems at day 2 post-weaning, and the pen was excluded from statistical analysis of growth performance.

3.2 Growth performance

During the periods 0–11 days and 0–32 days post-weaning, no significant differences were detected in ADG, ADFI, G:F, energy intake or energy utilisation between the diet groups (Table 3). No differences in small intestinal weight or length were detected at day 11 post-weaning in piglets fed the different experimental diets (data not shown).

3.3 Intestinal morphometry

The duodenal villi absorption area was significantly larger in piglets fed the Ve diet compared to piglets fed the PP diet (Table 4). By contrast analysis, piglets fed the SPH diets had a larger duodenal villi absorption area compared to piglets fed the PP diet (PP vs. SPH, $p = 0.02$). The duodenal villi tended to be longer in piglets fed the SPH diets compared to the piglets fed the PP diet (PP vs. SPH, $p = 0.09$), and the duodenal crypt tended to be deeper in the piglets fed the FM diet compared to the piglets fed the SPH diets (FM vs. SPH, $p = 0.06$). Therefore, the duodenal villi height-to-crypt-depth ratio tended to be increased in the SPH diet groups compared to both PP (PP vs. SPH, $p = 0.13$) and FM (FM vs. SPH, $p = 0.13$) diet groups. In the jejunum, there was a tendency towards deeper crypts in piglets fed the FM diet compared to piglets fed the SPH diets (FM vs. SPH, $p = 0.11$) and a higher villi height-to-crypt-depth ratio in piglets fed the SPH diets compared to piglets fed the PP diet (PP vs. SPH, $p = 0.14$). Small differences were detected in the ileum, but crypt depth tended to be deeper in the SPH diet groups compared to both the FM (FM vs. SPH, $p = 0.12$) and PP (PP vs. SPH, $p = 0.11$) diet groups. There was a significant positive linear correlation between duodenal villi height at day 11 and ADG in days 0–11 post-weaning ($r^2 = 0.36$; $p < 0.02$) (Figure 1), and jejunal crypt depth and ADFI in days 0–11 post-weaning ($r^2 = 0.47$; $p < 0.01$).

3.4 Microbiota

From sequencing on the Illumina MiSeq platform, 10 million reads were acquired. Following demultiplexing, pairing of forward and reverse reads, quality trimming and chimera removal, 3 million sequences were classified by the RDP-II database, resulting in 1.7 million mapped reads.

The microbiota of the ileal samples from all diets were notably similar, consisting of >99% of phylum *Firmicutes* of which 30–45% were genus *Lactobacillus* and 30–40% genus *Turicibacter* (Figure 2). The remaining bacteria were various members of the order

Clostridiales. A highly significant inverse correlation between *Turicibacter* and *Lactobacillus* (r^2 = 0.92; p <0.001) was found. The Shannon index at the genus level was significantly lower in piglets fed the FM diet compared to piglets in the PP diet group. In multivariate analysis, no differences were observed by CAP, and cluster analysis showed no distinct patterns. ANOSIM revealed no differences in ileal samples between the diets (P for difference = 0.99). Family *Enterobacteriaceae* comprised less than 1% in all diets, and no pathogenic bacteria were detected.

4 Discussion

In the present study, no significant differences were detected in ADG, ADFI, G:F, energy intake or energy utilisation between piglets fed the different experimental diets during the first 11 days post-weaning. This is in accordance with Tucker et al. (2011) who reported no difference in ADG, ADFI, or G:F during the first week post-weaning in piglets fed diets with inclusion of 1.5% and 3.0% SPH, and Nørgaard et al. (2012) who reported no differences in ADG, ADFI or feed conversion ratio in piglets fed diets containing FM, SPH or soy protein concentrate during the first 14 days of the trial. However, Nørgaard et al. (2012) introduced the experimental diets one week post weaning, and hence the experiments are not directly comparable. Hydrolysates from other sources, such as soybean meal and wheat gluten, are also reported not to influence growth performance in piglets during the first 14 days post-weaning compared to non-hydrolysed control diets (Vente-Spreeuwenberg et al. 2004). However, improved growth performance at two weeks post-weaning is reported in weaning piglets fed hydrolysed blood cells compared to piglets fed diets containing fishmeal or non-hydrolysed blood cells (Chen et al. 2013). Nørgaard et al. (2012) reported increased overall ADFI in piglets fed a SPH diet compared to a soy protein concentrate diet. This is in contrast to our results, as no differences in ADFI were observed at any time between the experimental diet groups.

The observed tendency towards higher duodenal villi height-to-crypt-depth ratio in piglets fed the SPH diets, compared to both the PP and FM diets, may indicate increased proximal intestinal absorption when peptides are supplied in the diet. In our study >74% of the water-soluble proteins of the hydrolysates had molecular weight <1000 Da corresponding to a peptide chain-length less than about 10 amino acids and free amino acids. A significant positive correlation between duodenal villi height and ADG during days 0–11 was detected. This is in line with a study by Pluske et al. (1996), who found a positive correlation between proximal intestinal villi height and weight gain in piglets fed ewe's milk or a standard starter diet.

However, the observed increase in absorption area and the tendencies towards increased villi height in the duodenum in piglets fed the SPH diets compared to the PP diet did not result in differences in ADG in the present study. This might be explained by the significant inter-individual variation in ADFI and ADG normally seen during the first 14 days post-weaning.

The main route of digestion and absorption of proteins is through peptides and amino acids that are normally hydrolysed from larger proteins in the stomach and intestine. Small peptides, below 4–5 amino acids in length, are able to be transported through specialised peptide transporters along the intestinal mucosa, co-transported with other components, and can diffuse through permeable tight junctions (Gilbert et al. 2008). In addition, peptides are reported to be digested and absorbed in the more proximal parts of the small intestine in dogs (Zhao et al. 1997) and more effectively absorbed in rats (Gilbert et al. 2010) compared to both intact protein and free amino acids. Additionally, in rats, a brief fasting period (Thamotharan et al. 1999) and malnutrition due to parasite infection (Barbot et al. 2003) are associated with high levels of intestinal peptide transporter (PepT1) expression, probably increasing the capacity for peptide absorption. SPH might therefore facilitate increased peptide and amino acid absorption compared to intact proteins during the critical post-weaning period and enteric diseases.

In ileum, there was a tendency towards increased crypt depths in the SPH diet groups compared to both the PP and FM diet groups. It is not known if this indicates that less protein reached the distal small intestine in the SPH diet groups or if other regulatory mechanisms were involved. The importance of undigested proteins reaching the hindgut has been investigated previously (Nyachoti et al. 2006; Heo et al. 2008) as this might facilitate microbial proliferation of proteolytic bacteria (Halas et al. 2007; Opapeju et al. 2009). However, the piglet microbiota community by 16S rRNA gene sequencing revealed small differences between the diets included in this study. This probably indicates small differences in the substrate available for microbial fermentation in the ileum content, suggesting that replacing SPH with FM or PP in piglet diets does not impair or negatively interact with the ileal luminal microbiota. Shifting the microbiota is mainly achieved through changes in intake of macronutrients like fibre, protein and fat (Wu et al. 2011; David et al. 2014). In our study, the experimental diets were comparable in these components and it is therefore likely that this has not influenced the intestinal microbiota community. In addition, a nutritional influence on the bacterial profiles in the ileum are reported to be more pronounced in mucosa-associated bacterial profiles compared to intestinal content profiles (Levesque et al. 2012). There was an inverse correlation between *Turicibacter* and *Lactobacillus*, which together comprised most of the bacterial genera found

in the ileal content. As different strains of *Lactobacillus* and *Bifidobacteria* are shown to suppress or inhibit microbial pathogens (Servin 2004), the knowledge about correlations between different bacterial genera may play an important role in the search for feed formulations that stimulate the piglets' robustness against post-weaning diarrhoea.

No differences were found between the two salmon protein hydrolysates on piglet growth performance, intestinal morphometry or intestinal microbiota composition. This indicate that the observed effects of SPH compared to the PP and FM diets are more related to the content of peptide and free amino acids, rather than differences in potential bioactivity between the two hydrolysates. Besides, the VHFe hydrolysate consisted of 50% viscera, which may have dominated and masked the potential effect from the head and frame rest raw material fractions.

5 Conclusion

The present study shows that inclusion of SPH in a piglet weaner diet increased the duodenal absorption area compared to the PP diet and tended to increase the duodenal villi height-to-crypt-depth ratio compared to both PP and FM diets. A significant positive correlation between duodenal villi height and ADG during the first 11 days post-weaning was detected. Only small differences in intestinal microbiota community and no differences in growth performance were detected between the experimental diets. Protein hydrolysates from salmon rest raw materials seem to be an interesting novel protein source with possible effects on intestinal health in weanling piglets.

Acknowledgements

The authors thank the staff at the Animal Production Experimental Centre at Aas for animal care and management of piglets during the feeding trial, Tore Remman at Nutrimar A/S for production and delivery of specified hydrolysates, and Hanne R. Skjelstad and Ketil Skår at the National Veterinary Institute Trondheim for lending microscopic facilities for the morphometric analysis.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the Regional Research Found Mid-Norway [ES486682/217393] and the National Research Council of Norway [221364/O30].

References

Barbot L, Windsor E, Rome S, Tricottet V, Reynes M, Topouchian A, Huneau JF, Gobert JG, Tome D, Kapel N. 2003. Intestinal peptide transporter PepT1 is over-expressed during acute cryptosporidiosis in suckling rats as a result of both malnutrition and experimental parasite infection. Parasitol Res. 89:364–370.

[CVB] Central Veevoederbureau. 2005.Veevoedertabel [Feedstuff table, nutritional value of feed ingredients]. Lelystad, The Netherlands.

Chen QS, Zhang H, Zheng YB, Shan AS, Bi ZP. 2013. Effects of enzymatically hydrolyzed blood cells on growth performance and intestinal characteristics of newly weaned piglets. Livest Sci. 157:514–519.

David LA, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, Ling AV, Devlin AS, Varma Y, Fischbach MA et al. . 2014. Diet rapidly and reproducibly alters the human gut microbiome. Nature. 505:559–563.

Espe M, Sveier H, Hogoy I, Lied E. 1999. Nutrient absorption and growth of Atlantic salmon (Salmo salar L.) fed fish protein concentrate. Aquaculture 174:119–137.

European Commission. 2009. Commission Regulation 2009/152/EC of 27 January 2009 laying down the methods of sampling and analysis for the official control of feed. OJEU. L 54 (2009):1–130.

Gilbert ER, Li H, Emmerson DA, Webb KE, Wong EA. 2010. Dietary protein composition influences abundance of peptide and amino acid transporter messenger ribonucleic acid in the small intestine of 2 lines of broiler chicks. Poultry Sci. 89:1663–1676.

Gilbert ER, Wong EA, Webb KE. 2008. Board-invited review: Peptide absorption and utilization: Implications for animal nutrition and health. J Anim Sci. 86:2135–2155.

Halas D, Heo JM, Hansen CF, Kim JC, Hampson DJ, Mullan BP, Pluske JR. 2007. Organic acids, prebiotics and protein level as dietary tools to control the weaning transition and reduce post-weaning diarrhoea in piglets. CAB Rev.: Perspect Agric Vet Sci Nutr Nat Resour. 2:1–13.

Harnedy PA, FitzGerald RJ. 2012. Bioactive peptides from marine processing waste and shellfish: A review. J Funct Foods. 4:6–24.

Heo JM, Kim JC, Hansen CF, Mullan BP, Hampson DJ, Pluske JR. 2008. Effects of feeding low protein diets to piglets on plasma urea nitrogen, faecal ammonia nitrogen, the incidence of diarrhoea and performance after weaning. Arch Anim Nutr. 62:343–358.

Hevroy EM, Espe M, Waagbo R, Sandnes K, Ruud M, Hemre GI. 2005. Nutrient utilization in Atlantic salmon (Salmo salar L.) fed increased levels of fish protein hydrolysate during a period of fast growth. Aquacult Nutr. 11:301–313.

Kim JC, Hansen CF, Mullan BP, Pluske JR. 2012. Nutrition and pathology of weaner pigs: Nutritional strategies to support barrier function in the gastrointestinal tract. Anim Feed Sci Technol. 173:3–16.

Kodera T, Hara H, Nishimori Y, Nio N. 2006. Amino acid absorption in portal blood after duodenal infusions of a soy protein hydrolysate prepared by a novel soybean protease D3. J Food Sci. 71:517–525.

Levesque CL, Yu H, Gong J, de Lange CFM. 2012. Ileal mucosa-associated—but not ileal digesta—bacterial profiles in grower pigs are influenced by nutrition and use of antibiotics for weaner pigs. J Anim Sci. 90:448–450.

Mahata ME, Dhama A, Ryanto HI, Rizal Y. 2008. Effect of substituting shrimp waste hydrolysate of Penaeus merguensis for fish meal in broiler performance. Pak J Nutr. 7:806–810.

[NRC] National Research Council. 1998. Nutrient Requirements of Swine: 10th rev ed. Washington DC: National Academy Press.

Ngo DH, Vo TS, Ngo DN, Wijesekara I, Kim SK. 2012. Biological activities and potential health benefits of bioactive peptides derived from marine organisms. Int J Biol Macrormolec. 51:378–383.

Nyachoti CM, Omogbenigun FO, Rademacher M, Blank G. 2006. Performance responses and indicators of gastrointestinal health in early-weaned pigs fed low-protein amino acid-supplemented diets. J Anim Sci. 84:125–134.

Nørgaard JV, Blaabjerg K, Poulsen HD. 2012. Salmon protein hydrolysate as a protein source in feed for young pigs. Anim Feed Sci Technol. 177:124–129.

Opapeju FO, Krause DO, Payne RL, Rademacher M, Nyachoti CM. 2009. Effect of dietary protein level on growth performance, indicators of enteric health, and gastrointestinal microbial ecology of weaned pigs induced with postweaning colibacillosis. J Anim Sci. 87:2635–2643.

401 Opheim M, Šližytė R, Sterten H, Provan F, Larssen E, Kjos NP. 2015. Hydrolysis of Atlantic
402 salmon (Salmo salar) rest raw materials–Effect of raw material and processing on
403 composition, nutritional value, and potential bioactive peptides in the hydrolysates.
404 Process Biochem. 50:1247–1257.
405 Peace RM, Campbell J, Polo J, Crenshaw J, Russell L, Moeser A. 2011. Spray-dried porcine
406 plasma influences intestinal barrier function, inflammation, and diarrhea in weaned
407 pigs. J Nutr. 141:1312–1317.
408 Pluske JR, Kerton DK, Cranwell PD, Campbell RG, Mullan BP, King RH, Power GN,
409 Pierzynowski SG, Westrom B, Rippe C et al. . 2003. Age, sex, and weight at weaning
410 influence organ weight and gastrointestinal development of weanling pigs. Aust J Agric
411 Res. 54:515–527.
412 Pluske JR, Williams IH, Aherne FX. 1996. Maintenance of villous height and crypt depth in
413 piglets by providing continuous nutrition after weaning. Anim Sci. 62:131-144.
414 Refstie S, Olli JJ, Standal H. 2004. Feed intake, growth, and protein utilisation by post-smolt
415 Atlantic salmon (Salmo salar) in response to graded levels of fish protein hydrolysate
416 in the diet. Aquaculture. 239:331–349.
417 Servin AL. 2004. Antagonistic activities of lactobacilli and bifidobacteria against microbial
418 pathogens. FEMS Microbiol Rev. 28:405–440.
419 Šidák Z. 1967. Rectangular confidence regions for the means of multivariate normal
420 distributions. J Am Stat Assoc. 62:626–633.
421 Standards Norway. 1994. NS 9402 Atlantic salmon - Measurement of colour and fat. Lilleaker:
422 Standards Norway.
423 Strube ML, Ravn HC, Ingerslev H-C, Meyer AS, Boye M. 2015. In situ prebiotics for weaning
424 piglets: In vitro production and fermentation of potato galacto-rhamnogalacturonan.
425 Appl Environ Microbiol. 81:1668–1678.
426 Thamotharan M, Bawani SZ, Zhou X, Adibi SA. 1999. Functional and molecular expression of
427 intestinal oligopeptide transporter (Pept-1) after a brief fast. Metabolism. 48:681–684.
428 Tucker JL, Naranjo VD, Bidner TD, Southern LL. 2011. Effect of salmon protein hydrolysate
429 and spray-dried plasma protein on growth performance of weanling pigs. J Anim Sci.
430 89:1466–1473.
431 Vente-Spreeuwenberg MAM, Verdonk J, Koninkx J, Beynen AC, Verstegen MWA. 2004.
432 Dietary protein hydrolysates vs. the intact proteins do not enhance mucosal integrity
433 and growth performance in weaned piglets. Livest Prod Sci. 85:151–164.

Wu G. 2014. Dietary requirements of synthesizable amino acids by animals: a paradigm shift in protein nutrition. J Anim Sci Biotechnol. 5:34–46.

Wu GD, Chen J, Hoffmann C, Bittinger K, Chen Y-Y, Keilbaugh SA, Bewtra M, Knights D, Walters WA, Knight R et al. . 2011. Linking long-term dietary patterns with gut microbial enterotypes. Science (New York). 334:105–108.

Zhao X-T, McCamish MA, Miller RH, Wang L, Lin HC. 1997. Intestinal transit and absorption of soy protein in dogs depend on load and degree of protein hydrolysis. J Nutr. 127:2350–2356.

442 Table 1. Composition of Atlantic salmon hydrolysates.

	Salmon protein hydrolysate (SPH)	
Hydrolysate	Ve*	VHFe†
Proximal composition (g/kg)		
Crude protein	291	304
Crude fat	27	13
Crude ash	28	24
Water content	605	642
Indispensible amino acids (g/kg)		
Arginine	14.7	17.1
Histidine	5.7	6.1
Isoleucine	10.3	9.8
Leucin	17.2	16.5
Lysine	18.0	19.0
Methionine	7.1	7.9
Phenylalanine	9.7	9.0
Threonine	11.7	11.3
Tryptophan	2.7	2.1
Valine	13.7	13.1
Dispensible amino acids (g/kg)		
Alanine	13.3	16.6
Aspartic acid	21.8	23.2
Cysteine	3.8	2.8
Glutamic acid	30.1	33.3
Glycine	15.7	25.2
OH-proline	1.7	5.2
Proline	11.2	14.4
Serine	11.5	11.6
Tyrosine	8.9	7.4
Molecular weight distribution (Dalton) (% of soluble protein)		
>20,000	0.2	0.1
20,000–15,000	<0.1	<0.1
15,000–10,000	0.2	0.2
10,000–8000	0.2	0.2
8000–6000	0.4	0.5
6000–4000	0.9	1.5
4000–2000	3.6	7.9
2000–1000	7.6	15.2
1000–500	10.9	15.6
<500	75.9	58.9
Degree of hydrolysis	50.1	33.7

443 Notes: *Ve, SPH from viscera obtained by use of endogenous enzymes; †VHFe, SPH from viscera,
444 head and frame (50%:25%:25% wet weight) obtained by use of endogenous enzymes
445

446 Table 2. Diet formulation, calculated and analysed composition of experimental diets.

			Salmon protein hydrolysate (SPH)	
	PP*	FM†	Ve‡	VHFe#
Ingredient (g/kg)				
LT Fishmeal+	-	40.0	-	-
SPH - Ve♦	-	-	100.0	-
SPH - VHFe◊	-	-	-	100.0
Wheat	500.0	500.0	500.0	500.0
Barley	199.5	223.7	211.4	215.5
HP 310§	122.1	70.0	70.0	70.0
Soybean meal	85.0	85.0	85.0	85.0
Soy oil	37.2	42.4	42.9	39.9
Limestone	10.4	8.5	11.3	11.0
Salmon oil	8.7	-	2.8	5.9
Mono calcium phosphate	8.3	5.7	6.9	7.5
Salt (NaCl)	6.9	5.6	6.1	6.0
Selenium premix$	1.1	0.8	0.6	0.6
Premix¶	11.4	11.4	11.4	11.4
L-Lysin	5.6	5.2	5.6	5.4
DL-Methionin	2.3	2.1	2.2	2.2
L-Threonin	1.6	1.6	1.7	1.7
L-Valin	0.3	0.3	0.4	0.4
L-Tryptophan	0.5	0.5	0.6	0.6
Formic acid 85%	8.1	8.1	5.3	5.3
Water evaporation£	-8.9	-11.1	-64.1	-68.4
Metabolisable energy (ME) (MJ/kg)ϕ	14.06	14.19	14.19	14.20
Analysed composition (g/kg)				
Crude protein	176	180	180	181
Crude fat	62	64	70	65
Crude ash	51	49	51	51
Starch	376	392	340	368
Water	122	126	105	111
Phosphorous	5.4	4.7	5.4	5.3
Calcium	7.8	6.2	8.6	7.8

447 Notes: *PP, Plant protein-based diet; †FM, Fishmeal diet; ‡Ve, SPH from viscera obtained by use of
448 endogenous enzymes; #VHFe, SPH from viscera, head and frame (50%:25%:25% wet weight)
449 obtained by use of endogenous enzymes; +LT Fishmeal, Norse-LT 94 Fishmeal
450 (http://www.norsildmel.no); ♦SPH-Ve, SPH from viscera obtained by use of endogenous enzymes;
451 ◊SPH-VHFe, SPH from viscera, head and frame (50%:25%:25% wet weight) obtained by use of
452 endogenous enzymes; §HP 310, Hamlet Protein 310, Horsens, Denmark, Soy protein concentrate;
453 $Selenium premix, 300 mg selenium/kg; ¶Premix, Vitamins, minerals, and enzymes; £Water
454 evaporation, Estimated water evaporation during feed production; ϕCalculated ME value from the
455 Dutch Central Veevoedertabel (CVB, 2005).

456 Table 3. Growth performance in piglets fed a plant protein-based diet (PP), a fishmeal diet (FM), and two diets with different Atlantic
457 salmon hydrolysates (Ve and VHFe) included.

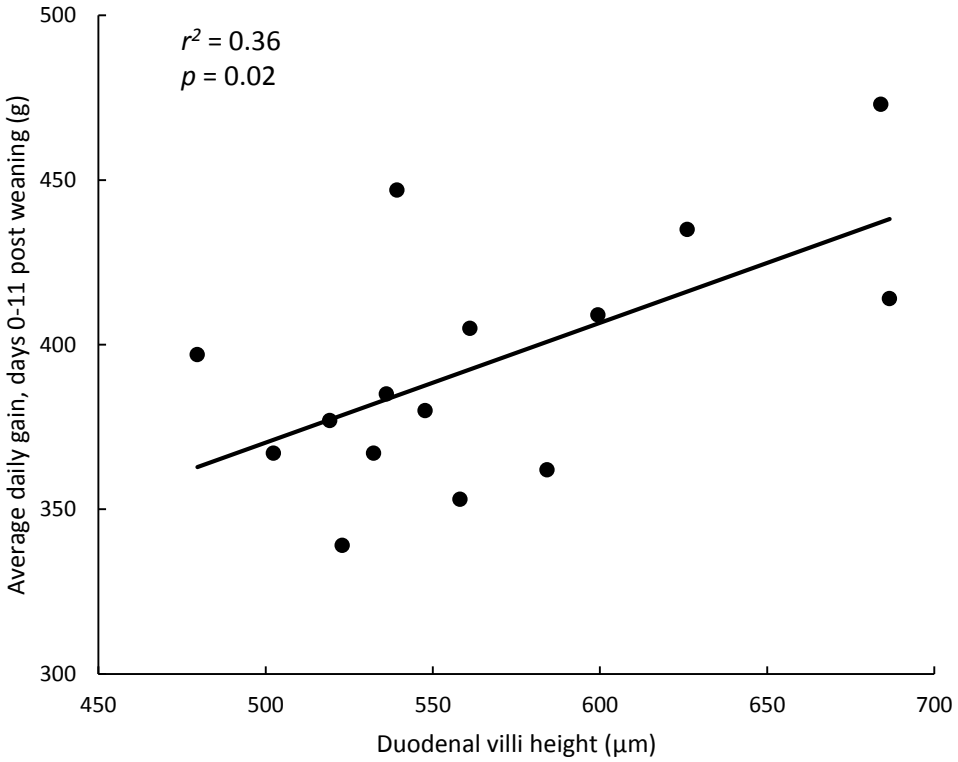
			Salmon protein hydrolysate (SPH)				Contrast analysis	
	PP	FM	Ve	VHFe	SEM[*]	*p*-value[†]	PP vs. SPH[‡]	FM vs. SPH[♯]
Days 0–11 post weaning								
Daily gain (g/d)	391	391	394	401	22	0.99	0.82	0.81
Daily feed intake (g/d)	462	480	467	469	24	0.95	0.84	0.68
Feed utilisation (gain/feed intake)	0.85	0.82	0.84	0.86	0.03	0.69	0.94	0.31
Energy intake (MJ)	6.50	6.82	6.62	6.66	0.34	0.92	0.74	0.68
Energy utilisation (gain/energy intake)	60.2	57.4	59.5	60.2	1.8	0.65	0.87	0.29
Days 0–32 post weaning								
Daily gain (g/d)	573	647	597	608	41	0.63	0.55	0.41
Daily feed intake (g/d)	828	933	858	874	58	0.64	0.59	0.39
Feed utilisation (gain/feed intake)	0.69	0.69	0.70	0.70	0.02	0.95	0.61	0.80
Energy intake (MJ)	11.65	13.23	12.18	12.40	0.82	0.59	0.52	0.39
Energy utilisation (gain/energy intake)	49.0	48.9	49.3	49.1	1.4	1.00	0.93	0.90

458 Notes: [*] Pooled standard error of mean; [†]*p*-value, all groups included; [‡]PP diet group vs. SPH (both Ve and VHFe) diet groups; [♯]FM diet group vs.
459 SPH (both Ve and VHFe) diet groups.

Table 4. Intestinal morphometric data, 11 days post-weaning, in piglets fed a plant protein-based diet (PP), a fishmeal diet (FM), and two diets with different Atlantic salmon hydrolysates (Ve and VHFe) included.

			Salmon protein hydrolysate (SPH)				Contrast analysis	
	PP	FM	Ve	VHFe	SEM[*]	*p*-value[†]	PP vs. SPH[‡]	FM vs. SPH[♯]
Duodenum								
Villi height (µm)	527	574	605	560	25	0.21	0.09	0.78
Vabs ($\mu m^2 *10^{-3}$)	70[b]	82[ab]	92[a]	78[ab]	5	0.03	0.02	0.58
Crypt depth (µm)	245	269	233	237	14	0.28	0.56	0.06
Villi:Crypt ratio	2.18	2.18	2.73	2.39	0.20	0.19	0.13	0.13
Jejunum								
Villi height (µm)	445	510	491	470	26	0.34	0.27	0.37
Vabs ($\mu m^2 *10^{-3}$)	50	60	56	52	4	0.30	0.44	0.20
Crypt depth (µm)	196	207	196	182	9	0.28	0.53	0.11
Villi:Crypt ratio	2.26	2.48	2.55	2.69	0.19	0.47	0.14	0.54
Ileum								
Villi height (µm)	387	381	411	365	24	0.61	0.97	0.82
Vabs ($\mu m^2 *10^{-3}$)	45	48	47	43	4	0.77	0.89	0.49
Crypt depth (µm)	148	149	158	169	7	0.18	0.11	0.12
Villi:Crypt ratio	2.66	2.59	2.63	2.20	0.18	0.25	0.27	0.44

Notes: [*] Pooled standard error of mean; [†]*p*-value, all groups included; [‡]PP diet group vs. SPH (both Ve and VHFe) diet groups; [♯]FM diet group vs. SPH (both Ve and VHFe) diet groups; [a,b]Values within a row with different superscripts differ significantly at $P<0.05$.



464

465 Figure 1**.** Significant positive correlation between duodenal villi height at day 11 and
466 average daily gain 0–11 days post-weaning.

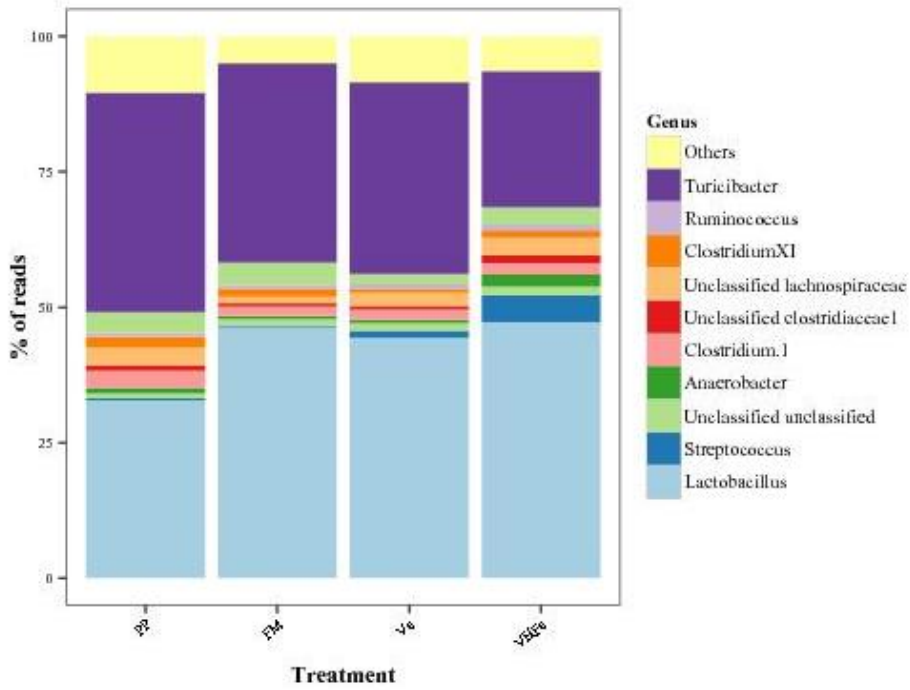


Figure 2**.** Genus-level composition of the piglet ileal microbiota community after 16S rRNA sequencing.

Notes: PP = Plant protein-based diet; FM = Fishmeal diet; Ve = salmon protein hydrolysate from viscera obtained by use of endogenous enzymes; VHFe = salmon protein hydrolysate from viscera, head and frame (50%:25%:25% wet weight) obtained by use of endogenous enzymes. Data are for eight samples each dietary treatment, 11 days post-weaning.