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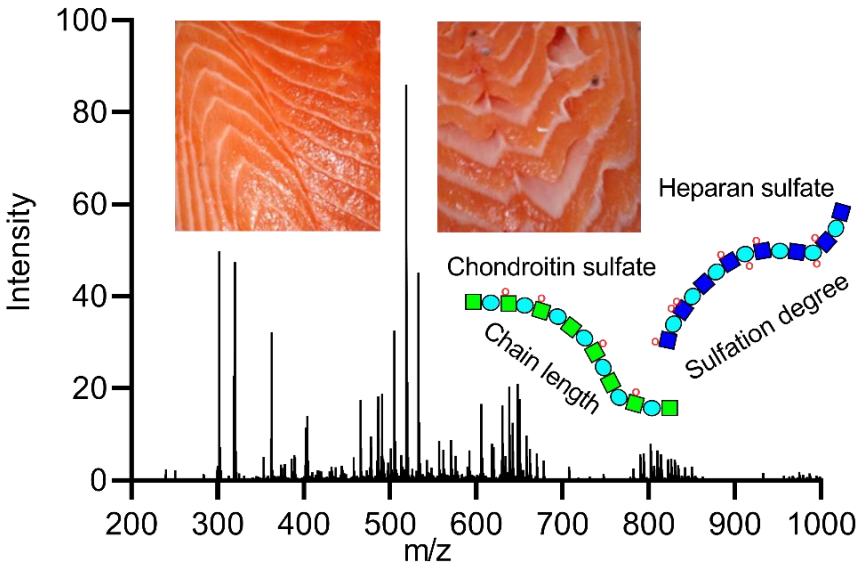
PhD Thesis

Exploring potential causes of gaping in salmon (*Salmo salar* L.) fillets

Ása Jacobsen



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Høvundar / Authors	**Ása Jacobsen**
Vegleiðari / Supervisor	Svein-Ole Mikalsen, Fróðskaparsetur Føroya Jonhard Eysturskarð, Fiskaaling
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✆ • @ +298 352 550 • nvd@setur.fo

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Preface

This PhD study was carried out at P/F Fiskaaling, Aquaculture Research Station of the Faroes, and the department of Science and Technology at the University of The Faroe Islands. The principal supervisor was Svein-Ole Mikalsen from the University of the Faroes Islands and external supervisor at P/F Fiskaaling was Jonhard Eysturskarð.

The PhD project was funded by The Faroese Research Council (grant no. 0421), The Fisheries Research Fund of the Faroe Islands (grant no. 201000728), and P/F Fiskaaling. Part of the laboratory work was performed at the Department of Biochemistry, Center for Mass Spectrometry at the Boston University School of Medicine and was funded by the NIH grant P41GM104603. In addition two Faroese salmon farming companies, Luna and Bakkafrost farming, were supportive in two experimental setups.

I would like to thank all these parties for their guidance, collaboration, and patience in making this study possible. Under adverse circumstances and necessary adjustments, I was always encouraged to continue and that is highly appreciated.

In addition, I would like to thank my colleagues at Fiskaaling and iNOVA for making every working day interesting and enjoyable. The technical discussions and laughters alike are integral parts of both my personal wellbeing at the workplace and my professional productivity. In particular, I would like to thank lab technicians Elin Jacobsen and Margreta Dam for their supportive work.

A special thanks to Hóraldur Joensen, Amanda Gratton Vang and Ian Salter for your constructive scientific input and collaboration. I hope we will have other opportunities for collaboration in the future.

At last, but not least, my family deserves a world of gratitude. Your importance to me is beyond description.

In memory of Jóna Jacobsen, Rói Magnussen and Eyðgerð Magnussen.

Summary

In recent years Atlantic salmon (*Salmo salar* L.) has become an increasingly important aquaculture species in the face of the growing world population and demand for animal proteins. Although aquaculture production has some resource requirement advantages over traditional farm animal production on land, there still are concerns to be addressed. The increasing production of Atlantic salmon requires that responsible measures be taken to ensure a sustainable intensification. One aspect is the reduction of loss and wastage both during processing and later in the supply chain at retailers and consumers. Quality issues are main causes to this form of loss and wastage and have been the focus of research and optimization for decades. Gaping, which is the disconnection between muscle segments and connective tissue in salmon fillets, is a complex quality issue where exact causes and mechanisms are still unresolved and this is a problem for both the aquaculture industry and the sustainability. In this study, we have investigated the effect of insufficient cleaning of the abdominal cavity of the salmon post-slaughter on gaping, any potential correlation between the abundance and structure of certain extracellular matrix components and gaping, and the possibility of microbial influence on gaping. This study has demonstrated the potential in an alternative route of investigation, which has the possibility to shed some additional light on the causes and mechanisms of gaping. The results are summarized below.

A positive correlation was detected between insufficient cleaning of the salmon abdominal cavity post-slaughter on the prevalence of gaping in the salmon fillets after cold storage. Results from the experimental setup indicated that the bodily fluids left in the abdominal cavity induced gaping in the fillets. Weather this was due to inherent enzymes in the blood or intestinal fluid or due to bacterial activity was not determined. However, the experiment suggested that investigating the effect on extracellular matrix components in the fillet's connective tissue might be informative. Samples of intact and gaping tissue were taken from these salmon for mass spectrometry analyses.

The extracellular matrix components chondroitin sulfate (CS) and heparan sulfate (HS) were extracted from samples of intact and gaping tissue and analyzed by mass spectrometry. The results showed differences in the HS chain composition between intact and gaping tissue samples. The gaping tissue samples contained less uniformly structured HS chains. Additional research is required to determine the significance of this finding. The results also showed that CS in gaping samples had a lower sulfation degree, which might have been caused by CS endosulfatases. No

eukaryotic CS endosulfatases have been discovered to date, and this suggested the involvement of bacterial CS endosulfatases, which are known to exist.

Next generation sequencing analyses of the microbial community in the intestines of salmon showed that *Mycoplasmataceae* was highly dominant and *Aliivibrio* was also relatively abundant. Bacterial species in the genus *Mycoplasma* can produce gelatinases, which can affect vital components in the extracellular matrix. A comparative analysis using two bioinformatic pipelines gave different taxonomical resolutions, meaning that many of the reads classified as *Mycoplasmataceae* in one pipeline were classified as *Mycoplasma* in the other. An experimental setup investigating the microbial communities in the post-slaughter environment of farmed salmon showed a correlation between intestinal microbial community composition in the abdominal cavity and an increased gelatinase activity. Samples from the abdominal cavity showing different microbial community compositions than in the intestines had low gelatinase activity. This suggested a potential bacterial involvement in extracellular matrix degradation and gaping. Other bacteria might also be relevant as well as inherent enzymes in the intestinal fluids from the salmon.

Overall, the study showed the potential in gaining more knowledge about gaping by investigating in detail the effect of intestinal fluids and microbiome in the abdominal cavity of salmon post-slaughter on gaping prevalence, enzymatic activity and extracellular matrix components. In addition, the results indicate that meticulous cleaning of the abdominal cavity of farmed salmon during processing might benefit the salmon farming industry and improve the sustainability.

Samandráttur á føroyskum (Faroese summary)

Við støðugt vaksandi tali av heimsíbúgvum og tørvi á djóra proteinum til føði er laksur (*Salmo salar* L.) vorðin ein alsamt meira týdningarmikil alifiskur. Aling á sjógvi krevur ofta minni av náttúrutilfeingi enn siðbundna framleiðslan av landbrúksdjórum, men hóast hetta eru ymisk viðurskifti, ið atlit má takast til. Tann vaksandi framleiðslan av laksi krevur, at átøk verða framd fyri at tryggja burðadygga menning. Eitt mál er, at so nógv av rávøruni sum gjørligt verður gagnnýtt. Í framleiðsluni ella í veitingarketuni hjá sølubúðum og hjá viðskiftafólki kemur ofta fyri at rávøra av laksi fer til spillis. Tílikt skyldast ofta vánaliga góðsku. Gaping, sum er skilnaður av bindivevnaðinum frá løgunum av vøddum í flakinum, er ein góðsku trupulleiki, sum hóast gransking og menning í nógv ár enn er óloystur. Orsøkir og grundleggjandi mekanismur eru ókendar, og hetta er ein trupulleiki bæði fyri vinnuna og fyri tí burðadyggu menningini. Í hesi verkætlan hava vit kannað ávirkanina av vantandi reinsing av búkholuni hjá laksi eftir slátur á gaping. Eisini varð kannað um samband var millum nøgd og bygnað av ávísum týdningarmiklum mýlum í bindivevnaðinum og gaping. Haraftrat var møguleikin fyri at bakteriur hava ávirkan á gaping kannaður. Hendan verkætlanin hevur víst á møguleikarnar at nýta ørvísi kanningar at geva innlit í orsøkir til gaping og mýlevnis mekanismurnar. Niðanfyri er samanumtøka av úrslitunum.

Ein royndaruppseting vísti samband millum vantandi reinsing av búkholuni hjá laksi eftir slátur og títtleika av gaping í laksafløkunum, tá laksurin hevði verið á goymslu í kølirúmi eina tíð. Úrslitini frá royndini bendu á, at okkurt í kropsvætunum, sum ikki blivu reinsaði burtur úr búkholuni við slátur, elvdi til gaping. Tað varð ikki staðfest, um hetta var orsakað av kveikum hjá laksinum í blóðinum ella tarmvætuni, ella um tað var orsakað av bakterium. Royndin gav tó møguleika fyri at kanna ávirkanina á ymisk mýl í bindivevnaðinum í laksaflakinum. Sýni av vødda og bindivevnaði við og uttan gaping vórðu tikin frá somu laksafløkum til massa spektrometri (MS) kanningar.

Mýlini chondroitin sulfate (CS) og heparan sulfate (HS), sum eru týdningarmiklir partar av bindivevnaðinum, vórðu vunnin burtur úr sýnunum av vøddum og bindivevnaði við og uttan gaping og kannaði við MS kanningum. Úrslitini vístu á munir í HS ketu samansetingini millum sýni við og uttan gaping. HS keturnar í sýnum við gaping sýntust at vera minni einstáttaðar enn í sýnunum við gaping. Víðari kanningar eru neyðugar at staðfesta týdningin av hesum. Úrslitini vístu eisini, at CS mýlini í sýnum við gaping høvdu lutvíst færri sulfatgruppur á ketunum, og hetta kann møguliga

vera orsakað av CS endosulfatasum. Ongar eukaryotiskar CS endosulfatasur eru enn staðfestar, og tað kundi møguliga týtt uppá ávirkan av CS endosulfatasum frá bakterium, tí tað er funnið áður.

Kanningar av bakteriu samfeløgunum í laksa tarmi vístu, at *Mycoplasmataceae* familjan var ein rættuliga dominerandi partur, og harumframt var *Aliivibrio* familjan til tíðir eisini lutfalsliga talrík. Bakteriur í *Mycoplasma* ættini kunnu gera gelatinasur, sum kunnu ávirka sera týdningarmikil mýl í bindivevnaðinum. Ein ørvísi dátaviðgerð, ið var gjørd til samanberingar, hevði nærri útgreining av *Mycoplasmataceae* familjuni og ávísti *Mycoplasma* í nógvum førum. Ein royndaruppseting, ið var framd beint eftir slátur og meðan laksurin var á kuldagoymslu, vísti samband millum at bakteriusamfeløg vanlig fyri tarmin vórðu staðfest í búkholuni og øktan gelatinasu aktivitet í búkholuni. Sýni úr búkholuni, sum ikki høvdu bakteriusamanseting eins og í tarmunum, høvdu lágan gelatinasu aktivitet. Hetta týddi á, at ávirkan av bakterium á niðurbróting av bindivevnaðinum og harvið á gaping er ein møguleika. Aðrar bakteriur umframt *Mycoplasma* eru møguliga eisini av týdningi eins og kveikir í tarmvætuni frá laksunum sjálvum.

Samanumtikið hevur verkætlanin víst á møguleikan at fáa meira vitan um gaping við at nágreiniliga kanna ávirkanina tarmvæta og bakteriusamanseting í búkholuni hjá laksi eftir slátur hevur á gaping, kveika aktivitet og mýl í bindivevnaðinum. Haraftrat benda úrslitini á, at nærløgd reinsing av búkholuni hjá laksi eftir slátur kann vera gagnlig bæði fyri alivinnuna og burðardyggu menningina.

List of papers

The thesis is based on the following papers. In the discussion, the papers will be referred to by their numeric assignment.

Paper I: Jacobsen Á, Joensen H, Eysturskarð J. (2017). Gaping and loss of fillet firmness in farmed salmon (*Salmo salar* L.) closely correlated with post-slaughter cleaning of the abdominal cavity. *Aquaculture Research* **48**: 321-331. Doi.org/10.1111/are.12884.

Paper II: Jacobsen Á, Shi X, Shao C, Eysturskarð J, Mikalsen S-O, Zaia J (2019). Characterization of glycosaminoglycans in gaping and intact connective tissue of farmed Atlantic salmon (*Salmo salar* L.) fillets by mass spectrometry. *ACS Omega* **4**, 15337-15247. Doi.org/10.1021/acsomega.9b01136.

Paper III: Jacobsen Á, Mikalsen S-O, Joensen H, Eysturskarð J (2019). Composition and dynamics of the bacterial communities present in the post-slaughter environment of farmed Atlantic salmon (*Salmo salar* L.) and correlations to gelatin degrading activity. *PeerJ* 7:e7040. Doi.org/10.7717/peerj.7040.

Paper IV: Jacobsen Á, Eysturskarð J, Mikalsen S-O. Initial metagenomic screening of microbiome communities in stomach and intestines of four fish species inhabiting coastal waters. *In prep*

Paper V: Jacobsen Á, Mikalsen S-O, Salter I. Comparison of custom made QIIME and standard pipeline SILVAngs bioinformatics for investigation of microbial communities in farmed salmon (*Salmo salar* L.) post-slaughter. *In prep*

List of symbols and abbreviations

Ac	Acetyl group
CS	Chondroitin sulfate
ΔHexA	Delta 4,5-unsaturated hexuronic acid
Dp(x)	Oligosaccharides containing x number of sugar residues
ECM	Extracellular matrix
EIC	Extracted ion chromatogram
GlcN	Glucosamine
HexA	Saturated hexuronic acid
HPLC	High performance liquid chromatography
HS	Heparan sulfate
QIM	Quality Index Method
GAGs	Glycosaminoglycans
LC	Liquid chromatography
MMPs	Metalloproteinases
MS	Mass spectrometry
NGS	Next Generation Sequencing
NRE	Non-reducing end
OTU	Operational Taxonomical Unit
PCA	Principal component analysis, PCoA based on Euclidian distances
PCoA	Principal coordinate analysis
PCR	Polymerase Chain Reaction
PGs	Proteoglycans

RFU	Relative fluorescence unit
RT	Retention time
SEC	Size exclusion chromatography
SO_3	Sulfate group
TIC	Total ion chromatogram
WGS	Whole genome sequencing

1. Introduction

1.1 Aquaculture

Aquaculture is believed to have had its beginning as early as 1000 – 2000 BC in China, where the common carp (*Cyprinus carpio*) is believed to be the first species, but farming of the Nile tilapia (*Oreochromis niloticus*) in Egypt also dates back to around that time (Teletchea 2018). However, it was only much later around 475 BC that the first records and descriptions of aquaculture practices were made by Fan Lai in his book "Fish breeding"[1]. The following millennia saw the dawn of aquaculture in other countries and regions, including Europe where the Romans utilized both fish and oysters. During the middle ages and beyond the aquaculture practices developed and more literature became available. In the 18th and 19th century, during the industrialization in Europe, research in artificial breeding emerged and hatcheries were introduced. In the 1950s, artificial granulated food was invented and this revolutionized the fish farming industry. Moreover, in the 1970s, the focus on marine species increased due to development of new materials and this branch of aquaculture has since increased in volume and importance.

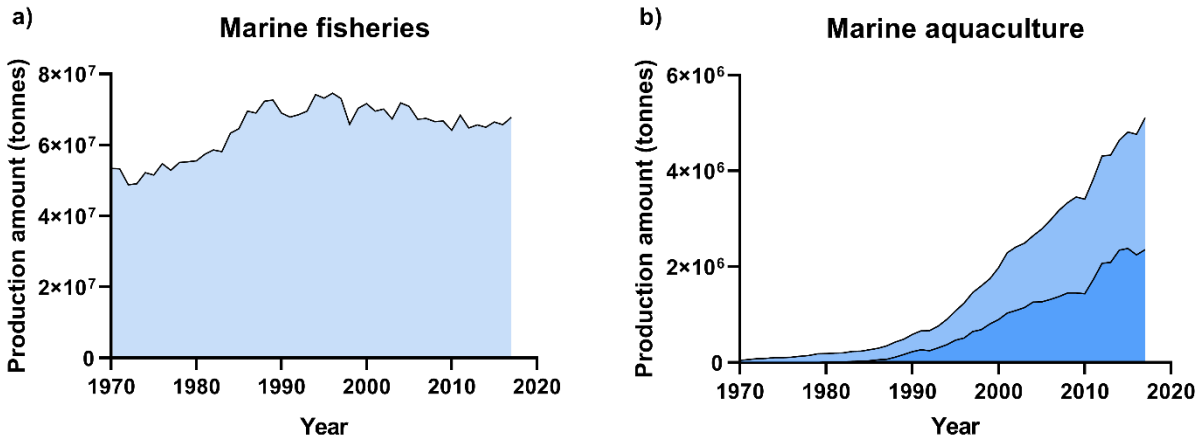

Figure 1.1. Production amounts of marine finfish. Statistics extracted from the FAO Fishstat database www.fao.org/fishery/statistics. Yearly fisheries production amount of marine finfish from 1970 to 2017 (a), and yearly aquaculture production amount of marine finfish (light blue) and Atlantic salmon (dark blue) from 1970 to 2017 (b).

[1] The author's name and title of the book vary in the litterature, but the date is consistent.

Since the late 1980s, global marine finfish fisheries have remained relatively stable at about 70 mio tonnes (Figure 1.1a) while the state of the world's marine fisheries resources has continued to decline (FAO 2018). On the other hand, marine finfish aquaculture has continually increased its production over the last decades (Figure 1.1b) passing 5 mio tonnes in 2017 (FAO 2018). This trend is a reflection of the overall fisheries and aquaculture production rates over the last decades. With the current trends, aquaculture will soon surpass fisheries as the main supplier of marine and freshwater animal proteins for human consumption. In 2016, the global fisheries production was 90.9 mio tonnes while aquaculture production was 80 mio tonnes of which marine and freshwater finfish aquaculture production combined was 54.1 mio tonnes (FAO 2018). The rise of aquaculture as a major supplier of animal proteins for the global population has been met with optimism as an attractive alternative to both the overexploitation of fisheries as well as to terrestrial farming. One reason is that most farmed finfish are highly efficient in feed conversion compared to most terrestrial livestock animals (Torrison *et al*. 2011; Waite *et al*. 2014). Another reason is the relatively modest requirements of the limited resources, land and fresh water, used for production. On the other hand, there are also concerns about the environmental impact of the expansion and intensification of aquaculture (Troell *et al*. 2014; Quiñones *et al*. 2019). These concerns include the usage of marine material in feed, pollution and mixture with wild fish stocks. In response to these dual challenges, there is currently an increasing focus on sustainable intensification of aquaculture systems (Aubin *et al*. 2019).

1.2 Sustainable intensification

FAO defines sustainable intensification of aquaculture as: "Aquaculture production system or technologies or management practices that contribute to at least one of the following: 1) Improved production and resource use efficiency, namely land, water, feed and energy; 2) Improved environmental benefits; 3) Strengthened economic viability and farmer's resilience; 4) Improved social acceptance and equality and do not compromise the rest" (FAO, 2016). Because this is a relatively new vision and other definitions of the subject have been put forward, attempts are currently being made to establish how to measure and evaluate this concept (Ellis *et al*. 2016; Henriksson *et al*. 2018) and how to implement it (Jones *et al.* 2015; Philis *et al.* 2019). However, the core issue is that the aquaculture production and efficiency need to increase while the relative consumption of resources and negative environmental impacts need to be reduced. More product for

less environmental impact, while retaining or improving the high dietary value of farmed seafood and ensuring high welfare for the animals produced, are critical components of sustainable intensification (Little *et al.* 2016). In the definition by FAO, stated above, improving production and resource use efficiency is vital and within this statement lies the desire to reduce food loss and wastage. The loss during the whole production period of the salmon from egg to slaughter has many causes. Illnesses, escapes, and adverse environmental conditions are all challenges that can lead to substantial losses. In the processing part of the production, where the slaughtered salmon is processed, shipped to retailers and sold to consumers, there is a great need for reducing the loss or wastage of aquaculture food products which is especially large late in the supply chain at retailers and at the consumers (Love *et al.* 2015; Ytrestøyl *et al.* 2015). Spoilage and poor quality products are partly at fault. However, in the advanced aquaculture practices and regions, the increasing consumer awareness of sustainability and quality acts as an incentive for the industry to improve their product variation and quality (The World Bank 2013). In recent years, the fish food sector has become more heterogeneous and dynamic. In more advanced economies fish processing has diversified particularly into high value fresh and processed products and ready and/or portion controlled uniform quality meals. Maintaining focus on the importance of product quality during the expansion of aquaculture production is vital for the reduction of loss and wastage, which can lead to higher overall profitability and less negative environmental impact.

1.3 Atlantic salmon aquaculture

Farming of Atlantic salmon in sea cages started in Norway in the 1960s and the first family based breeding program was initiated in 1970 (Teletchea 2018). Today, Atlantic salmon is considered to be one of the few successfully domesticated fish species farmed for food out of the approximately 250 species people have attempted to farm since 1950 (Teletchea 2014). The general increase in aquaculture production is also reflected in an increasing production of farmed Atlantic salmon (figure 1.1b). In 2017, the worldwide production of Atlantic salmon was approximately 2.3 mio tonnes slaughtered weight (FAO 2018). The main producing countries of farmed Atlantic salmon include Norway, Chile, Scotland, Ireland, Canada and The Faroe Islands. In comparison with other aquaculture species and land based farm animals, salmon is highly efficient at converting feed to animal biomass for human consumption according to both the feed conversion ratio (FCR) and to more extensive calculations considering other factors such as nutrient retention and yield (Fry *et al.*

2018). Salmon also has a relatively low carbon footprint and low occupation of agricultural land (Hognes *et al.* 2011). In addition, salmon is versatile fish that can be used for a large variety of food products. These attributes make the Atlantic salmon an interesting aquaculture species considering the demand for production intensification.

1.4 The salmon fillet

The most valuable part of the salmon is the fillet. The salmon fillet is structured with layered w-shaped muscle sheets, named myomeres or myotomes, arranged in rows down along each side of the salmon. The salmon's flesh has a very distinct pink/orange color, which is in clear contrast to the white connective tissue, making the structure of the fillet very visible (Figure 1.2).

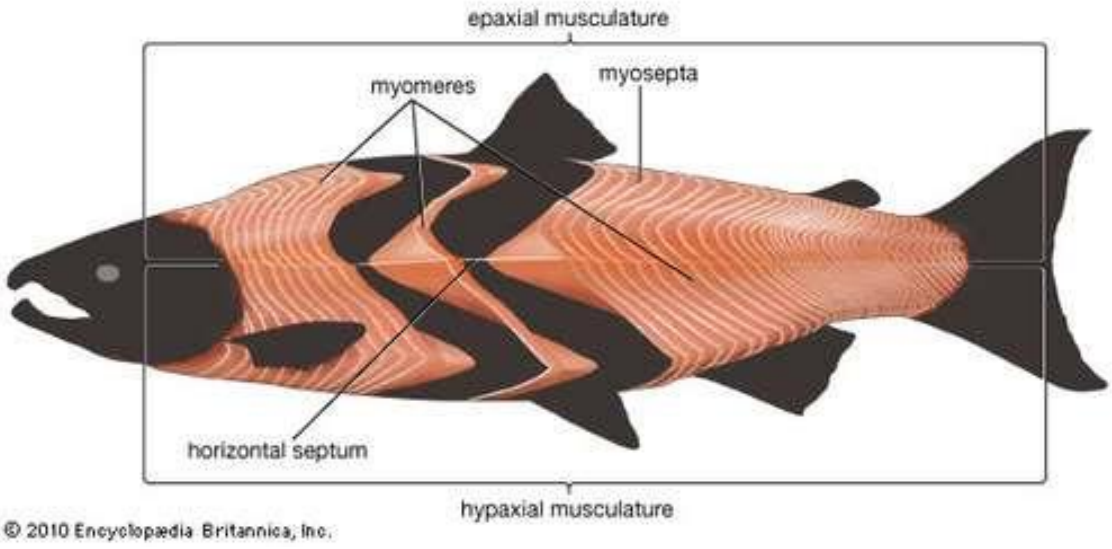

Figure 1.2. Salmon fillet structure. The salmon image from Encyclopedia Britannica illustrates the structure in the fillet with the myomeres in the characteristic pink/orange color and the white myocommata (myosepta) between the myomeres.

The connective tissue, named myocommata or myosepta, is clearly visible as the white lines between the pink/orange muscle segments. The myocommata hold the myomeres together ensuring that they can function together and the contraction forces are transferred as appropriate. In addition, there are horizontal and ventral septa organizing the musculature into four segments, the epaxial

(dorsal) and hypaxial (ventral) segments on left and right side of the salmon. Each myomere is composed of muscle fibers organized into bundles or fascicles. The connective tissue surrounding each fascicle, named perimysium, is thinner than the myocommata and the connective tissue surrounding each muscle fiber, called endomysium, is thinner still. Unlike birds and mammals, most fish species continue growing throughout their lifetime, a phenomenon called indeterminate growth (Hariharan *et al*. 2016). The muscle growth is a combination of formation of new cells (hyperplasia), and the increasing size of already existing cells (hypertrophy). Both muscle fiber and connective tissue characteristics are important features in fillet quality (Taylor *et al.* 2002; Kiessling *et al*. 2006).

1.5 Fillet quality

Fillet quality of farmed Atlantic salmon has been studied relatively intensely over the last few decades, especially those attributes that cause problems for selling the product and for producing specialized food products. However, the focus on sustainable intensification makes it ever more important to get as much premium quality food from every fillet as possible in order to reduce loss, wastage and potential financial loss. In this context, consumer awareness and preferences have been and still are highly important issues to use as a guidance to focus research and development in the industry. For example, the Marine Harvest Salmon farming industry handbook 2018 states that the risk factors, research and development focus is: "1) Fish welfare and robustness; 2) Product quality and safety; 3) New growth; 4) Production efficiency (…identify best harvesting methods, fillet yield optimization…); 5) Footprint" (Marine Harvest 2018). Research in fillet quality with the aim of reducing loss and wastage is therefore one important part of the multi-faceted approach needed in making the expanding salmon aquaculture industry more sustainable, variable and profitable (Figure 1.3).

Figure 1.3. Research in fillet quality in the context of sustainability and profitability in aquaculture

Fillet quality research can roughly be divided into four aspects: 1) nutritious value, such as content of omega-3 fatty acids; 2) freshness or shelf-life, including packaging methods; 3) appearance, such as color; and 4) texture, important for added-value production possibilities. There are many methods by which to study these parameters, making it a wide and not easy comparable field of research. However, in 2003 various sensory quality measurements were standardized for salmon as a Quality Index Method (QIM) as they had been for other fish species before (Sveinsdottir *et al*. 2003). These and a few other standardizations have improved the possibility of making comparisons between studies. The standardization of various measurements is important, as many farming procedures can have a potential negative or positive effect on fillet quality. These procedures include feed composition (Rørå *et al*. 2005; Kousoulaki *et al*. 2015; Sissener *et al*. 2016), handling stress (Mørkøre *et al.* 2008; Bahuaud *et al*. 2010; Hansen *et al*. 2012) and slaughter processes (Borderíaz and Sánchez-Alonso 2011; Møretrø *et al*. 2016). Over the years, research as well as optimizations in the industry has improved the state of some of the quality issues while other quality issues have proven too complicated to relate to any one cause and effect and persist in spite of various optimizations. Gaping is one of them.

1.6 Gaping

Gaping is the phenomenon where the myocommata fails to hold together the myotomes and slits and holes appear in the fillet as a result (Figure 1.4ab). Gaping is a serious quality issue for the salmon aquaculture industry. A survey performed by Nordberg (2018) showed that gaping was one of the main quality concerns in the Norwegian industry. In a review by Pittman *et al.* (2013), it was estimated that gaping could cause a downgrade of up to 38 % of the farmed salmon. However, there are large variations in the severity of the problem at the different salmon producers (Pittman *et al*. 2013).

No holes	= score 0
< 5 small holes	= score 1
< 10 small and/or 5 large holes	= score 2
> 10 small and/or 5 large holes	= score 3

Table 1.1. Gaping scores applied in this study

The unappealing appearance of gaping fillets makes it less desirable for consumers, causing a reduction in prize. In addition, the fillet is less suited for specialized food products as further processing is hampered by the weakness of the connective tissue (Garber *et al.* 2019). The severity of gaping is conventionally scored by observations of amounts and sizes of slits and holes, as defined by Andersen *et al.* (1994) or variations thereof. The scoring system used in this study is illustrated in Table 1.1.

a) b)

c)

Figure 1.4. Gaping assessment. Intact fillet (a), fillet with gaping (b), and gaping score assessments of salmon fillets by trained personnel (c). Photographs (a,b) taken by Durita Nielsen and reprinted with permission from (Jacobsen *et al.* 2019) © ACS Omega 2019. Photograph (c) property of Fiskaaling.

The fillets are handled carefully during examination as trained personnel only slide their hands below the fillet revealing any potential tears and holes (Figure 1.4c). The measurement is normally performed some days post-mortem as gaping is seldom apparent in newly slaughtered salmon. Although the problem has been known for many years and addressed by industrial optimizations as well as academic research, the causes of gaping and the molecular mechanisms are still not fully understood.

1.7 Previous studies of gaping

Previous studies analyzing potential causes of gaping have had widespread focus (Figure 1.5), examining various factors' influence on the presence or severity of gaping. There is a general agreement that heritability is not the determining factor (Kause *et al*. 2011; Garber *et al*. 2019), but rather that one or several external factors are more influential. An effect of season and location on gaping severity has been suggested although any consistent pattern is difficult to establish (Mørkøre and Rørvik 2001; Bjørnevik *et al*. 2004; Espe *et al*. 2004; Mørkøre and Austreng 2004; Margeirsson *et al*. 2007).

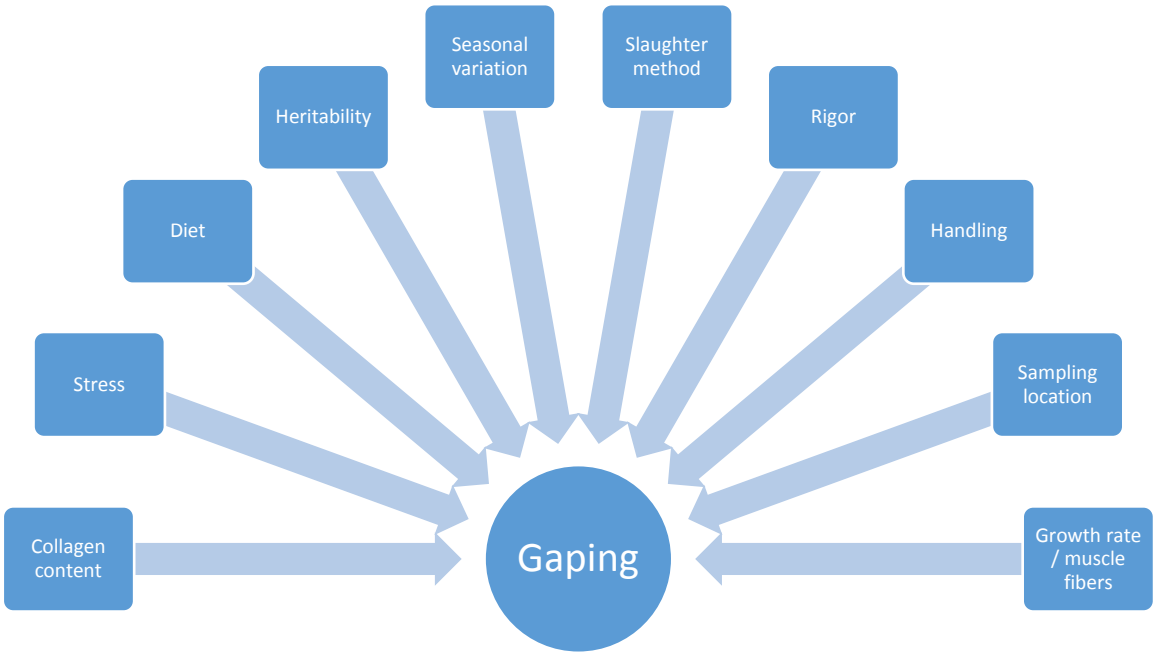

Figure 1.5. Parameters tested for potential correlation to gaping

In addition, the relationship between growth rate or muscle fibers and the occurrence of gaping has been studied, and again contradictory results have been reported (Kissing *et al*. 2006).

Experimental studies investigating effects of different diets or additives have had various results (Mørkøre and Austreng 2004; Hatlen *et al.* 2013; Kousoulaki *et al.* 2015; Kousoulaki *et al.* 2016), and practical application of this knowledge is somewhat restricted to feed manufacturers. On the other hand, stress and handling methods at slaughter have a greater potential for transfer of knowledge from experimental studies to the farming facilities. It is reasonably established that stress prior to and at slaughter of farmed salmon has a negative effect on gaping in the fillets (Lavéty *et al.* 1988; Roth *et al.* 2006; Bauhaud *et al*. 2010) and optimizations in the industry has improved the fillet quality. However, some studies have not found any correlation (Skjervold *et al.* 2001; Kissing *et al.* 2004), again making it difficult to discern the actual process of gaping, although rigor and pH are suspected to play a role in stress induced gaping (Lavéty *et al*. 1988; Huss 1995). Overall, any fulfilling explanation is lacking.

Although the process leading to gaping is illusive, the location has been established as being in the sarcolemma, the cell membrane of the muscle cells, and in the basement membrane, the layer of the myocommata adjacent to the sarcolemma. On this site the muscle fibers are connected to the myocommatal sheets and an increased degree of disconnection in this interface leads to increased gaping (Bremner & Hallett 1985; Hallett and Bremner 1988; Ofstad *et al.* 1996; Fletcher *et al*. 1997). The structural components in the sarcolemma and the myocommata as well as their interactions are therefore an essential focus in the analysis of the molecular processes involved in gaping.

1.8 The extracellular matrix

The myocommata is an extracellular matrix (ECM) that is composed of a wide array of different molecules interacting with each other and the cells, providing structural support, adhesiveness, and means of biochemical communication within the tissue. Both in the ECM and in the sarcolemma the major components are collagens, proteoglycans (PGs) and glycosaminoglycans (GAGs). Together they form a comprehensive structural network that greatly influences the functions and textural properties of these connective tissue types.

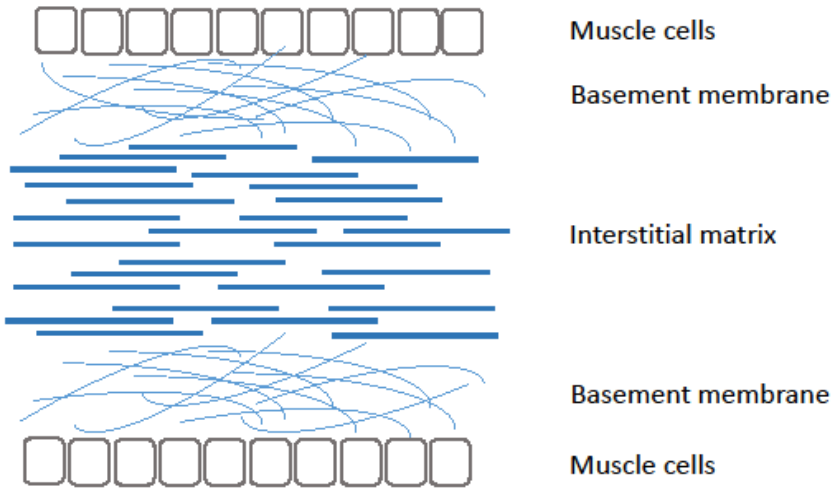

Figure 1.6. Schematic structure across the myosepta. Muscle cells are attached to the basement membrane, on both sides of the interstitial matrix.

The mechanisms of adhesion between muscle cells and the ECM is still not fully understood, but due to the major importance of the dynamic interactions between the cells and the ECM there is currently a major focus on this research issue (Goody *et al.* 2015).

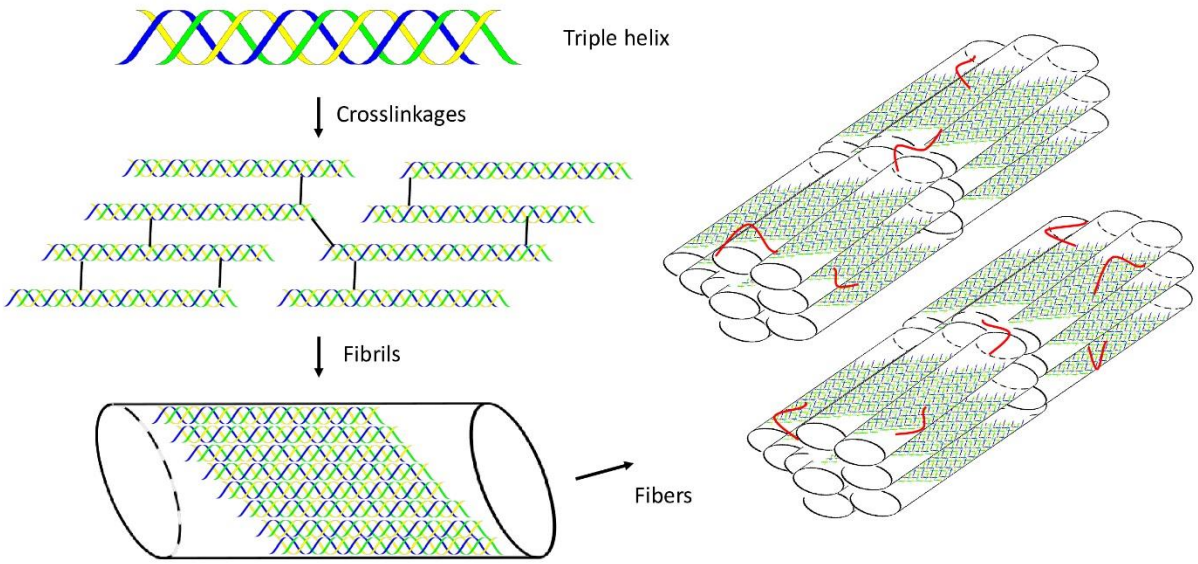

Figure 1.7. Schematic illustration of fibrillar collagen structure. The red strings depicted on the fibers are proteoglycans.

The ECM is effectively divided into two sections with quite different characteristics and functions: the interstitial matrix and the basement membrane (Figure 1.6). The interstitial matrix is mainly composed of fibrillar collagens and proteoglycans (Figure 1.7) and determines physical characteristics such as strength and elasticity of the tissue. The basement membrane lies between the interstitial matrix and the cell surface and contains other types of collagens and proteoglycans (Figure 1.8) which link the cell and the ECM together, providing attachment and possibility of interaction between the cell and its microenvironment. Research on ECM components in relation to fillet quality has mainly focused on other quality issues than gaping (e.g. Johnston *et al*. 2006; Hannesson *et al.* 2007; Moreno *et al*. 2012; Torgersen *et al.* 2014). However, some studies have focused on ECM components in relation to gaping in salmon fillets (Andersen *et al.* 2003; Espe *et al*. 2004). The ECM component receiving most attention in this context has been collagen.

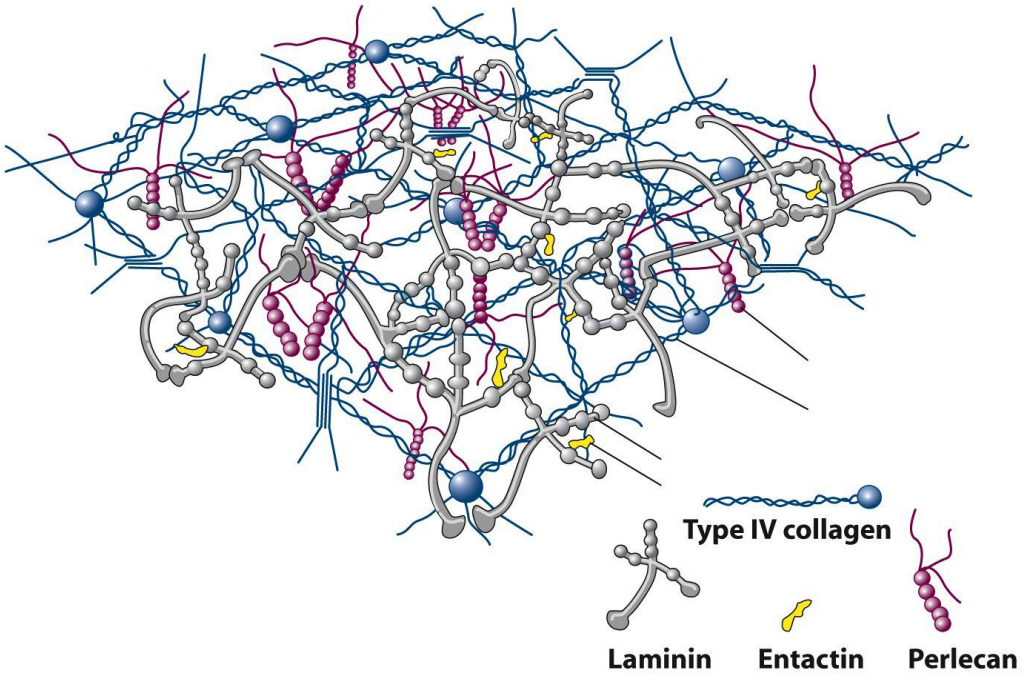

Figure 19-20
Molecular Cell Biology, Sixth Edition

Figure 1.8. The basement membrane. Major components are illustrated. The illustration is from Molecular Cell Biology, 6th edition (Lodish *et al.* 2008)

1.9 Collagen

Collagen is the most abundant protein in the extracellular matrix and is highly important for the function and properties of the ECM. Collagen is composed of three polypeptide chains intertwining to form a triple helix (Figure 1.7). Collagen is a protein with an atypical amino acid composition as it contains many hydroxyprolines and it is formed as a triple helix. It has two common repeating motifs consisting of glycine-proline-X and glycine-X-hydroxyproline. Collagen is a very stable molecule and insoluble in water due to intra- and intermolecular covalent linkages. Characterization of the collagen content often involves measuring the acid-, and pepsin soluble as well as insoluble fractions (e.g. Zhang *et al*. 2007; Eysturskarð *et al*. 2009; Matmaroh *et al*. 2011; Bhuimbar *et al*. 2019).

There are many types of collagen with various characteristics. The most common collagen types in connective tissue are types I, II, III, V and XI (Gelse *et al*. 2003). These are termed fibrillar collagens as they aggregate to form long fibrils (Figure 1.7). Collagen type I (Figure 1.9a) is the most abundant type of collagen and the most abundant protein in vertebrates. It is the main structural component in tissues such as skin, bone, artery walls and the endomysium surrounding the muscle cells (Gelse *et al*. 2003). In contrast, collagen type IV forms a sheet like two-dimensional network. It is much less abundant but is an important component of the basement membrane in the ECM (Figure 1.9b), and is therefore of high interest in connection with gaping.

a) b)

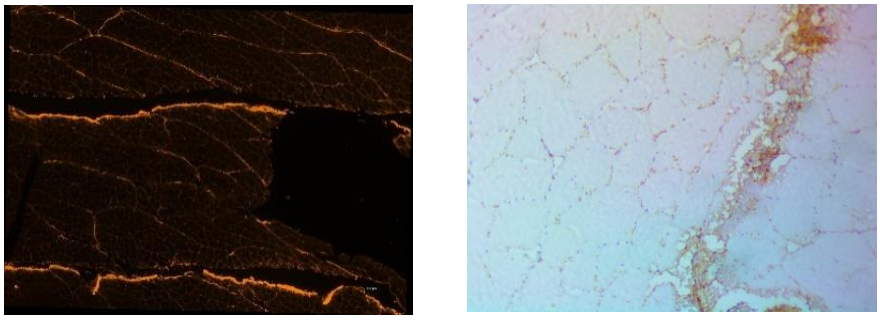

Figure 1.9. Collagen I and IV in salmon fillets. IHC staining with antibodies against collagen I (a), DAB staining with antibodies against collagen IV (b).

Studies investigating the potential correlation between collagen and gaping have revealed that the amount of collagen does not seem to be critical but that the composition and dynamics of the

collagen in the ECM is linked to gaping in fish fillets (Espe *et al.* 2004; Pittman *et al*. 2013; Hagen and Johnsen 2016). This would suggest that the collagen in the ECM somehow becomes unraveled and disorganized in connection with gaping. Other studies have shown that collagen IV is essential to the structural integrity of the basement membrane, and a deficiency of collagen IV causes a patchier and weaker basement membrane (Pöschl *et al.* 2004). However, collagen is not a solitary structure, but is intertwined with proteoglycans and glycosaminoglycans, which are highly important for its structural development and integrity (Costell *et al.* 1999; Geng *et al.* 2006; Bastiaansen-Jenniskens *et al*. 2009).

1.10 Proteoglycans and glycosaminoglycans

Glycosaminoglycans (GAGs) are carbohydrate polymers usually modified with sulfate groups giving them a highly negative charge.

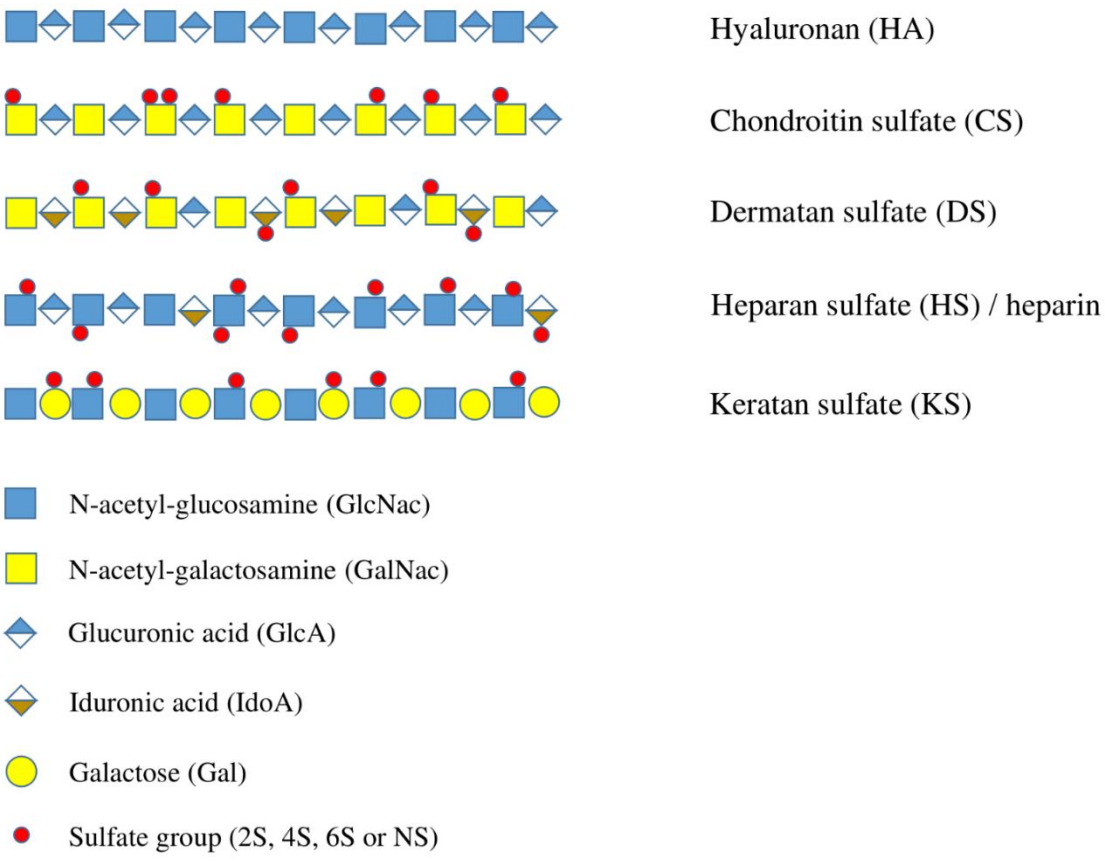

Figure 1.10. Glycosaminoglycan types and structure. Positioning of the sulfate group indicator varies as the compounds can be sulfated at positions C2, C4 and C6 or be N-sulfated.

There are four main types of GAGs: Chondroitin sulfate/dermatan sulfate, heparan sulfate and keratan sulfate which all carry sulfate groups and hyaluronan, which is unsulfated. All are linear polysaccharides composed of repeating units of disaccharides, an amino sugar (N-acetylglucosamine or N-acetylgalactosamine) and an uronic acid (glucuronic acid or iduronic acid) or galactose (Figure 1.10). The sulfated GAGs are usually attached to extracellular proteins forming proteoglycans (PGs). Because the GAGs are relatively large and highly charged, they can dominate the chemical properties of the PGs (Ly *et al*. 2010; Mouw *et al.* 2014). Each GAG type can vary in both chain length and sulfation degree. In addition, the sulfation degree is often not uniform along the entire chain.

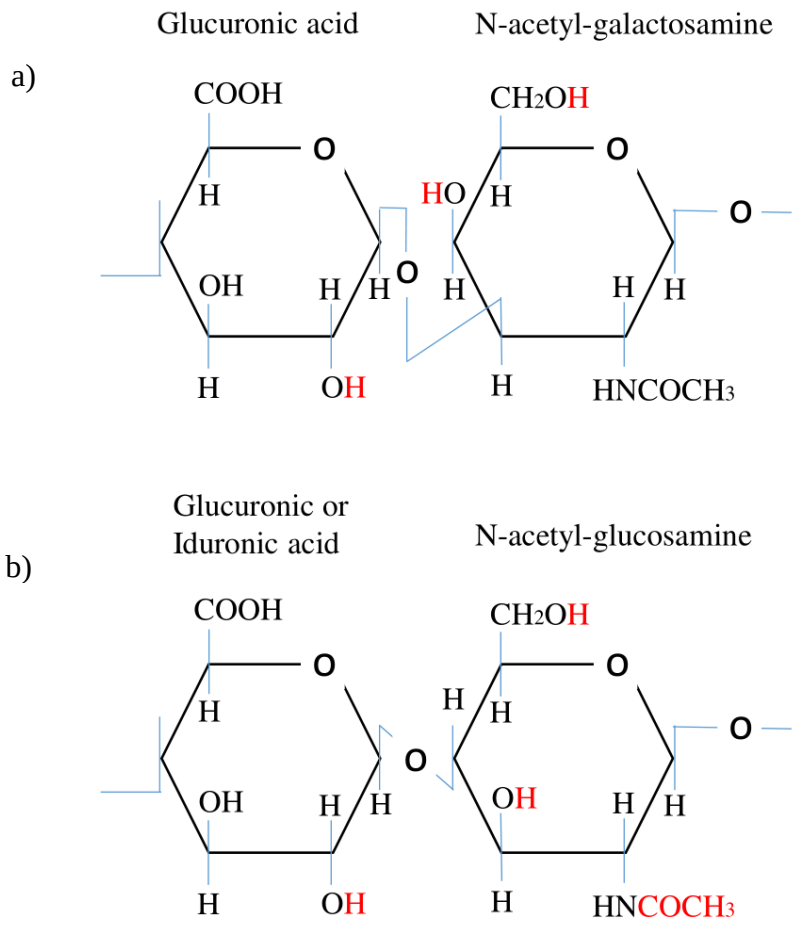

Figure 1.11. Chemical structures of chondroitin sulfate and heparan sulfate. Chondroitin sulfate (a), heparan sulfate (b). Potential sulfation sites are marked with red letters.

These molecular variations influence the functional properties of the GAG. Therefore, the GAGs are very versatile compounds and still the different variants have their own characteristics. In the connective tissue of muscle, chondroitin sulfate and heparan sulfate are the most abundant sulfated GAGs. Chondroitin sulfate is composed of N-acetylgalactosamine and glucuronic acid disaccharide units. The N-acetylgalactosamine commonly carry sulfate on position C4 (chondroitin 4-sulfate), position C6 (chondroitin 6-sulfate) or both (chondroitin 4,6-sulfate) (Figure 1.11a). Sulfation of the C2 position on the glucuronic acid is not as common. Heparan sulfate is composed of the disaccharide units N-acetylglucosamine and glucuronic acid. HS is more versatile than CS as the N-acetylglucosamine can also be N-sulfated (Figure 1.11b). Heparan sulfate is known to have high and low sulfated domains along the chain, which are important for the properties of the HS. Proteoglycans are present in all bilateral animals and integral components of most tissues indicating a long evolutionary history (Couchman and Pataki 2012). Although the number of GAG types and the number of proteins that can be substituted with GAGs is limited, the PGs have evolved into many forms due to the variability of the GAG chains as well as variation in the number of GAGs attached to the protein. This flexibility of the PGs means they have many important functions in development and homeostasis (Chakravarti *et al*. 1998; Costell *et al*. 1999; Farach-Carson and Carson 2007; Bastiaansen-Jenniskens *et al*. 2009). The roles of PGs and GAGs in the ECM are many as some are located in the matrix itself, some on the cell surfaces and some are transmembrane PGs. One major function of the PGs is structuring the collagens in both the interstitial matrix and in the basement membrane and ensuring attachment between these and the cells (Danielson *et al*. 1997; al Jamal *et al*. 2001; Tingbø *et al*. 2006; Kalamajski and Oldberg 2010; Iozzo and Schaefer 2015).

1.11 Degradation of the extracellular matrix

The ECM, crucial for maintaining an array of tissue structures, physiological states and events, is a surprisingly dynamic entity. Remodeling of the ECM is therefore vital for its various functions (Mott and Werb 2004). The remodeling is regulated by various proteolytic enzymes that can degrade the extracellular components and make modifications. The main group of enzymes capable of degrading collagens and other ECM components are the matrix metalloproteinases (MMPs). There are many types of MMPs grouped into subfamilies based on the historical assessment of their main substrate. For instance, gelatinases were considered most effective on gelatins and basement

membrane collagens while collagenases were classified as most effective on interstitial and fibrillar collagens (Vargová *et al*. 2012). The classification has its flaws and one should be careful not to rely solely on this as a description of a MMPs range of activity. MMP2 and MMP9 are classified as gelatinases because gelatin was considered their main substrate. However, they are also fully capable of degrading collagen IV (Zeng *et al*. 1999; Odaka *et al*. 2005; Monaco *et al.* 2006) and MMP2 is in addition capable of degrading collagen type I (Aimes and Quigley 1995). The gelatinases also bind to various other collagen types, proteoglycans, proteins and glycoproteins in the ECM (Murphy *et al*. 1994; Shipley *et al*. 1996; Gianelli *et al*. 1997; Krekoski *et al*. 2002). In living tissue, the production, activation and inhibition of these enzymes is controlled to maintain the tissue in a functional equilibrium (Birkedal-Hansen *et al*. 1993) and in sound connective tissues PG and GAG chains also protect the collagen (Geng *et al.* 2006; Tatara *et al*. 2017). However, failure to keep this balance can have detrimental physiological effects (Vargová *et al*. 2012). The increased amount of soluble collagen in gaping fillets of salmon might suggests the action of such enzymes post-mortem when the equilibrium is no longer upheld. As gelatinases have been shown to be present in salmon blood (Eysturskarð *et al.* 2017), they could potentially cause basement membrane degradation post-mortem if the blood is not drained and cleaned properly from the slaughtered salmon. However, it is also worth noticing that MMPs might not be the main enzymes degrading ECM components (Gill and Parks 2011) and that some studies suggest that the gelatinases actually are weak type IV collagenases (Rowe and Weiss 2008).

Degradation of the ECM might also be caused by other enzymes acting on the proteoglycans and glycosaminoglycans. An example are the ADAMTS (A Disintegrin And Metalloproteinase with ThromboSpondin motifs), a family of enzymes related to the MMPs. The ADAMTS are secreted proteinases that act on extracellular substrates (Porter *et al.* 2005). They are capable of degrading proteoglycans and can have detrimental effects on cell-matrix communication and cause impaired fibrillar ECM networks (Stanton *et al.* 2011). However, as with MMPs it can be difficult to distinguish between situations where the activity of the ADAMTS is a necessity for normal function and when it has an adverse effect on the tissue (Stanton *et al.* 2011). The activity of ADAMTS on various proteoglycans can also be influenced by the presence or absence of glycosaminoglycans (Pratta *et al*. 2000; Kashiwagi *et al.* 2004; Poon *et al*. 2005). Catabolism of glycosaminoglycans themselves usually takes place in the lysosomes after endoenzymes release them from the protein core of the PG and partially degrade the polysaccharide to oligosaccharides. The oligosaccharides are endocytosed and transferred to the lysosomes where a number of exoenzymes reduce them to

monosaccharides and inorganic sulfate (Fuller et al. 2004). Under normal physiological conditions, the lysosomal enzymes are active only in the acidic environment inside the lysosome. However, a rapid decline in muscle pH post-mortem might compromise this barrier and allow these enzymes to be active outside the lysosome (Bahuaud *et al.* 2010). The endoenzymes performing the initial degradation of the glycosaminoglycans include endoglycosidases heparanase and hyaluronidase. The heparanase releases heparan sulfate from the protein core of the PG and cleaves the polysaccharide to shorter oligosaccharides (Bame 2001). Heparanase cleaves the heparan sulfate chains at specific sites generating fragments of 10 to 20 disaccharides (Masola *et al*. 2018). In addition to ECM turnover, heparanase has also been associated with various pathological conditions (Masola *et al*. 2018). Despite their name, hyaluronidases can degrade chondroitin sulfate as well as hyaluronan, and they are thought to be involved in the initial stage of the CS degradation process (Honda *et al.* 2012). However, degradation of glycosaminoglycans can also be performed by bacterial enzymes (Schmidtchen *et al.* 2001; Han *et al.* 2014; Ulmer *et al.* 2014; Wang *et al.* 2015; Kawai *et al.* 2018).

1.12 Bacterial enzymes

Bacteria are present everywhere and in food production there usually is a high awareness of this fact. Hygienic conditions in production facilities are of vital importance as some bacteria can cause illnesses to consumers or spoil food products needlessly fast. However, there has not been much focus on the possibility that bacteria might partly be responsible for the degradation of connective tissue in salmon fillets resulting in gaping or other quality issues not characterized as spoilage. Bacteria are capable of degrading chondroitin sulfate (Prabhakar *et al.* 2005) and heparan sulfate (Tripathi *et al.* 2012; Gu *et al.* 2017) which is commonly exploited in various laboratory methods. However, it is only recently that the true diversity of bacterial GAG degrading enzymes has started to emerge. Recent discoveries have characterized bacterial enzymes deviating from the traditional degradation pathways (Ulmer *et al*. 2014; Ndeh *et al*. 2018; Wang *et al*. 2019) revealing several potential GAG degradation pathways. Various typical soil bacteria are known to produce GAG degrading enzymes, but bacteria with such capabilities have also been detected in the marine environment and in digestive tracts (Han *et al*. 2014; Ulmer *et al*. 2014; Wang *et al*. 2015; Kawai *et al*. 2018). Some bacteria can also produce collagenolytic proteases (Harrington 1996). Similar to the bacterial GAG degrading enzymes, some of these collagenases have been exploited in laboratory

methods. In addition, there has recently been an increase in new identifications of bacterial collagenases (Zhang *et al*. 2015) and application of collagenases derived from marine bacteria has also been studied (Yang et al. 2017). Bacteria, which can degrade the major basement membrane component, collagen IV, have also been detected in the human digestive tract (Pruteanu et al. 2011). Although bacterial collagenases are largely unknown and understudied, it seems that collagenase-producing bacteria are present in the marine environment and potentially in digestive systems of animals. Because the known bacterial collagenases have demonstrated a broad specificity (Duarte *et al*. 2016), the possibility of bacterial influence on degradation of the ECM in salmon fillets is certainly present.

2. Purpose and objectives

The purpose if this study was to gain insight into potential causes of gaping in farmed salmon fillets and possible molecular mechanisms of this process.

The research objectives were to:

1) Establish any potential effect of two production parameters on the occurrence of gaping in farmed salmon fillets. The tested parameters were cleaning of the abdominal cavity and the temperature between slaughter and processing. (Paper I).

2) Determine if analysis of glycosaminoglycan structure in the connective tissue of salmon fillet with and without gaping can reveal potential molecular mechanisms of degradation causing gaping (Paper II).

3) Determine if bacterial enzymes potentially play a role in post-mortem degradation of the connective tissue causing gaping in salmon fillets (Paper III and IV)

4) Estimate the applicability of the used methods for research in causes of gaping in salmon fillets and the level of reproducibility (Paper V and Synoptic discussion).

3. Summaries of included papers

3.1 Paper I

Jacobsen Á, Joensen H, Eysturskarð J. (2017). Gaping and loss of fillet firmness in farmed salmon (*Salmo salar* L.) closely correlated with post-slaughter cleaning of the abdominal cavity. *Aquaculture Research* 48, 321-331. Doi.org/10.1111/are.12884.

The paper describes the results from an experimental setup examining the potential effects of cleaning of the abdominal cavity post-slaughter and the temperature between slaughtering and processing of farmed Atlantic salmon (*Salmo salar* L.) on the level of gaping in the salmon fillets. The study revealed a positive correlation between the amount of blood and bodily fluids left in the abdominal cavity of the salmon during cold storage and occurrence of gaping in the fillets 7 days post-mortem. This finding was supported by a multivariate analysis including all 28 parameters measured.

3.2 Paper II

Jacobsen Á, Shi X, Shao C, Eysturskarð J, Mikalsen S-O, Zaia J (2019). Characterization of glycosaminoglycans in gaping and intact connective tissues of farmed Atlantic salmon (*Salmo salar*) fillets by mass spectrometry. *ACS Omega* 4, 15337-15247. Doi.org/10.1021/acsomega.9b01136.

Following the quality measurements of the salmon from the experimental setup described in Paper I, samples were taken of connective tissue in the fillets in areas with and without gaping. Mass spectrometry analysis of chondroitin sulfate and heparan sulfate were performed on digested GAG pools extracted from these connective tissue samples.

This paper describes the results from the SEC-LC/MS analysis of the two glycosaminoglycans, chondroitin sulfate (CS) and heparan sulfate (HS), extracted from these samples. The results showed differences in the distribution of CS disaccharides with the gaping samples having a lower CS sulfation degree than the intact tissue samples. There were also differences in the HS chain composition between intact and gaping samples.

3.3 Paper III

Jacobsen Á, Mikalsen S-O, Joensen H, Eysturskarð J (2019). Composition and dynamics of the bacterial communities present in the post-slaughter environment of farmed Atlantic salmon (*Salmo salar* L.) and correlations to gelatin degrading activity. *PeerJ* 7:e7040. Doi.org/10.7717/peerj.7040.

The results presented in Paper I and II suggested the possibility of bacterial involvement in the level of gaping in the salmon fillets. This paper describes the results of a next-generation sequencing analysis of the microbial community present in the intestines of farmed Atlantic salmon at slaughter and in skin mucus, abdominal cavity fluids and storage ice during cold storage. Gelatinase activity was also measured in the same samples and comparisons made. The results showed a connection between transfer of intestinal material to the abdominal cavity and the increase in gelatinase activity there.

3.4 Paper IV

Jacobsen Á, Eysturskarð J, Mikalsen S-O. Initial metagenomic screening of microbial communities in stomach and intestines of four fish species inhabiting coastal waters. *In prep.*

The paper describes a small pilot study performed during method development. Samples from intestine and abdomen of four marine fish species inhabiting coastal waters in the Faroe Islands, including farmed Atlantic salmon, were sequenced by the 454 next generation sequencing technology. The results showed microbial communities in Atlantic salmon and cod (*Gadus morhua* L.) similar to other studies. In particular, the bacterial community in the salmon intestines were dominated by *Mycoplasmataceae*, which was also detected in the analysis presented in Paper III. It also revealed a relatively high abundance of potentially pollutant bacteria in the free coastal living species. In addition, it contains the first next-generation sequencing studies of two marine fish species, haddock (*Melanogrammus aeglefinus* L.) and ling (*Molva molva* L.), which had clearly distinct microbiomes.

3.5 Paper V

Jacobsen Á, Mikalsen S-O, Salter I. Comparison of custom made QIIME and standard pipeline SILVAngs bioinformatics for investigation of microbial communities in farmed salmon (*Salmo salar* L.) post-slaughter. *In prep*.

The paper describes a comparison of the custom made bioinformatics analysis applied to the next-generation sequencing study described in paper III with a standardized SILVAngs pipeline analysis of the same data. Differences were detected in obtained reads and OTUs formed. This was reflected in a difference in the alpha diversity estimate Chao1. Bacterial community composition was similar at family level for all sample types, while there were discrepancies at higher resolution.

Aquaculture Research

Aquaculture Research, 2015, 1–11 doi: 10.1111/are.12884

Gaping and loss of fillet firmness in farmed salmon (*Salmo salar* L.) closely correlated with post-slaughter cleaning of the abdominal cavity

Ása Jacobsen[1], Hóraldur Joensen[2] & Jonhard Eysturskarð[1]

[1]Fiskaaling, Aquaculture Research Station of the Faroes, Hvalvík, Faroe Islands

[2]Department of Science and Technology, University of the Faroe Islands, Tórshavn, Faroe Islands

Correspondence: Á Jacobsen, Fiskaaling, Aquaculture Research Station of the Faroes, Við Áir, Hvalvík 430, Faroe Islands. E-mail: asa@fiskaaling.fo

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Abstract

This study analysed the effect of cleaning intensity of the abdominal cavity and storage temperature from slaughter to the end of processing on the quality of farmed salmon (*Salmo salar* L.) fillets. These two parameters were manipulated in an experimental setup using in total thirty salmon with an average weight of 4.2 kg. The experiment was designed to imitate realistic scenarios in a normal production process in the Faroe Islands. The salmon stored at low temperatures had an average muscle temperature of 4.65°C, whereas the salmon stored at ambient temperature had an average muscle temperature of 11.27°C. After the salmon were gutted to remove all viscera except the kidney, the abdominal cavity was either rinsed lightly or meticulously cleansed of kidneys, all blood and bodily fluids. A wide range of quality and production parameters were measured either straight after cleaning or after the salmon had been stored in chipped ice at 1.5°C for 7 days. All measured parameters were analysed for possible correlations by principal component analysis (PCA). Blood and remains left in the abdominal cavity were shown to have a significant negative effect on fillet firmness ($P < 0.01$) and gaping ($P < 0.01$). The different storage temperatures between slaughter and gutting, tested in this experiment, did not significantly affect fillet firmness or gaping. However, the fillet colour showed significant negative correlation ($P < 0.01$) with the storage temperatures applied.

Keywords: fillet quality, fillet gaping, fillet firmness, Atlantic salmon

Introduction

Quality issues related to farmed Atlantic salmon (*Salmo salar* L.) fillets have been studied quite intensively over the last few decades. There are many quality parameters to take into account, but some of the main concerns are gaping and loss of fillet firmness, which according to Mørkøre and Rørvik (2001) are not inherently interrelated. Gaping is the tearing of the connective tissue between muscle layers and hence causes holes and slits in the fish fillet. This leads to the downgrading of the food product, and hence economic loss, because of the rejection by consumers due to its unappealing appearance. This also causes limitations for specialized food production (Pittman, Grigory & Brandebourg 2013). Fillet firmness is measured manually by pressing a finger on the fillet to estimate the elasticity and firmness. This should not be confused with fillet texture, which is measured mechanically by a Texture Analyser. Loss of fillet firmness poses the same problems as gaping for the industry due to difficulty in further processing, downgrading of the product, and consumer rejection is also a very important sensory criterion (Veland & Torrisen 1999; Torgersen, Koppang, Stien, Kohler, Pedersen & Mørkøre 2014). Relating these quality issues to one specific reason has proven to be difficult. Although the extensive research has shed light on several key elements and optimization possibilities, there are still contradictory results, which indicate that the underlying causes are not yet fully understood, especially so for gaping. The most recognized cause of gaping and soft fillets is the increased

level of stress caused by handling prior to and at slaughter (Roth, Slinde & Arildsen 2006; Bahuaud, Mørkøre, Østbye, Veiseth-Kent, Thomassen & Ofstad 2010). This is believed to be linked to a decrease in pH (Lavety, Afolabi & Love 1988; Skjervold, Fjæra, Braarød & Einen 2001). Acidic conditions also cause an increased activity of Cathepsin L in the muscle tissue, which degrades collagen and is linked to the softening of the fillets (Bahuaud, Gaarder, Veiseth-Kent & Thomassen 2010). However, stress cannot always be correlated with gaping and loss and fillet firmness (Kiessling, Espe, Ruohonen & Mørkøre 2004). Fillet firmness has also been shown to be heritable (Bahuaud, Gaarder *et al.* 2010) whereas gaping, on the contrary, does not seem to be genetically determined (Kause, Quinton, Airaksinen, Ruohonen & Koskela 2011). On the other hand, gaping has been shown to vary with season, although a consistent pattern seems difficult to deduce (Mørkøre & Rørvik 2001; Espe, Ruohonen, Bjørnevik, Frøyland, Nordtvedt & Kiessling 2004). In addition, location of catch has been shown to have an effect (Margeirsson, Jonsson, Arason & Thorkelsson 2007). However, it is clear that the loss of strength in the connective tissue, which reveals itself as gaping, is connected to a higher percentage of soluble collagen in the extracellular matrix (ECM) compared to the fillets with intact connective tissue (Espe *et al.* 2004). The location of tearing has been found to be in the sarcolemma, the cell membrane of the muscle cells, which connects the muscle fibres to the myocommatal sheets of the ECM, and in the innermost layer of the myocommata, the endomysium. The myocommatal–muscle fibre interface has been shown to have an increased degree of disconnection with increased gaping (Bremner & Hallett 1985; Hallett & Bremner 1988; Ofstad, Egelandsdal, Kidman, Myklebust, Olsen & Hermansson 1996; Fletcher, Hallett, Jerrett & Holland 1997). Both in the extracellular matrix and in the sarcolemma, the components: collagen, proteoglycans (PGs) and glycosaminoglycans (GAGs) interact to form a structural network, which greatly influences the textural properties of various connective tissue types. Collagenases present in the tissue can cause harmful proteolytic degradation of the collagen but in sound connective tissues PG and GAG chains protect the collagen (Geng, McQuillan & Roughley 2006). In living tissue, the expression, activation and inhibition of these collagenases is controlled to maintain tissue homoeostasis including necessary turnover and restructuring of collagen (Birkedal-Hansen, Moore, Bodden, Windsor, Birkedal-Hansen, DeCarlo & Engler 1993). The increased amount of soluble collagen in gaping tissue therefore suggests that these normal conditions of equilibrium are somehow disturbed. Dr Nielsen, QC Manager at Hiddenfjord (Faroe Islands), states that apart from handling stress, two production parameters seem to be critical factors responsible for the gaping severity of farmed Atlantic salmon fillets. One factor is the initial storage temperature from the time the salmon are killed and until they are further processed. In their experience, chilling the salmon immediately after slaughter seems to have a positive effect on the quality of the fillets. The other factor is the thoroughness of the post-mortem cleaning of the abdominal cavity. Leaving parts of the organs or intestines, blood and/or other bodily fluids in the abdominal cavity was suspected to induce gaping. Changing procedures to meticulously cleaning the abdomen reduced occurrences of gaping (personal communication). This article reports the results of an experiment focusing on these two production parameters, which we will attempt to relate to the corresponding firmness and gaping level of Atlantic salmon fillets. A broad range of other production and quality parameters were also included in the study, and principal component analysis (PCA) was performed to reveal possible correlations.

Materials and methods

Material background and experimental setup

Thirty Atlantic salmon, with an average size of 4.2 kg, were kindly provided by the aquaculture farming company Hiddenfjord (Faroe Islands). All individuals measured were taken from the same net pen on an aquaculture site located in Sørvágur, Faroe Islands. On the day of sampling, 7 July 2011, the seawater temperature in the area was 9.8°C. Fish from the same net pen are usually of the same age and all the biotic and abiotic factors have been identical from fry to slaughter. None of the salmon used in the experiment had reached maturity. The standard process of slaughter laid emphasis on avoiding handling and crowding stress as much as possible. The net pen with salmon was tugged very carefully from the on-growing site in the fjord to the slaughter

facility by the shore. They were subsequently pumped up to the slaughter processing line, where they were killed by a blow to the head in an automated system. The gills were thereafter slit open, before the salmon were released from the automated system to an inspection table. All individuals were inspected manually to ensure the gills had been cut well enough for the salmon to bleed out properly. The first fifteen specimens to be used in the experiment were taken off the inspection table at this stage. They were carefully held with the head angled down until having bled out and thereafter stored at ambient temperature, approximately 11°C. All other individuals were submerged in iced seawater in a transport container. At the time of the experiment, the cooling system unfortunately was not functioning optimally, and the temperature of the ice water in the container was approximately 3.5°C. When full, the container was moved to another facility where the salmon were processed further. The time span between slaughtering and gutting was approximately 2 h. At the first stage on the processing line all salmon were gutted. Intestines and organs were removed, though the kidney was not cut/scraped out completely. The fifteen salmon taken earlier off the inspection table were, at the same time as the others, put through the same process of gutting. At the second stage the salmon were rinsed and their abdominal cavity cleaned thoroughly, removing all traces of organs, intestines, blood and bodily fluids. Ten of the fifteen salmon previously stored at ambient temperature were cleaned well, according to standard procedure, whereas the other five were only rinsed lightly, not cleaned properly. All 15 salmon, previously stored at ambient temperature, were thereafter stored in transport boxes with chipped ice. Fifteen of the salmon that had been stored and transported in the iced seawater were also taken to be used in the experiment. Ten of these 15 individuals were only rinsed lightly, not cleaned properly, whereas the other five were rinsed and the abdominal cavity thoroughly cleaned. Again, all fifteen individuals were subsequently stored in transport boxes with chipped ice. Table 1 provides an overview of the various treatments.

Table 1 The various experimental treatments and number of salmon subjected to investigation

	Well cleaned	Not cleaned	Total
Low temp.	5	10	15
High temp.	10	5	15
Total	15	15	30

Quality measurements and evaluations

Three hours after the beginning of the slaughtering process, all thirty salmon taken aside for the experiment were stored in transport boxes with chipped ice. At this time a few measurements were taken. The objective measurements were length, weight and muscle temperature. Sensory evaluations included stage of rigour mortis, skin condition, colour of gills and cleaning of the abdomen. The sensory evaluations were each categorized into groups 0–3, where 0 was the best condition and 3 the worst. Following the measurements, every individual was again stored in a transport box with chipped ice, and all were subsequently transported to a cooling facility for storage at 1.5°C for 7 days.

On the 7th day, standardized quality measurements and evaluations (Table 2) were carried out by trained personnel, unaware of the experimental changes made to the initial storage temperature and cleaning of the abdominal cavity. The salmon were filleted by hand, and additional sensory evaluations were conducted categorizing each individual according to appearance in the same manner as on day one. These evaluations included the Quality Index Method (QIM) for salmon, developed by Sveinsdottir, Hyldig, Martinsdottir, Jorgensen and Kristbergsson (2003). The number of holes and slits observed in the fillet determined the gaping score. Fillet firmness score was determined by pressing with the forefinger on the loin right in front of the dorsal fin. Objective measurements performed on day seven included analysis of a standard section of the fillet, the Norwegian Quality Cut (NQC – Norwegian standard procedure – NS 9401 1994). The fillet texture was measured as breaking strength with a TA.XT plus Texture Analyser (Stable Micro System Ltd., Surrey, UK), equipped with a flat-ended cylinder (12.5 mm diameter, type P/0.5). The trigger force was 0.1 N and the test speed was 1 mm s^{-1}. The force–time graph was recorded by a computer, equipped with the Texture Expert software for Windows (version 1.15, SMS, Surrey, UK). Three recordings per fillet were made perpendicular to the muscle fibres, and mean values, expressed as the force (N) required puncturing the surface of the sample, were calculated.

Fat content and pigmentation (astaxanthin concentration) in the NQC was analysed using PhotoFish^{MT} (Nofima, Ås, Norway). An overview of all measurements and parameters categorizing the salmon is given in Table 2.

Statistical analysis

Fisher's exact test for Count data (Fisher 1922) was used for testing differences in gaping and firmness scores between the groups with differing cleaning and storage conditions (Table 1). The Fisher's exact test was applied because it is useful for categorical data and, contrary to approximation tests, is valid for small sample sizes. Strictly speaking, the test requires both the column and row totals in the data matrix to be fixed in order for it to be exact. In cases where not all of the row or column totals are conditioned, the Fisher's exact test instead provides a conservative *P*-value. The 4×4 factorial matrixes, four treatment groups and four categories of either gaping or firmness, were analysed. The Fisher exact tests were also applied to two groups at a time, in order to compare groups with only one of the treatment parameters varying. If a treatment parameter was shown to have no significant effect on either gaping or firmness scores, the groups, which differed in only that parameter, were combined. The Fisher exact test was thereafter performed on the 2×2 factorial matrix for the other treatment parameter. The tests were performed in the statistical software package R (http://www.r-project.org).

Table 2 Parameters included in the study

Day	Parameters	Units/Grades
Sensoric evaluations		
1	Loss of scales	0–3
1	Rigour status	0–3
1	Cleaning of the abdominal cavity	0–3
1	Gill colour	0–3
7	Fillet – gaping	0–3
7	Fillet – firmness	0–3
7	Fillet – colour	*
QIM		
7	Skin – appearance	0–2
7	Skin – slime	0–2
7	Skin – smell	0–3
7	Skin – firmness	0–2
7	Eye – pupils	0–2
7	Eye – shape	0–2
7	Gills – colour	0–2
7	Gills – slime	0–2
7	Gills – smell	0–3
7	Abdomen – blood	0–1
7	Abdomen – smell	0–3
Objective measurements		
1	Weight	kg
1	Length	cm
1	K-factor	†
1	Temperature	°C
7	Temperature	°C
7	NQC colour – PhotoFish	‡
7	NQC pigmentation – PhotoFish	mg kg^{-1}
7	NQC fat content§ – PhotoFish	%
7	Fillet texture – Texture Analyzer	*N*

*A *Salmo*Fan™ Ruler was used for measuring.
†K-factor was calculated as $K = 10^{N}W/L^{3}$, $N = 5$.
‡PhotoFish measures expressed as SalmoFan values.
§Group average.

For multivariate analysis the data matrix with results from the extensive sensory analysis was scaled first by normalization and secondly by log-transformation. The number of variables and samples was 28 and 30 respectively. Normalization is obligatory for a subsequent proper comparison of the sample data, and this part of the pre-processing was carried out by expressing each variable as a percentage of the sum of values for each sample. The normalized data were then log-transformed to avoid domination of the variables with comparatively large entries. The data were since subjected to Principal Component Analysis, PCA (Wold 1976) using the software package SIRIUS (Kvalheim & Karstang 1987). In the PCA process the samples or objects are placed in a 28-dimensional vector-space, i.e. one coordinate for each variable and new orthogonal vectors or principal components, PCs, are generated through the centroid of all the objects in the multidimensional space. The courses of the new PCs are in the direction of the largest and second largest variation of the objects. In this manner the dimensionality is reduced from 28 dimensions to two without loosing much of the total variance. The relation among the objects is displayed by projecting them on the plane spanned by the two PCs, that is PC1 and PC2 describing the largest variation and next largest variation respectively.

Results and discussion

Cleaning of the abdominal cavity

The Fisher's exact test applied to the 4×4 factorial matrixes, four treatment groups and four categories of either gaping or fillet firmness, showed

highly significant differences between the groups; $P < 0.01$ for both gaping and firmness. However, this does not state what effect each of the treatment parameters, temperature and level of cleaning, had on the quality estimates. The Fisher exact tests applied to two groups at a time, with the same cleaning treatment but different initial storage temperatures, were not significant for neither gaping nor fillet firmness (data not shown). The Fisher exact tests applied to the groups with different cleaning treatments kept at same temperatures, on the other hand, were significant in most cases. Concerning gaping, the Fisher's exact test of the high temperature groups showed significant differences between the two cleaning treatments ($P < 0.01$), but not for the low temperature groups ($P = 0.077$), whereas the tests for fillet firmness were significant for both high and low initial storage temperatures ($P < 0.01$). Because the temperatures tested in the experiment did not seem to have different effects, the salmon were grouped according to how well the abdominal cavity was cleaned only, for further analyses.

The salmon with well-cleaned abdominal cavity and the salmon, which were not cleaned, had different group distributions of gaping scores (Fig. 1a). The salmon that were not cleaned ($n = 15$) had a very high occurrence of gaping with 14 individuals having the highest gaping score. In the group of salmon with well-cleaned abdominal cavity ($n = 15$), there were equally many with gaping scores 1 and 3. Fisher's exact test for Count data showed a significant difference between the distribution of gaping scores of the two groups ($P < 0.01$).

Fillet firmness did not vary much, as only scores 1 and 2 were detected. This indicated a more moderate effect, but still, the level of fillet firmness in the group of salmon with well-cleaned abdominal cavity was markedly different from that in the group of salmon with not cleaned abdominal cavity (Fig. 1b). Thirteen of the 15 individuals, cleaned well, had a firmness score of 1, whereas all but one of the not cleaned individuals had a firmness score of 2. Again Fisher's exact test for Count data showed a significant difference ($P < 0.01$) between these two groups.

This experiment reveals that bodily fluids, remnants and blood left in the abdominal cavity during storage have a highly negative effect on the fillet quality of Atlantic salmon. The strong correlation detected between not cleaned abdominal

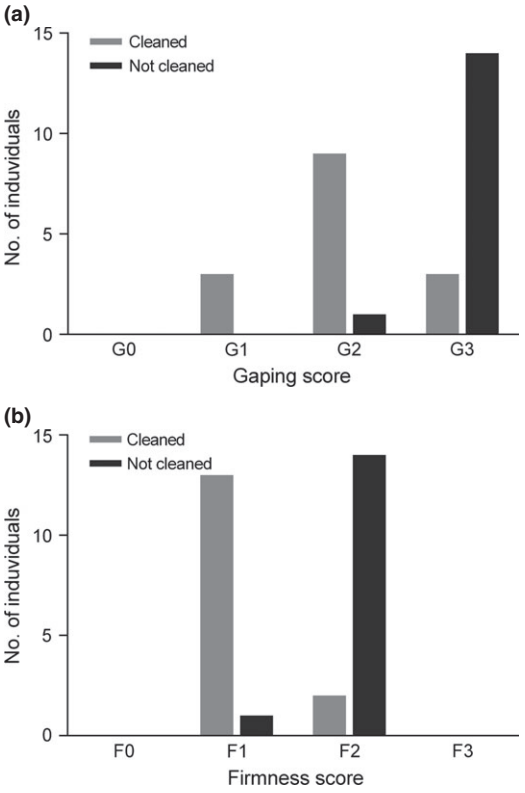

Figure 1 (a,b) Fillet gaping and firmness. Distribution of (a) gaping scores and (b) firmness scores of the individuals with either cleaned or not cleaned abdominal cavities. Gaping score is determined by number of holes and slits in the fillet. G0: no gaping, G1: < 5 small, G2: < 10 small and/or 5 large, G3: > 10 small and/or 5 large. Small slits: < 1 cm, large slits: > 1 cm. Firmness score is determined by pressing with the forefinger on the loin right in front of the dorsal fin. F0: firm and elastic, F1: firm but not elastic, F2: soft, F3: very soft.

cavities and a higher occurrence of gaping and loss of fillet firmness has to our knowledge not been previously reported. However, in a review by Borderias and Sánchez-Alonso (2011) it was recommended to clean gutted fish thoroughly in order to remove traces of blood and intestinal content, as this has been shown to reduce the microbial load (Erkan 2007). For the same reason starvation was highly recommended in order to reduce the amount of digestive enzymes produced by bacteria in the intestines. In fresh, chilled fish the bacteria generally do not invade the muscle tissue, as their activity is mainly on the surface. On the other hand, the bacterial enzymes diffuse from the surface into the muscle tissue, releasing compounds into fluids and watery substances,

which diffuse outwards (Huss 1995). It is also possible for the bacteria to degrade the collagen network in the ECM with their collagenases. These differ from vertebrate collagenases, as they exhibit broader substrate specificity (Peterkofsky 1982; Birkedal-Hansen 1987). They can attack almost all collagen types, and are able to make multiple cleavages within triple helical regions (Mookhtiar & Van Wart 1992). Bacterial collagenases have shown the ability to disrupt the extracellular matrix of arterial walls in vertebrates, where collagenases present in the blood did not cause such degradation (Rosenberg, Estrada, Kelley & Kornfeld 1993). Brown, Hook and Tragakis (1972) reported the same phenomenon, where corneal collagen was protected by proteoglycans from degradation by collagenases present, but was degraded by bacterial collagenases, as they also broke down the proteoglycans. However, bacterial activity is not the only possible explanation for the ECM degradation detected in this experiment, as polymorphonuclear leucocytes in the blood contain collagenases, that are also able to degrade proteoglycans and hence the collagen structure. These lysosomal collagenases, also named neutrophilic collagenase or Matrix Metalloproteinase 8 (MMP-8), have been connected to various diseases, where the collagen structure is damaged (Harris, Faulkner & Brown 1975; Gangbar, Overall, McCulloch & Sodek 1990; Herman, Sukhova, Libby, Gerdes, Tang, Horton, Kilbride, Breitbart, Chun & Schönbeck 2001). Matrix Metalloproteinase 8 is mainly produced in polymorphonuclear granulocytes and preferentially degrade collagen type I. Matrix Metalloproteinase 8 degrades collagen type I much more effectively than the other interstitial collagenases, MMP-1 and MMP-13, which preferentially degrade collagen types III and II respectively (Herman *et al.* 2001). As type I collagen is a major constituent of the ECM in salmon, MMP-8 could very likely cause damage to the connective tissue resulting in gaping of the fillet. Coagulation of the blood also induces an enhanced release and activation of MMP-8 (Jung 2008; Manello 2008), and the remains of the blood present in the abdominal cavity will thus contain a high concentration of activated MMP-8, capable of degrading the ECM in the salmon fillet. As mentioned in the introduction some lysosomal cysteine proteases also have the ability to disrupt the collagen network, but their activity is generally thought to be restricted to the lysosomes, as they are inhibited by the neutral pH and oxidative extra lysosomal environment. However, cathepsin L, which previously has been associated with post-mortem autolysis of fish muscle, has fairly recently been detected in blood cells of certain fish species (Ahimbisibwe, Inoue & Aoki 2010). Blood with lowered pH, caused by stress prior to and at slaughter, left in the abdominal cavity could, thus contain active forms of cathepsins, capable of degrading the ECM structure. Degradation by both bacterial and neutrophilic collagenases can also be enhanced by stress. Hansen, Rødbotten, Eie, Lea, Rudi and Mørkøre (2012) showed that crowding stress had a significant impact on the bacterial load in salmon fillets. Others have discovered that stress causes an increase in polymorphonuclear leucocytes in the blood (Sasagawa, Matsubara & Satow 1993; Suzuki, Totsuka, Nakaji, Yamada, Kudoh, Liu, Sugawara, Yamaya & Sato 1999). Several components may also operate in concert, as various bacteria have the ability to activate host MMPs and neutrophil interstitial procollagenases as well as inactivate proteinase inhibitors (Lähteenmäki, Kuusela & Korhonen 2001).

Further research is required to describe the degradation process properly and determine the enzymes involved in the loss of fillet firmness and increase in fillet gaping.

Temperature between slaughter and gutting

In contrast to the cleaning of the abdominal cavity, the storage temperature between slaughter and gutting did not show an effect on gaping or fillet firmness (data not shown), and Fisher's exact test for Count data was not significant ($P > 0.05$). The muscle temperature in the salmon stored at ambient temperature was on average 11.27°C, SD = 0.08, measured straight after gutting and cleaning. Under optimized conditions, the salmon are stored in iced seawater between slaughter and gutting in order to keep the salmon chilled at all times. However, at the time of sampling, the cooling system was not operating at full effect due to a malfunction. Muscle temperatures measured straight after gutting and cleaning of the fifteen salmon which were chilled was on average 4.65°C, SD = 0.65. This is slightly higher than the desired threshold of max 4.0°C, and the variation within the group is fairly large. These limitations in the comparisons of temperature differences between the various experimental groups, caused

by relatively high and varying muscle temperatures in the low temperature group, have to be taken into account, when considering the results. As the results show no difference in the occurrence of gaping or loss of fillet firmness between the two initial storage temperatures tested, two options are indicated. One option is that the muscle temperature has to be above 11.3°C, during an approximately two to three hour period in the production process before proper storage, to induce gaping or loss of fillet firmness. The other option is that the muscle temperature has to be kept below the approximate 4.5°C, measured in this experiment during initial storage and processing, in order to avoid temperature-induced gaping and loss of fillet firmness. If this latter option would be the case, then only a small elevation of initial storage temperature could have an effect on quality. Purely speculative, the overall high gaping scores found in this study might be an indication of this. Furthermore, it should be kept in mind that many of the possible mechanisms behind the specific tissue degradation that causes gaping or loss of fillet firmness are temperature dependent.

Multivariate analysis

A comprehensive simultaneous evaluation of the samples and variables was obtained by multivariate analysis, PCA, visualized in a PC-plot (Fig. 2), which illustrates the relationship between the manipulated variables, temperature (Temp) and cleaning of the abdominal cavity (Abd) and the sampled individuals as well as their mutual relationship. The reciprocal relationship between all relevant quality variables measured is also illustrated in the plot. The variables centred around origo and a few others without relevancy to the plot were eliminated for clarity.

It is evident that the two manipulated variables, Temp and Abd, have the largest influence on the positioning of both the sampled individuals and the other variables in the PC plot. First, all the other measured variables are much closer to the origo and second all the individual salmon samples are neatly grouped according to temperature and cleaning treatment of the abdominal cavity, positioned in relation to the manipulated variables. Individuals stored at low and high temperatures, respectively, are separated along the axis connecting Temp and origo and the cleaned and not cleaned individuals are separated along the axis

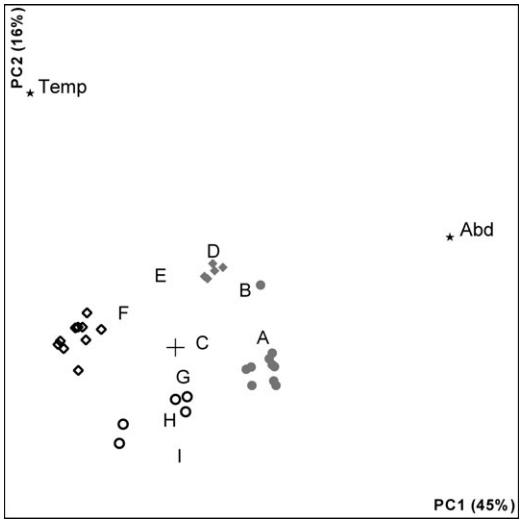

Figure 2 PC plot of variables and samples. The represented variables are: temperature in the abdominal cavity between gutting and storage (* Temp), remains left in the abdominal cavity (* Abd), fillet gaping day 7 (A), fillet firmness day 7 (B), fillet texture day 7 (C), rigour status day 1 (D), smell of skin day 7 (E), abdominal cavity temperature day 7 (F), fillet colour day 7 (G), and colour of gills day 7 (H) and day 1 (I). The samples are salmon with well-cleaned abdominal cavity and kept chilled between slaughter and gutting (○), well cleaned but not chilled (◇), not cleaned but chilled (●), and not cleaned and not chilled (◆). The odd one out sample near variable B had a relatively high temperature (>6°C). The first principal component (PC1) and the second principal component (PC2) describe 45% and 16% of the variance respectively.

connecting Abd and origo. The odd one out had a relatively high storage temperature compared to the other salmon from the same treatment group.

The quality parameters gaping (A) and firmness (B) are drawn towards Abd, which means higher scores are positively correlated with the lack of cleaning of the abdominal cavity. Gaping seems to be slightly more affected as it is drawn a bit closer towards Abd and at a slightly narrower angle.

In order to see how well gaping was correlated with the level of cleaning, a new calculation was carried out including the not cleaned individuals only. The resulting biplot has Abd scores as abscissa and gaping score as ordinate (Fig. 3a).

With one sample excluded, as it turned out to be an outlier, the two variables were perfectly correlated ($R^2 = 1.000$). The exact correlation is of course influenced by the fact that the variables are discrete data, prior to normalization and

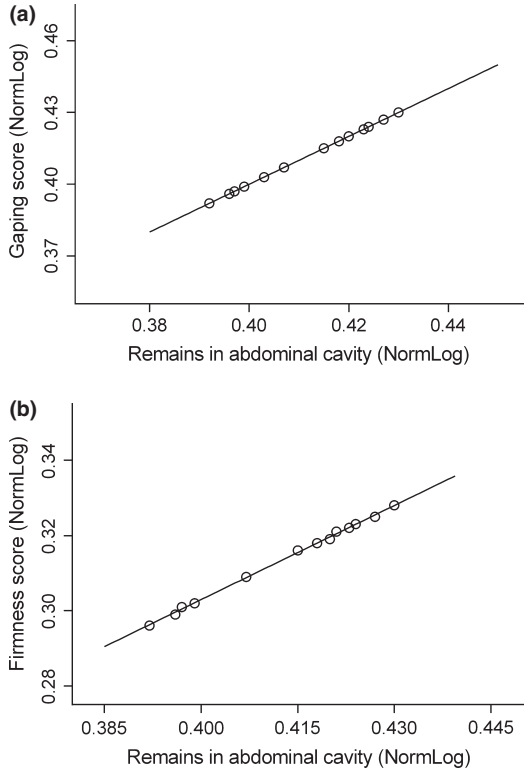

Figure 3 (a,b) Biplot of gaping scores (a) and firmness scores (b) correlated with level of remains in the abdominal cavity after normalization and log-transformation. Not cleaned individuals only.

Log-transformation. In the same way, an analysis of fillet firmness as a function of cleaning of the abdominal cavity was conducted. Again the variables were perfectly correlated ($R^2 = 0.999$) (Fig. 3b), when another different outlier sample was excluded.

Mørkøre and Rørvik (2001) found out that gaping could well occur even though firmness was high. This assessment that there is no linear relationship between gaping and fillet firmness is not disputed by our results. However, it is clearly demonstrated that both quality parameters are negatively affected by a not cleaned abdominal cavity during storage; something researchers should have in mind for future experiments including these parameters.

In the PC plot (Fig. 2) the mechanical measurement of fillet texture (C) is also drawn towards Abd, albeit not as far as gaping and firmness, but in an almost straight line, a clear indication of correlation. As a consequence, the not cleaned salmon thus have fillets with higher breaking strength at the same time as they have more gaping and loss of firmness. The lack of intuitive correlation between texture and gaping has been demonstrated previously by Kiessling *et al.* (2004). Likewise, Stien, Hirmas, Bjørnevik, Karlsen, Nortvedt, Bencze Rørå, Sunde and Kiessling (2005) did not find any significant effect of stress on fillet texture in cod, when measured by a texture analyser. Also rigour (D) is affected by the manipulated parameters, as it is positioned close to the samples neither cleaned nor cooled, which are positively correlated with both Temp and Abd. The correlation between rigour and storage temperature is consistent with previous findings (Borderias & Sánchez-Alonso 2011), but a mutual relationship between rigour and lack of post-slaughter cleaning of the abdominal cavity has to our knowledge not been reported previously.

Along with the high temperature groups, the skin smell (E) and temperature (F) on day 7 are drawn towards Temp (Fig. 2), and thus positively correlated with the initially elevated storage temperatures. The colour of the fillet and gills (G, H, I) on the other hand seems to be negatively correlated with the elevated temperature and not affected by the cleaning of the abdominal cavity, as these are at an almost 90 degree angle to Abd and 180 degree angle to Temp from the Origo. The difference in fillet colour for salmon stored at high and low temperature between slaughter and gutting is illustrated in a boxplot (Fig. 4) with the SalmoFan values (NormLog). A Wilcoxon test,

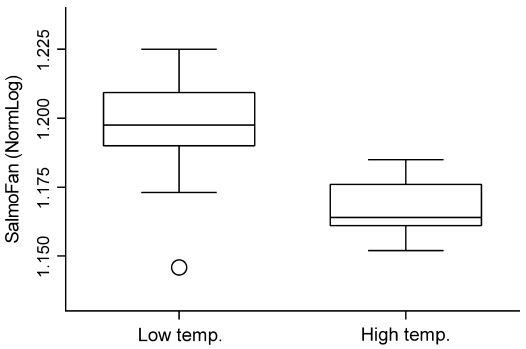

Figure 4 Fillet colour. Boxplot of normalized and log-transformed values of fillet colour comparing salmon chilled (4.65°C average) with salmon kept at ambient temperature (11.27°C average) from slaughter to end of gutting and cleaning. Fillet colour was measured using a *Salmo*Fan™ Ruler and values ranged from 25.5 to 29.0.

performed in Excel, showed a significant difference between the two groups ($P < 0.01$). Fillet colouration is generally caused by carotene deposition in the muscle pre-mortem, but it has been shown to be affected by slaughtering methods (Robb, Kestin & Warris 2000; Erikson & Misimi 2008). However, the effect of temperature differences on fillet colour straight after slaughter has to our knowledge not been reported previously.

Fat content and harvest weight showed no connection to either gaping or loss of fillet firmness (data not shown). This is consistent with previously published findings (Andersen, Strømsnes, Steinsholt & Thomassen 1994; Andersen, Thomassen & Rørå 1997; Johnston, Bickerdike, Li, Dingwall, Nickell, Alderson & Campbell 2007; Kause *et al.* 2011).

Conclusion

In conclusion, the results show that the severity of gaping as well as loss of fillet firmness is significantly increased by leaving blood and/or other remains in the abdominal cavity after slaughter and gutting. The multivariate analysis also verifies that gaping and loss of fillet firmness is not connected to any of the other quality parameters. Furthermore, the experiment shows that an elevated storage temperature of the salmon, for just two to three hours following slaughter, causes loss of fillet and gill colour.

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References

Ahimbisibwe J.B., Inoue K. & Aoki T. (2010) Detection of cathepsin L in red cell membranes from fish blood. *Fisheries Science* **76**, 155–159.

Andersen U.B., Strømsnes A., Steinsholt K. & Thomassen M.S. (1994) Fillet gaping in farmed Atlantic salmon (*Salmo salar*). *Norwegian Journal of Aquaculture Sciences* **8**, 165–179.

Andersen U.B., Thomassen M.S. & Rørå A.M.B. (1997) Texture properties of farmed Rainbow trout (*Oncorhynchus mykiss*): effects of diet, muscle fat content and time of storage on ice. *Journal of the Science of Food and Agriculture* **74**, 347–353.

Bahuaud D., Gaarder M., Veiseth-Kent E. & Thomassen M. (2010) Fillet texture and protease activities in different families of farmed Atlantic salmon (*Salmo salar* L.). *Aquaculture* **310**, 213–220.

Bahuaud D., Mørkøre T., Østbye T.-K., Veiseth-Kent E., Thomassen M.S. & Ofstad R. (2010) Muscle structure response and lysosomal cathepsin B and L in farmed Atlantic salmon (*Salmo salar* L.) *pre-* and *post-rigor* fillets exposed to short and long-term crowding stress. *Food Chemistry* **118**, 602–615.

Birkedal-Hansen H. (1987) Catabolism and Turnover of Collagens: *Collagenases*. *Methods in Enzymology* **144**, 140–171.

Birkedal-Hansen H., Moore W.G.I., Bodden M.K., Windsor L.J., Birkedal-Hansen B., DeCarlo A. & Engler J.A. (1993) Matrix metalloproteinases: a review. *Critical Reviews in Oral Biology and Medicine* **4**, 197–250.

Borderias A.J. & Sánchez-Alonso I. (2011) First processing steps and the quality of wild and farmed fish. *Journal of Food Science* **76**, R1–R5.

Bremner H.A. & Hallett I.C. (1985) Muscle fiber–connective tissue junctions in the fish blue grenadier (*Macruronus novaezelandiae*). A scanning electron microscopy study. *Journal of Food Science* **50**, 975–980.

Brown S.I., Hook C.W. & Tragakis M.P. (1972) Presence, significance, and inhibition of lysosomal proteoglycans. *Investigative Ophthalmology* **11**, 149–152.

Erikson U. & Misimi E. (2008) Atlantic salmon skin and fillet color changes effected by perimortem handling stress, rigor mortis and ice storage. *Journal of Food Chemistry* **73**, C50–C59.

Erkan N. (2007) Sensory, chemical, and microbial attributes of sea bream (Sparus aurata): effects of washing and ice storage. *International Journal of Food Properties* **10**, 421–434.

Espe M., Ruohonen K., Bjørnevik M., Frøyland L., Nordtvedt R. & Kiessling A. (2004) Interactions between ice storage time, collagen composition, gaping and textural properties in farmed salmon muscle harvested at different times of the year. *Aquaculture* **240**, 489–504.

Fisher R.A. (1922) On the interpretation of χ^2 from contingency tables, and the calculation of P. *Journal of the Royal Statistical Society* **85**, 87–94.

Fletcher G.H., Hallett I.C., Jerrett A.R. & Holland A.J. (1997) Changes in the fine structure of the myocommata-muscle fibre junction related to gaping in rested and exercised muscle from king salmon (*Oncorhynchus tchawytacha*). *LWT – Food Science and Technology* **30**, 246–252.

Gangbar S., Overall C.M., McCulloch C.A. & Sodek J. (1990) Identification of polymorphonuclear leukocyte collagenase and gelatinase activities in mouthrinse samples: correlation with peridontal disease activity in

adult and juvenile peridontits. *Journal of Periodontal Research* **25**, 257–267.

Geng Y., McQuillan D. & Roughley P.J. (2006) SLRP interaction can protect collagen fibrils from cleavage by collagenases. *Matrix Biology* **25**, 484–491.

Hallett I.C. & Bremner H.A. (1988) Fine structure of the myocommata-muscle fibre junction in hoki (*Macruronus novaezelandiae*). *Journal of the Science of Food and Agriculture* **44**, 245–261.

Hansen A.Å., Rødbotten M., Eie T., Lea P., Rudi K. & Mørkøre T. (2012) The effect of crowding stress on bacterial growth and sensory properties of chilled Atlantic salmon fillets. *Journal of Food Science* **71**, s84–s90.

Harris E.D. Jr, Faulkner C.S. 2nd & Brown F.E. (1975) Collagenolytic systems in rheumatoid arthritis. *Clinical Orthopaedics and Related Research* **110**, 303–316.

Herman M.P., Sukhova G.K., Libby P., Gerdes N., Tang N., Horton D.B., Kilbride M., Breitbart R.E., Chun M. & Schönbeck U. (2001) Expression of neutrophil collagenase (matrix Metalloproteinase–8) in human atheroma. A novel collagenolytic pathway suggested by transcriptional profiling. *Circulation* **104**, 1899–1904.

Huss H.H. (1995) *Quality and Quality Changes in Fresh Fish*. FAO fisheries technical paper 348. FAO Press, Rome.

Johnston I.A., Bickerdike R., Li X., Dingwall A., Nickell D., Alderson R. & Campbell P. (2007) Fast growth was not associated with an increased incidence of soft flesh and gaping in two strains of Atlantic salmon (*Salmo salar*) grown under different environmental conditions. *Aquaculture* **265**, 148–155.

Jung K. (2008) Measurements of matrix metalloproteinases and their tissue inhibitors in serum produces doubtful results. *The Journal of Infectious Diseases* **198**, 1722–1723.

Kause A., Quinton C., Airaksinen S., Ruohonen K. & Koskela J. (2011) Quality and production trait genetics of farmed European whitefish, *Coregonus lavaretus*. *Journal of Animal Science* **89**, 959–971.

Kiessling A., Espe M., Ruohonen K. & Mørkøre T. (2004) Texture, gaping and colour of fresh and frozen Atlantic salmon flesh as affected by pre-slaughter iso-eugenol or CO_2 anaesthesia. *Aquaculture* **236**, 645–657.

Kvalheim O.M. & Karstang T.V. (1987) A general purpose program for multivariate data-analysis. *Chemometrics and Intelligent Laboratory Systems* **2**, 235–237.

Lähteenmäki K., Kuusela P. & Korhonen T. (2001) Bacterial plasminogen activators and receptors. *FEMS Microbiology Reviews* **25**, 531–552.

Lavety J., Afolabi O.A. & Love R.M. (1988) The connective tissues of fish. IX. Gaping in farmed species. *International Journal of Food Science and Technology* **23**, 23–30.

Manello F. (2008) Serum or plasma samples? The "Cindarella" role of blood collection procedures. Preanalytical methodological issues influence the release and activity of circulating matrix metalloproteinases and their tissue inhibitors, hampering diagnostic trueness and leading to misinterpretation. *Arteriosclerosis, Thrombosis, and Vascular Biology* **28**, 611–614.

Margeirsson S., Jonsson G.R., Arason S. & Thorkelsson G. (2007) Influencing factors on yield, gaping, bruises and nematodes in cod (*Gadus morhua*) fillets. *Journal of Food Engineering*, **80**, 503–508.

Mookhtiar K.A. & Van Wart H.E. (1992) Clostridium histolyticum collagenases: a new look at some old enzymes. *Matrix. Supplement* **1**, 116–126.

Mørkøre T. & Rørvik K.-A. (2001) Seasonal variation in growth, feed utilisation and product quality of farmed Atlantic salmon (*Salmo salar*) transferred to seawater os 0 + smolts or 1 + smolts. *Aquaculture* **199**, 145–157.

Ofstad R., Egelandsdal B., Kidman S., Myklebust R., Olsen R.L. & Hermansson A.M. (1996) Liquid loss as effected by post mortem ultrastructural changes in fish muscle: cod (*Gadus morhua* L.) and salmon (*Salmo salar*). *Journal of the Science of Food and Agriculture* **71**, 301–312.

Peterkofsky B. (1982) Bacterial collagenase. *Methods in Enzymology* **82**, 453–471.

Pittman K., Grigory V.M. & Brandebourg T. (2013) Bridging the gap to sustainable salmon farming: overcoming the gaping problem. *Journal of Fisheries & Livestock Production* **1**, 104.

Robb D.H.F., Kestin C.S. & Warris P.D. (2000) Muscle activity at slaughter: I. Changes in flesh colour and gaping in rainbow trout. *Aquaculture* **182**, 261–269.

Rosenberg G.A., Estrada E., Kelley R.O. & Kornfeld M. (1993) Bacterial collagenase disrupts extracellular matrix and opens blood-brain barrier in rat. *Neuroscience Letters* **160**, 117–119.

Roth B., Slinde E. & Arildsen J. (2006) Pre or post mortem muscle activity in Atlantic salmon (*Salmo salar*). The effect on rigor mortis and the physical properties of flesh. *Aquaculture* **257**, 504–510.

Sasagawa S., Matsubara J. & Satow Y. (1993) Stress-related Induction of Hepatic Metallothionein Synthesis and Increase in Peripheral Polymorphonuclear Leukocytes in Mice. *Immunopharmacology and Immunotoxicology* **15**, 217–226.

Skjervold P.O., Fjæra S.O., Braarød Østby P. & Einen O. (2001) Live-chilling and crowding stress before slaughter of Atlantic salmon. *Aquaculture* **192**, 265–280.

Stien L.H., Hirmas E., Bjørnevik M., Karlsen Ø., Nortvedt R., Bencze Rørå A.M., Sunde J. & Kiessling A. (2005) The effects of stress and storage temperature on the colour and texture of pre-rigor filleted farmed cod (*Gadus morhua* L.). *Aquaculture Research* **36**, 1197–1206.

Suzuki K., Totsuka M., Nakaji S., Yamada M., Kudoh S., Liu Q., Sugawara K., Yamaya K. & Sato K. (1999) Endurance exercise causes interaction among stress

hormones, cytokines, neutrophil dynamics, and muscle damage. *Journal of Applied Physiology* **87**, 1360–1367.

Sveinsdottir K., Hyldig G., Martinsdottir E., Jorgensen B. & Kristbergsson K. (2003) Quality Index Method (QIM) scheme developed for farmed Atlantic salmon (Salmo salar). *Food Quality and Preference* **14**, 237–245.

Torgersen J.S., Koppang E.O., Stien L.H., Kohler A., Pedersen M.E. & Mørkøre T. (2014) Soft Texture of Atlantic Salmon Fillets Is Associated with Glycogen Accumulation. *PLoS ONE* **9**(1), e85551.

Veland J.O. & Torrisen O.J. (1999) The texture of Atlantic salmon (*Salmo salar*) muscle is measured instrumentally using TPA and Warner-Brazler shear test. *Journal of the Science of Food and Agriculture* **79**, 1737–1746.

Wold S. (1976) Pattern recognition by means of disjoint principal component models. *Pattern Recognition* **8**, 127–139.

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Characterization of Glycosaminoglycans in Gaping and Intact Connective Tissues of Farmed Atlantic Salmon (*Salmo salar*) Fillets by Mass Spectrometry

Ása Jacobsen,*[,†] Xiaofeng Shi,[‡] Chun Shao,[‡] Jonhard Eysturskarð,[†] Svein-Ole Mikalsen,[§] and Joseph Zaia[‡]

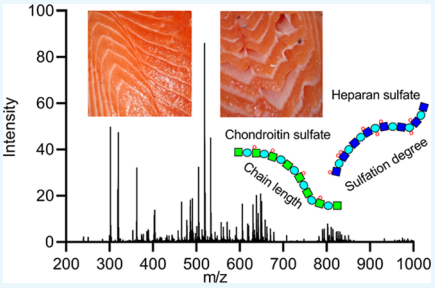
[†]Aquaculture Research Station of the Faroe Islands, Við Áir, FO-430 Hvalvík, The Faroe Islands

[‡]Department of Biochemistry, Center for Biomedical Mass Spectrometry, Boston University School of Medicine, 670 Albany Street, Boston, Massachusetats 02118, United States

[§]Department of Science and Technology, University of the Faroe Islands, Vestara Bryggja 15, FO-100 Tórshavn, The Faroe Islands

Supporting Information

ABSTRACT: In the Atlantic salmon (*Salmo salar*) aquaculture industry, gaping (the separation of muscle bundles from the connective tissue) is a major quality problem. This study characterized chondroitin sulfate (CS) and heparan sulfate (HS) in the connective tissue of intact and gaping salmon fillets from 30 salmon by mass spectrometry. Statistical difference was detected between gaping and intact tissues only when comparing pairwise samples from the same individual (n = 10). The gaping tissue had a lower content of monosulfated CS disaccharides (p = 0.027), and the relative distribution of CS disaccharides was significantly different (p < 0.05). The HS chains were short (average = 14.09, SD = 4.91), and the intact tissue seemed to have a more uniform HS chain structure compared to the gaping tissue. Time-series samples from the same individuals are recommended for future research to improve the understanding of reasons and implications of these differences.



INTRODUCTION

The fish muscle myotomes are arranged as folded sheets that are joined together in layers along the longitudinal axis of the fish by connective tissue, the myocommata. Gaping is defined as the phenomenon in which the connective tissue of fish fillets is weakened and fails to hold the muscle myotomes together, resulting in holes and slits in the fillet. Gaping poses a major problem for the aquaculture and fisheries industries due to the unappealing appearance and limitations in specialized food production of the fillets, which causes down-rating of the quality classification and hence affects pricing.[1]

The location of tearing has been found to be in the sarcolemma (the cell membrane of the muscle cells), which connects the muscle fibers to the myocommatal sheets of the extracellular matrix (ECM) and, in the innermost layer of the myocommata, the basement membrane.[2,3] The myocommata−muscle fiber interface has a lower degree of connection as gaping increases.[4,5] Both in the ECM and in the sarcolemma, the components collagen, proteoglycans (PGs), and glycosaminoglycans (GAGs) interact to form a structural network, which is known to influence strongly the textural properties of various tissue types including connective tissue.[6,7] The main structural component of the ECM is collagen, and the solubility of major collagen types (I and V) has long been suspected to be higher in gaping tissue.[8−10] However, collagen is not the only factor affecting the strength of the ECM. Research in many different fields have revealed that GAG chains of PGs contribute significantly to the formation of a functional collagen network and thus to the structural integrity of connective tissue.[11−14]

There are four main groups of GAGs: chondroitin sulfate (CS)/dermatan sulfate (DS), heparin/heparan sulfate (HS), keratan sulfate (KS), and hyaluronan. They are linear polysaccharides whose disaccharide building blocks consist of an amino sugar (*N*-acetylglucosamine or *N*-acetylgalactosamine) and an uronic acid (glucuronic acid or iduronic acid) or galactose. PGs are glycoproteins covalently modified with heparin, HS, CS/DS, and/or KS chains (see study of Iozzo and Schaefer[7] for the nomenclature). The GAG chains are much larger than other types of glycans typically found on PGs, and they therefore tend to dominate the chemical properties of the PGs.[6,15] Many PGs also exhibit differences in the number of GAG chains, their lengths, and the arrangement of sulfated residues along the chains depending on the PG's tissue location and function.[16,17] A study examining the effect of GAGs on matrix production, distribution, and functionality by inhibiting GAG incorporation[18] showed that an incomplete proteoglycan network resulted in a decrease in collagen deposition and less cross-linked collagen. This work suggested that the GAG incorporation in PGs contributes to the

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Table 1. Chondroitin Sulfate Saccharides Included in the Comparative Analysis[a]

saccharide units	m/z $[M - H]^{-1}$	retention time (min)	composition					ion species
			ΔHexA	HexA	GalN	SO_3	Ac	
dp1	302.29	102.5	0	0	1	1	1	[0,0,1,1,1]
dp2	378.1	106	1	0	1	0	1	[1,0,1,0,1]
	468.01	106	1	0	1	0	1	modification[b]
	757.216	106	1	0	1	0	1	dimer
dp2	458.06	97	1	0	1	1	1	[1,0,1,1,1]
	917.13	97	1	0	1	1	1	dimer
dp2	537.02	90.5	1	0	1	2	1	[1,0,1,2,1]
	268.51	90.5	1	0	1	2	1	$z = 2$
	458.06	90.5	1	0	1	2	1	[1,0,1,2,1][c]
dp3	634.09	93.5	1	1	1	1	1	[1,1,1,1,1]
	839.07	86.5	0	1	2	3	2	sat. [0,1,2,3,2]
	419.03	86.5	0	1	2	3	2	$z = 2$
dp4	757.22	96	1	1	2	0	2	[1,1,2,0,2]
	837.17	86	1	1	2	1	2	[1,1,2,1,2]
	997.09	105.5	1	1	2	3	2	[1,1,2,3,2]
	498.04	105.5	1	1	2	3	2	$z = 2$
dp5	568.65	91	0	2	3	2	3	sat. [0,2,3,2,3], $z = 2$
dp6	1136.33	106	1	2	3	0	3	[1,2,3,0,3]
dp8	757.22	92	1	3	4	0	4	[1,3,4,0,4], $z = 2$

[a]Description of composition by shorthand adapted from ref 30. Abbreviations: ΔHexA, Δ-4,5-unsaturated hexuronic acid; HexA, saturated hexuronic acid; GalN, galactosamine; SO_3, sulfate group; Ac, acetyl group; sat., saturated. [b]Ion with unknown modification assigned as [1,0,1,0,1] based on chromatographic retention time. [c]Loss of SO_3.

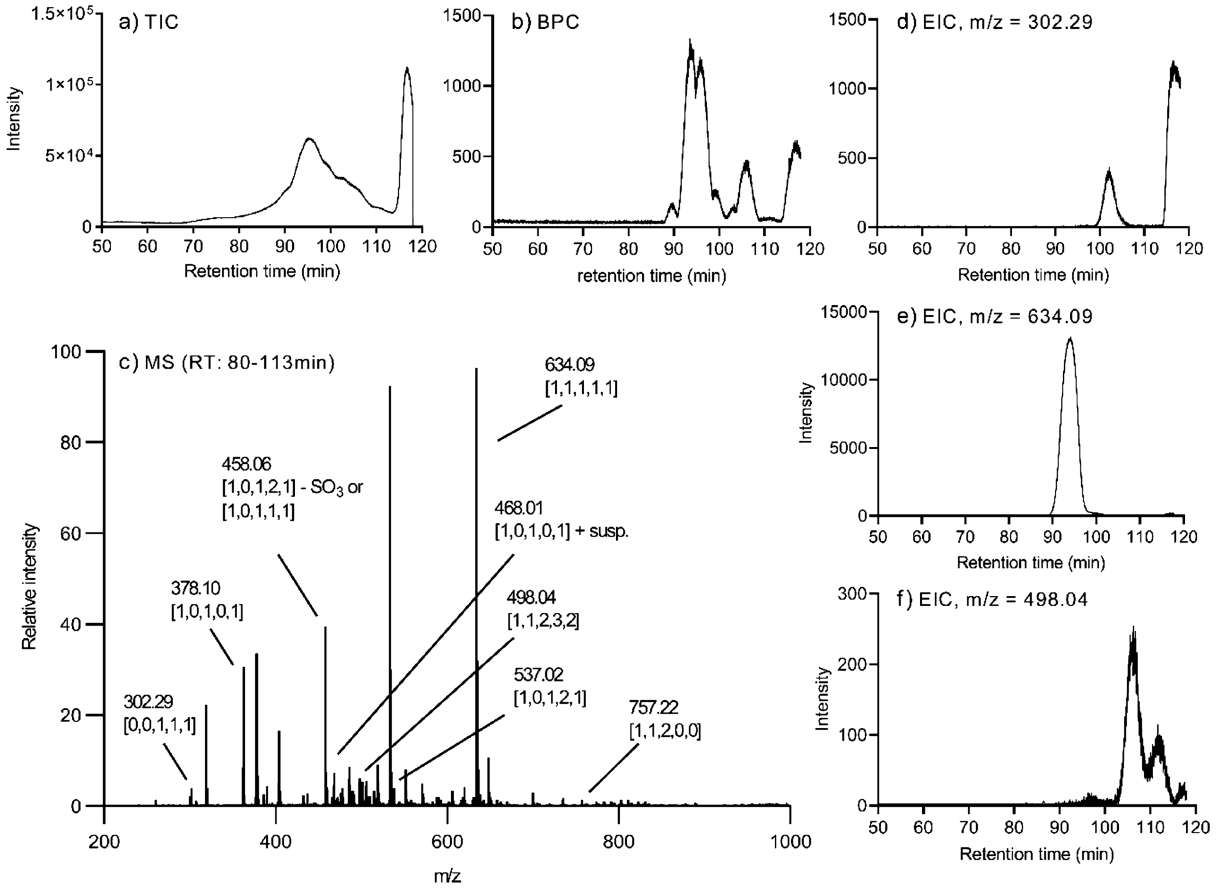

Figure 1. Representative extracted MS spectrum and chromatograms for chondroitinase ABC-digested CS extracted from salmon muscle and connective tissues. (a) TIC, (b) BPC, and (c) extracted MS spectrum from SEC-LC/MS analysis. (c) Various peaks analyzed are labeled with m/z values and description of composition using the nomenclature described previously.[30] EICs of relatively abundant CS oligosaccharides are illustrated in (d) dp1 [0,0,1,1,1], (e) dp3 [1,1,1,1,1], and (f) dp4 [1,1,2,3,2].

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Table 2. Chondroitin Sulfate Measurements[a]

	all samples (n = 32) (μg/mg dried tissue)		intact samples (n = 14) (μg/mg dried tissue)		gaping samples (n = 18) (μg/mg dried tissue)		
chondroitin sulfate	average	SD	average	SD	average	SD	statistics (Welch's t-test)
total dp2	0.9148	0.2683	0.9833	0.317	0.8615	0.1983	p = 0.238
dp2 [1,0,1,0,1]	0.3573	0.0994	0.3541	0.953	0.3598	0.1023	p = 0.876
dp2 [1,0,1,1,1]	0.4786	0.1706	0.5419	0.2192	0.4293	0.0939	p = 0.081
dp2 [1,0,1,2,1]	0.0798	0.0452	0.0894	0.0546	0.0722	0.0343	p = 0.331
dp1	0.032	0.011	0.0321	0.0129	0.032	0.0093	p = 0.985
dp3 [0,1,2,3,2]	0.00111	0.00137	0.00103	0.00101	0.00126	0.0016	p = 0.786
dp3 [1,1,1,1,1]	1.505	0.2203	1.4917	0.1269	1.5154	0.2755	p = 0.749
dp4	0.0494	0.0309	0.0398	0.0281	0.057	0.031	p = 0.123
dp5	0.00201	0.00112	0.0079	0.00111	0.00206	0.00111	p = 0.807
dp6	0.00026	0.00032	0.0003	0.00043	0.00022	0.00019	p = 0.515
dp8	0.00066	0.00103	0.00041	0.00099	0.00086	0.00103	p = 0.230
chain length	99.8	23.65	104.3	29.57	96.6	18.82	p = 0.385
			dp2 distribution (%)				
[1,0,1,0,1]	39.6	7.0	37.2	7.1	41.5	6.5	p = 0.088
[1,0,1,1,1]	52.0	5.7	54.1	5.5	50.4	5.5	p = 0.071
[1,0,1,2,1]	8.4	2.3	8.8	2.5	8.1	2.2	p = 0.446

[a]Welch's t-test was used to test for differences between intact (n = 14) and gaping (n = 18) samples. The chain length was estimated by dividing the total CS saccharide measurements adjusted to disaccharide units with the amount detected of saturated dp1, dp3, and dp5, which originate from the nonreducing end.

formation of a functional collagen network. In addition, a major function of PGs is binding collagen fibers together and anchoring cells to collagen or basement membranes.[7,13,19−22] Some researchers claim that the degradation of the myocommata is caused mainly by the degradation of the PGs.[23] The content of sulfated GAGs has been shown to differ between fish species, which are differently prone to gaping.[24] Also, content of various PGs known to interact with collagen were shown to vary among these species,[21] and the degradation of the intramuscular connective tissue in Pacific rockfish (*Sebastes* sp.) has also been related to both increased solubility of collagen and degradation of the GAGs.[11] However, most published studies that analyzed the PG and GAG content in connection with fillet quality in other fish species and salmon fillets (e.g., refs 25 and 26) have not focused on gaping.

The aim of this study was to examine the content and structure of GAGs in gaping and intact salmon fillets in detail. Although all GAG types are of importance, CS and HS were of most interest in this instance as they are parts of PGs and they were estimated to be present in the tissue in adequate amounts for MS measurements. Liquid chromatography−mass spectrometry (LC−MS) methods are capable of providing detailed knowledge of the structural diversities of GAGs in tissue.[27] These methods provide insight into variations in phenotypic distribution of GAGs in tissue of different origin, developmental stage, or disease stage.[27−35] The research presented here reveals the content and distribution of structural phenotypes of the two GAGs, chondroitin sulfate (CS) and heparan sulfate (HS), as well as comparisons between gaping and intact connective tissues in farmed salmon (*Salmo salar*) fish fillets.

RESULTS AND DISCUSSION

Chondroitin Sulfate. When digested with chondroitinase ABC, the average amount of CS disaccharides detected was 0.9147 μg/mg (SD = 0.4559) dried tissue. By comparison, extraction from fish heads, bones, or other organs containing a higher content of GAGs than muscle tissue generally resulted in higher amounts of extracted CS.[36,37] The amounts of CS recovered from various diamond squid tissue types were between 0.021 and 3.482 μg/mg defatted tissue,[38] while another study[39] detected 0.11 μg/mg sulfated CS disaccharides in rat muscle. Therefore, the amounts recovered from salmon muscle tissue in this study were at levels comparable with those found in similar tissue of other animal species. In addition to disaccharides, the digestion of CS resulted in a variety of mono- and oligosaccharides. In-source dimer formation of nonsulfated and singly sulfated CS disaccharides during ionization was also registered and included in the analysis. The dimers were identified by having the same size exclusion chromatography (SEC) retention times as the respective disaccharides but with m/z values corresponding to dimers (Table 1). To our knowledge, there have not been any previous reports on in-source dimer formation of glycosaminoglycans. However, multimer formation in MS analyses of various compounds is not uncommon.[40,41] All chondroitin sulfates detected and included in the comparative analysis are listed in Table 1, with m/z values, retention time, description of composition by shorthand (adapted from ref 42), and ion species detected.

The total ion chromatograms (TICs) showed a considerable amount of noise when samples were examined by SEC-HPLC, as illustrated by a representative CS TIC in Figure 1a. An attempt to reduce the noise by precipitating CS in chilled ethanol prior to chondroitinase digestion proved unsuccessful. However, the base peak chromatograms (Figure 1b) show that relevant MS peaks could be analyzed without any problems. A representative MS spectrum with various peaks labeled is illustrated in Figure 1c. Representative extracted ion chromatograms (EICs) of the most abundant dp1, dp3, and dp4 ion species are illustrated in Figure 1d−f.

EICs of every ion species listed in Table 1 were measured for all samples, and comparisons were made between gaping and intact tissue samples. No significant differences were found in the abundances of the measured CS ion species, the CS chain

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Table 3. Pairwise Comparisons of Intra-Individual Gaping and Intact Samples

	intact samples (n = 10) (μg/mg dried tissue)		Gaping samples (n = 10) (μg/mg dried tissue)		
chondroitin sulfate	average	SD	average	SD	statistics (paired t-test)
total dp2	1.0161	0.362	0.8866	0.1656	p = 0.212
10101	0.3438	0.1058	0.3845	0.0928	p = 0.285
10111	0.5537	0.1994	0.4336	0.0893	p = 0.027[a]
10121	0.0244	0.0646	0.0692	0.0982	p = 0.098
dp1	0.0296	0.0118	0.0342	0.0104	p = 0.265
dp3 [0,1,2,3,2]	0.0012	0.0011	0.001	0.0012	p = 0.803
dp3 [1,1,1,1,1]	1.4584	0.1257	1.537	0.2821	p = 0.507
dp4	0.0407	0.0264	0.045	0.0353	p = 0.636
dp5	0.0009	0.0011	0.0023	0.0017	p = 0.284
dp6	0.00033	0.00052	0.00024	0.0002	p = 0.663
dp8	0.00057	0.00119	0.00091	0.00109	p = 0.578
chain length	109.6	28.75	94.64	19.91	p = 0.172
dp2 distribution (%)					
[1,0,1,0,1]	35.2	7.03	43.33	6.17	p = 0.002[b]
[1,0,1,1,1]	55.49	5.72	49.04	5.05	p = 0.006[b]
[1,0,1,2,1]	9.3	2.52	7.63	1.5	p = 0.03[a]

[a]Significant at $p < 0.05$. [b]Significant at $p < 0.01$.

length, or the relative dp2 distribution when comparing all gaping and intact samples. All measurements and statistical values for the comparison between intact and gaping tissues are listed in Table 2.

Chondroitin sulfate has a normal chain length ranging from 40 to 120 disaccharide units, depending on tissue context,[43] although shorter chains also have been reported.[30] Therefore, the average CS chain length of 99.8 disaccharide units estimated in this study is within the expected range. The length of the CS chain and molecular weight distribution have been reported to have a considerable effect on CS function and interaction with other molecules.[44] The average CS chain length of intact tissue was higher than that of gaping tissue, but the difference was not significant (Table 2).

The dp3 composition [1,1,1,1,1] (see Table 1) accounted for between 93.3 and 99.8% of the various mono- and oligosaccharides, excluding the dp2s. An earlier study[45] reported the presence of CS trisaccharides resistant to chondroitinase digestion and concluded that they originated from the reducing terminal end of CS chain and their release caused by either alkaline treatment of the PG or presence of tissue endo-β-D-glucuronidases. Another study[46] also detected these trisaccharides in shark cartilage and suggested that they were generated by breakdown of the CS chain or during commercial processing. Because sampling for this study took place 7 days postmortem, some degradation might well have occurred, although it is difficult to estimate to what degree since no such analysis has been made of fresh salmon muscle and connective tissues for comparison. The cause of such a high concentration of these trisaccharides does not appear to be related to the connective tissue degradation causing gaping as no significant difference was detected between their content in intact and gaping tissues.

To our knowledge, no comparable quantitative analysis of GAGs in fish muscle has been reported. However, the relative CS dp2 distribution detected in this study was considerably different from that detected in other studies analyzing the CS content in various fish species,[37,47−50] although these studies focused on other tissues such as bone, whole embryo or adult, fins, swim bladder, and cartilage. The present CS sulfation degree was much lower as nearly 40% of the CS disaccharides were nonsulfated, while these other studies reported from undetectable amounts to ∼33% at the most of nonsulfated CS disaccharides. On the other hand, the relative content of disulfated CS disaccharides was at a more comparable level to these other studies at around 8 to 10% (Table 2). There were no significant differences detected in the relative distribution of the three dp2 ion species when comparing all gaping and intact samples (Table 2).

Pairwise Comparisons of Intra-Individual Gaping and Intact Tissue Samples. Comparisons of the intact and gaping sample pairs taken from the same individuals (n = 10) showed a significant difference in the relative distribution of the dp2 phenotypes (Table 3). Each dp2 phenotype was tested for discrimination between the two sample types by the paired t-test and by the paired Wilcoxon test (not shown). Both tests showed significant difference between intact samples and gaping samples for all three dp2 phenotypes.

Comparisons of the measured CS amounts showed significant difference in the monosulfated dp2 [1,0,1,1,1] only (Table 3). Representative EICs of the dp2 phenotypes for intact (Figure 2a) and gaping (Figure 2b) samples illustrate the relation between the peak sizes.

The comparisons of the relative dp2 values of the intact and gaping sample pairs from each individual were illustrated in a

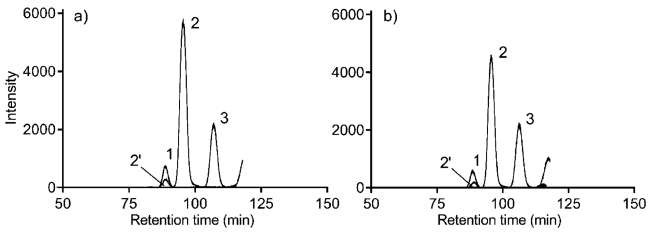

Figure 2. Representative combined EICs of the three major CS disaccharide compositions from (a) intact and (b) gaping tissues. Peak 1, disulfated CS dp2 [1,0,1,2,1]; peak 2, monosulfated CS dp2 [1,0,1,1,1]; peak 2′, in source loss of SO_3 from monosulfated CS; peak 3, nonsulfated CS dp2 [1,0,1,0,1].

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dot plot showing connections between the samples taken from the same individual (Figure 3). Intact samples nearly always

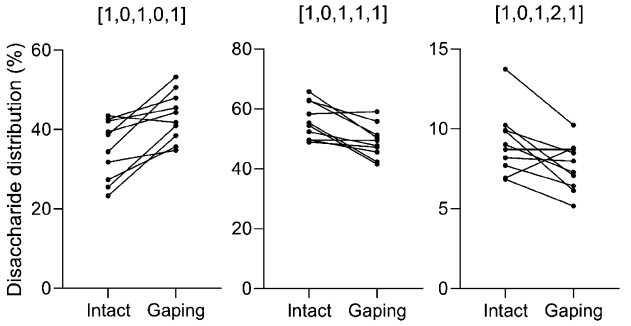

Figure 3. Dot plot comparing the relative distribution of the three CS disaccharide compositions in intact (n = 10) and gaping (n = 10) sample pairs from the same individuals. The sample pairs are illustrated with connecting lines.

had lower relative content of nonsulfated CS dp2s compared to gaping samples from the same individual and a higher relative content of both mono- and disulfated CS dp2s.

This means that a difference was found in the CS sulfation degree between intact and gaping samples from the same fillet, while no difference was seen in total dp2 content or any CS saccharide other than the monosulfated disaccharide. This indicates that CS in gaping tissue has been more subject to desulfation. A higher degree of desulfation but not degradation might suggest the action of endosulfatases. Although the endogenous CS catabolism has not been fully established, the most recognized path of CS degradation in vertebrates is by fragmentation of the CS chain into oligosaccharides followed by sequential degradation from the nonreducing end by exoglycosidases and sulfatases working in tandem.[51] A study showed that both total CS content and CS4 were significantly reduced by higher galactosamine-4-sulfatase levels in humans.[52] Therefore, it might be possible that the different degradation pattern in gaping tissue is caused by bacterial endosulfatase activity.[53] Many bacteria in the human intestines are capable of digesting GAGs,[54] and chondroitin sulfate 4-*O*-endosulfatases from marine bacteria have also been identified.[55] These bacterial enzymes might potentially display a higher activity or a wider range of CS-degrading pathways. To our knowledge, no eukaryotic CS endosulfatases have yet been reported. The bacterial CS 4-*O* endosulfatases can remove 4-*O*-sulfate from CS polysaccharide chains but are inhibited by the 6-*O*-sulfation of GalNAc.[55] This corresponds well with the findings in this study showing that the differences between the singly sulfated and nonsulfated forms in gaping and intact tissues are highly significant, while the difference in relative content of the doubly sulfated CS disaccharides is less significant (Table 3). An earlier study showed a correlation between intestinal fluids and/or blood left in the abdominal cavity post-slaughter and a higher degree of gaping.[56] Potentially, bacteria present in salmon intestines are capable of producing endosulfatases acting on chondroitin sulfate. On the other hand, the desulfation of CS polysaccharides by bacterial sulfatases might also promote the digestion of CS by bacterial lyases, which could cause a difference in CS content.

Alternatively, there could be some inherent variation in the sulfation degree in the connective tissue of the salmon fillets, leaving the integrity of some areas more vulnerable to disintegration by the action of sulfatases from the blood and/or intestinal fluids. Further research into the CS desulfation potential of fish blood and intestinal bacteria is needed to clarify any potential effect of these substances on the connective tissue and chondroitin sulfate composition. Comparisons to fresh tissue are also recommended.

Another implication of these findings is that future research might benefit from comparing samples from the same individuals over a time period postmortem instead of comparing different individuals. The results from this study imply that the total content and relative distribution of CS dp2 in the tissue vary from individual to individual and it might be

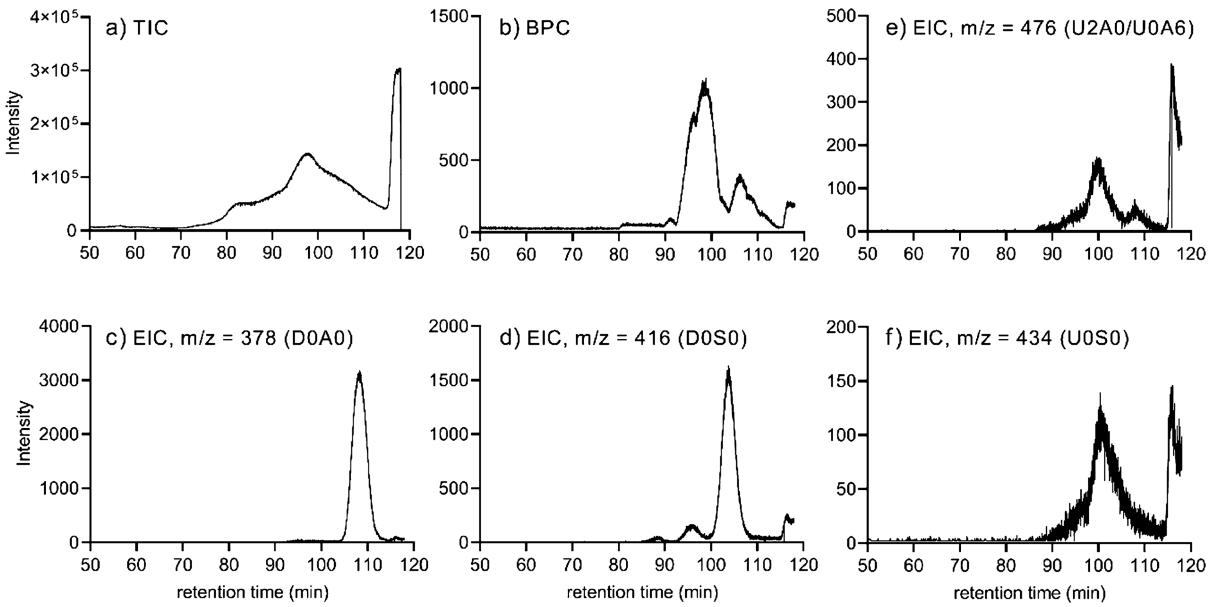

Figure 4. Representative chromatograms for heparanase I, II, and III-digested HS extracted from salmon muscle and connective tissues. (a) TIC, (b) BPC, and EICs of the relatively abundant Δ-unsaturated disaccharides (c) D0A0 and (d) D0S0 and the relatively abundant saturated disaccharides (e) U2A0/U0A6 and (f) U0S0.

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Table 4. HS Measurements and Comparisons of Amounts Detected, Chain Length, and Sulfation Degree

	all samples (n = 32)		intact samples (n = 14)		gaping samples (n = 18)		statistics[a]	
parameter	average	SD	average	SD	average	SD	Welch's t-test	Paired t-test
total amount of HS (μg/mg dried tissue)	0.194	0.065	0.209	0.058	0.189	0.069	0.411	0.552
chain length	14.09	4.91	14.6	4.8	13.7	5.11	0.598	0.744
Δ-unsat. sulf. degree	0.473	0.054	0.467	0.06	0.478	0.05	0.579	0.28
Δ-unsat. N-sulf. degree	0.323	0.018	0.322	0.02	0.326	0.016	0.572	0.037[b]
sat. sulf. degree	0.945	0.137	0.96	0.118	0.939	0.154	0.676	0.964
sat. N-sulf. degree	0.27	0.137	0.256	0.123	0.281	0.15	0.604	0.76

[a]Welch's t-test included all samples, while the paired t-test only included those intact (n = 10) and gaping (n = 10) sample pairs retrieved from the same individuals. [b]Significant at $p < 0.05$.

difficult to find a specific threshold amount or dp2 distribution indicative of gaping tissue. On the other hand, the results indicate that changes within the same individual are more informative.

Heparan Sulfate. The TICs of the HS samples showed some noise (Figure 4a), but as with CS, the BPC was better resolved (Figure 4b) and it was possible to quantify disaccharides using the SEC-MS data. In addition, Figure 4 illustrates representative EICs of relatively abundant HS Δ-unsaturated disaccharides (Figure 4c,d) and saturated disaccharides (Figure 4e,f).

The amount of HS disaccharides measured after GAG extraction and digestion by heparanase I, II, and III was, on average, 0.209 μg/mg dried tissue. A similar amount of HS, 0.2 μg/mg dried tissue, was detected in swim bladders of groupers (subfamily Epinephelinae).[49] For comparison, there was 0.34 μg/mg sulfated HS detected in rat muscle,[39] while the amounts detected in various defatted and dried murine tissue types were between 0.120 and 3.30 μg/mg.[57] As with the CS analysis, the HS values were compared between all intact and gaping samples as well as between intact and gaping sample pairs from the same individual (n = 10). An overview of all HS dp2 compositions and ion species measured with m/z values and retention times can be found in Table S1. There was no difference detected in the total amount of HS disaccharides recovered from intact and gaping tissues by either comparison method (Table 4). In this study, the amount of CS dp2s detected (Table 2) was 4.7 times more abundant than that of HS dp2s (Table 4). A study of cod and wolffish, the first prone to gaping and the other not,[23] reported that the sulfated CS/HS ratios in the fillets were 0.58 and 4.14, respectively.[24] However, these measurements were performed using different techniques than applied in this study.

The HS chain length, which was calculated by dividing the total amount of disaccharides with the saturated amount, was 14.09 dp2s on average (Table 4), which is short compared to the 25−200 disaccharides considered to be the common range.[58] In comparison, various tissue types in rat contained an HS chain length between ∼30 and 60 disaccharide units.[27] It is difficult to estimate to what extent the relatively short chain length is caused by postmortem degradation as there are no data of this kind from live or newly slaughtered fish. However, as the salmon was in storage for 7 days postmortem before sampling, it is likely that some degradation has taken place. In addition, no significant differences were detected in the HS chain length between intact and gaping tissues (Table 4). This indicates that gaping is not caused by a difference in rate of the usual form of degradation where HS chains are first cleaved into smaller fragments by endo-β-glucuronidase followed by a well-ordered sequential degradation including several enzymes. Endolytic heparanase activity can cause shorter HS chains[59] as they seem to have larger affinity for cleaving glycosidic bonds closest to the nonreducing end.[60]

The short chain length was most likely also the reason that the HS dp2 distribution detected in this study, as illustrated in Figure 5 using shorthand description,[61] was different and that

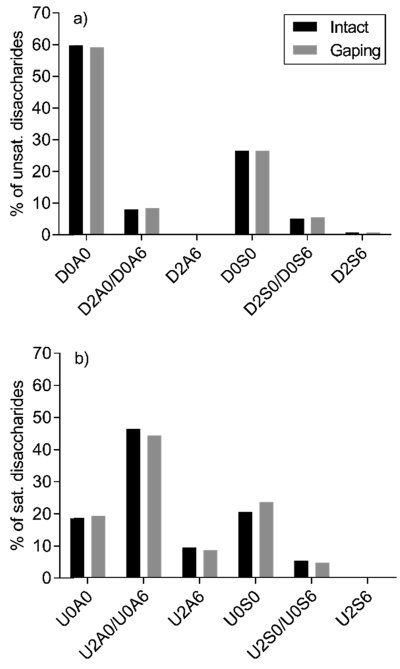

Figure 5. Relative distribution of (a) Δ-unsaturated and (b) saturated HS disaccharides in intact and gaping tissues. The HS dp2s are abbreviated using the nomenclature described previously.[63]

the HS sulfation degree (Table 4) was lower than that detected in other species and organs.[27,42,47] HS chains are arranged in domains of high sulfation, low sulfation, and mixed regions. The specific arrangement of domains seems to be important for the function of HS.[62] Salmon muscle might contain less sulfated HS chains, or the comparatively low sulfation degree might be a reflection of postmortem desulfation or loss of more densely sulfated regions due to postmortem degradation. However, rat skeletal muscle has also been demonstrated to have a relatively low sulfation degree of HS, both internally in the chain and at the nonreducing end, compared to various other organs.[27] No significant difference was detected in the overall sulfation degree of the HS chains between gaping and intact tissues in our samples. However, a significant difference

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was found in the Δ-unsaturated *N*-sulfation degree between intact and gaping tissues ($p = 0.034$) sampled from the same individual ($n = 10$). The gaping samples generally had a higher Δ-unsaturated *N*-sulfation degree. No difference was detected when comparing all samples (Table 4).

Consistent with the findings of others,[42] there was a clear difference in the sulfation pattern of the internal chain and nonreducing end (Figure 5). The unsaturated HS disaccharides had a low level of *O*-sulfation (Figure 5) as they were mainly nonsulfated (~59.5%) or just *N*-sulfated (~26.5%), while the saturated disaccharides had a much higher sulfation degree with only ~19% nonsulfated dp2s. Whether this is a reflection of the original chain structure or it is due to postmortem degradation of the HS chains is difficult to estimate. A comparison with fresh salmon tissue samples is needed to clarify that issue.

From the saturated and unsaturated dp2 distribution (Figure 5), it is also possible to estimate the probability of where the various disaccharide phenotypes are positioned. U2A6 dp2s are much more likely to be at the NRE than internally in the chain. This is in stark contrast to various bovine and murine organs,[42] while some rat organs also show this tendency, albeit to a lesser degree.[27] In humans, the HS nonreducing end seems to be very important for binding affinity of HS to fibroblast growth factor-2 (FGF2), which regulates, among other things, cell adhesion.[63]

Comparisons between NRE dp2 distribution and chain length showed a gradual change in the composition as the chain length increased or decreased (Figure 6). The proportion of *N*-sulfated disaccharides at the nonreducing end increases with increasing chain length, consistent with findings of others.[42] The three NRE dp2 phenotypes, U0A0, U2A0/U0A2, and U2S0/U0S2, seem to have a correlated pattern, while the other dp2 phenotypes do not seem to have a structured pattern (data not shown). Interestingly, there is a difference between intact and gaping samples in the stringency of this structure. While the intact samples can be regressed to a trend line with R^2 values between 0.44 and 0.77 (Figure 6a), the gaping samples are less uniform, and the trend lines are less valid with R^2 values between 0.05 and 0.56 (Figure 6b). Whether a larger variation in the HS chain structure means a less organized and weaker connective tissue is uncertain but might be worth the attention.

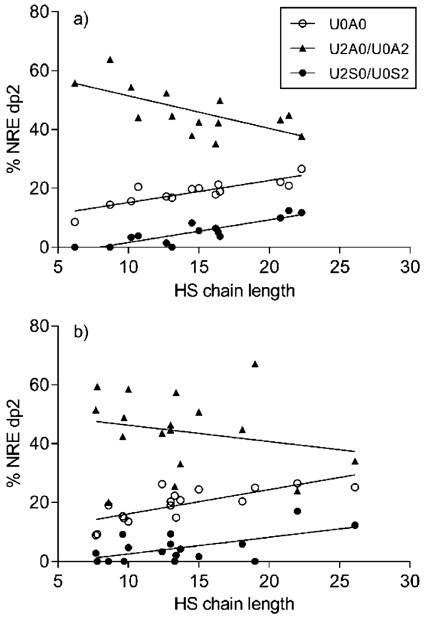

Figure 6. Distribution of HS disaccharides at the NRE compared to chain length for (a) intact and (b) gaping samples. Linear regression values for (a) intact tissue were as follows: U0A0, $R^2 = 0.73$, $p < 0.0001$; U2A0/U0A2, $R^2 = 0.44$, $p = 0.0094$; U2S0/U0S2, $R^2 = 0.77$, $p < 0.0001$. Linear regression values for (b) gaping tissue were as follows: U0A0, $R^2 = 0.56$, $p < 0.0005$; U2A0/U0A2, $R^2 = 0.05$, $p = 0.4103$; U2S0/U0S2, $R^2 = 0.35$, $p < 0.0128$.

CONCLUSIONS

There were significant differences detected in the relative content of CS dp2 phenotypes in gaping and intact tissue samples when comparing samples taken from the same individual. The CS dp2s in gaping tissue had a lower sulfation degree. In addition, the amount of singly sulfated CS dp2 was significantly lower in gaping tissue. This difference in CS sulfation degree could be due either to an inherent difference in the tissue or endosulfatases in which bacteria are known to produce because there was no difference in degradation of CS otherwise. The HS analysis revealed overall short chain lengths potentially caused by the action of endogenous heparanases. The only significant difference in the HS structure between intact and gaping samples was that gaping samples had a higher Δ-*N*-sulfation degree in terms of the internal chain. Gaping samples also had a less uniform chain structure. Further elucidation of reasons and implications of these differences requires additional research including samples of fresh tissue for comparison. Differences in the GAG structure between intact and gaping tissues were mainly detected when comparing intact and gaping samples taken from the same individuals. Potential threshold values for GAG function failure in relation to gaping might thus be difficult to ascertain for a species in general. Instead, it might be beneficial for future research to compare postmortem time series of samples from the same individuals.

EXPERIMENTAL SECTION

Samples. Thirty farmed Atlantic salmon were kindly provided by the farming company Luna (Faroe Islands). The salmon, taken at an aquaculture facility during normal slaughtering procedures, were ~2.5 years of age and had an average weight of 4.2 kg. All salmon were from the same net pen and had experienced the same conditions from fry to slaughter. None of the salmon had reached maturity. The 30 salmon were used in a postmortem experimental setup (detailed in ref 64) for analyzing the effect of cleaning of the abdomen and initial storage temperature in the presence of gaping. After 7 days in cold storage, all 30 salmon were evaluated for the presence of gaping. At the same time, one gaping and/or one intact tissue sample was taken from each of the 30 salmon ($n = 43$). The samples consisted of connective tissue, myocommata, and the adjacent muscle tissue. Illustrations of intact and gaping fillets as well as sampling area are provided in Figure S1. The samples were stored at −18 °C and later homogenized into powder while frozen by sterile mortar and pestle. The samples were subsequently lyophilized.

Reagents. Heparin lyases I, II, and III from *Flavobacterium heparinum* were purchased from Ibex Pharmaceuticals (Montreal, Canada). Chondroitinase ABC from *Proteus*

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vulgaris and benzonase were purchased from Sigma-Aldrich (St. Louis, MO, USA). Pronase was purchased from Roche Applied Science.

GAG Extraction. GAGs were extracted from dry tissue (~60 mg) of the 43 samples by a slightly modified version of the extraction procedure previously described.[27] The tissue was suspended in 0.75 mL of protein digest buffer (50 mM Tris/HCl (pH ≈ 8), 1 mM $CaCl_2$, 1% Triton X-100) and incubated with end-over-end mixing for 48 h at 55 °C. Pronase (1 mg/sample) was added initially and again after 24 h. Following heat inactivation of pronase by suspending the sample tubes into boiling water for 5 min, the buffer was adjusted to 2 mM $MgCl_2$. Benzonase (400 mg/sample) was added, and samples were incubated at 37 °C for 3 h. Then, the samples were adjusted to 0.5 M NaOH and incubated overnight with end-over-end mixing at 4 °C. Thereafter, the samples were acidified with acetic acid to pH ≈ 5.5 and centrifuged at 15.000*g* for 15 min. The supernatants were transferred to new tubes, and 3 mL of MilliQ water was added. DEAE-Sephacel columns were prepared for GAG purification by adding 2.0 mL of slurry (Sigma-Aldrich) to the 10 mL columns (Bio-Rad) with frits only at the bottom. The resins were flushed with 10 mL of 0.1 M NaCl and 20 mM NaOAc (pH = 6.0) and eluted by gravity. The samples were added and washed with 25 mL of the same solution. The samples were since eluted, and GAGs were recovered by eluting with 2.5 mL of 1 M NaCl and 29 mM NaOAc (pH = 6.0). For desalting, PD-10 (GE Healthcare) columns and buffer reservoirs were washed with 30 mL of MilliQ water before the samples were added and eluted by gravity with 3.5 mL of MilliQ water. Each of the eluted samples was divided into two tubes and freeze-dried overnight for further analysis.

GAG Digestion. CS and HS were chosen for MS analysis as these are the most abundant GAGs in the muscle connective tissue that otherwise has a low concentration of GAGs. Forty percent of the total extracted GAG pool was digested with heparin lyases I, II, and III, targeting heparan sulfate, while 15% was digested with chondroitinase ABC, targeting chondroitin sulfate. Each of the two tubes with extracted and dried GAG pool samples was rehydrated with 5.0 μL of H_2O. For HS digestion, 4.0 μL of GAG pool was incubated at 37 °C for 24 h with 0.5 μL of 100 mM $Ca(OAc)_2$, 2.0 μL of 100 mM Tris/HCl (pH = 7.4), and 7.0 μL of a mixture of HS lyases I, II, and III. For CS digestion, 1.5 μL of GAG pool was incubated at 37 °C for 24 h with 1.0 μL of 100 mM NH_4OAc, 3.0 μL of 100 mM Tris/HCl (pH = 8.0), and 6.5 μL of CH ABC. Because digested GAGs can be separated from GAG polysaccharides with size exclusion chromatography used in connection with LC−MS, no further treatment was needed. A simplified overview of GAG extraction and digestion is provided in Figure S2. For optimal use of laboratory facilities, some of the extracted GAG samples were pooled in equivalent amounts, resulting in a final sample size of $n = 32$ for SEC-LC/MS analysis (Table 5). Pooled samples were of either gaping or intact tissue and from fish within the same experimental group and with the same gaping classification.[64] Pooling of samples was performed without interfering with the possibility of comparing intact and gaping tissue samples from the same individual.

Liquid Chromatography−Mass Spectrometry Analysis. For the disaccharide analysis of HS and CS, the technical design of the SEC-LC/MS system used was the same as reported by Shi and Zaia.[27] The mobile phase (12.5 mM formic acid, pH adjusted to 4.4 by ammonia, in 10% acetonitrile) was delivered isocratically at 0.015 mL/min. Disaccharides and oligosaccharides eluting from the SEC column were analyzed using the Applied Biosystems QSTAR Pulsar-I (Q-ToF) mass spectrometer operating in negative polarity using the enhanced mode. The MS analyses were performed with an internal standard (ΔHexA2S-GlcNS6S), making quantification possible. HS and CS saccharides were identified according to m/z values and retention times, as described.[27] Throughout the HS and CS analyses, 10 randomly chosen samples were run in triplicate to confirm consistent technical performance.

Table 5. Tissue Samples for Glycosaminoglycan Extraction and Mass Spectrometry Analysis[a]

salmon no.	gaping tissue	intact tissue	number of samples
1	X		1
2 and 9 (pooled)		X	1
3	X		1
4	X		1
5	X	X	2
6	X		1
7	X	X	2
8 and 10 (pooled)	X		1
11		X	1
12 and 15 (pooled)	X		1
13	X	X	2
14	X	X	2
16 and 18 (pooled)	X	X	2
17	X	X	2
19 and 20 (pooled)	X		1
21	X	X	2
22 and 25 (pooled)		X	1
23 and 24 (pooled)	X	X	2
26	X	X	2
27 and 28 (pooled)	X		1
29	X	X	2
30		X	1
total	18	14	32

[a]Some samples were pooled in equivalent amounts following GAG extraction. Pooled samples were of same tissue type, gaping or intact, from two individuals, as indicated.

Statistical Analysis. All statistical analyses were performed using R (http://www.r-project.org). The significance level was set at p-values below 0.05. Welch's t-test[65] was used when comparing all gaping and intact tissue samples ($n = 32$). The paired t-test[66] and paired Wilcoxon test,[67] a distribution-free method that replaces the data with corresponding order statistics, were applied to all pairwise comparisons of HS and CS measurements from gaping ($n = 10$) and intact ($n = 10$) sample pairs taken from the same 10 individuals.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.9b01136.

Table S1, compositions, m/z values, ion species, and retention times of all HS disaccharides measured; Figure S1, illustration of intact and gaping fillets and sampling area; Figure S2, overview of GAG extraction and digestion procedures (PDF)

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AUTHOR INFORMATION

Corresponding Author

*E-mail: asa@fiskaaling.fo.

ORCID

Ása Jacobsen: 0000-0002-2574-7448

Joseph Zaia: 0000-0001-9497-8701

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Notes

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ABBREVIATIONS USED

CS	Cchondroitin sulfate
dp2	disaccharide
ECM	Eextracellular matrix
EIC	Eextracted ion chromatogram
GAGs	Gglycosaminoglycans
HPLC	Hhigh- performance liquid chromatography
HS	Hheparan sulfate
MS	Mmass spectrometry
PGs	Pproteoglycans
SEC	Ssize exclusion chromatography
TIC	Ttotal ion chromatogram

REFERENCES

(1) Pittman, K.; Grigory, V. M.; Brandebourg, T. Bridging the gap to sustainable salmon farming: overcoming the gaping problem. *J. Fisheries Livest. Prod.* **2013**, *01*, 104.

(2) Bremner, H. A.; Hallett, I. C. Muscle Fiber−Connective tissue junctions in the fish blue grenadier (*macruronus novaezelandiae*). A scanning electron microscope study. *J. Food Sci.* **1985**, *50*, 975−980.

(3) Hallett, I. C.; Bremer, H. A. Fine structure of the myocommata-muscle fibre junction in hoki (*Macruronus novaezelandiae*). *J. Sci. Food Agric.* **1988**, *44*, 245−261.

(4) Ofstad, R.; Egelandsdal, B.; Kidman, S.; Myklebust, R.; Olsen, R. L.; Hermansson, A.-R. Liquid loss as effected by Post mortem ultrastructural changes in fish muscle: cod (*Gadus morhua* L) and salmon (*Salmo salar*). *J. Sci. Food Agric.* **1996**, *71*, 301−312.

(5) Fletcher, G. C.; Hallett, I. C.; Jerrett, A. R.; Holland, A. J. Changes in the fine structure of the myocommata-muscle Fibre junction related to gaping in rested and exercised muscle from king salmon (*Oncorhynchus* tshawytscha). *LWT−Food Sci. Technol.* **1997**, *30*, 246−252.

(6) Mouw, J. K.; Ou, G.; Weaver, V. M. Extracellular matrix assembly: a multiscale deconstruction. *Nat. Rev. Mol. Cell Biol.* **2014**, *15*, 771−785.

(7) Iozzo, R. V.; Schaefer, L. Proteoglycan form and function: A comprehensive nomenclature of proteoglycans. *Matrix Biol.* **2015**, *42*, 11−55.

(8) Bjørnevik, M.; Espe, M.; Beattie, C.; Nortvedt, R.; Kiessling, A. Temporal variation in muscle fibre area, gaping, texture, colour and collagen in triploid and diploid Atlantic *salmon*(*Salmo salar* L). *J. Sci. Food Agric.* **2004**, *84*, 530−540.

(9) Espe, M.; Ruohonen, K.; Bjørnevik, M.; Frøyland, L.; Nordtvedt, R.; Kiessling, A. Interactions between ice storage time, collagen composition, gaping and textural properties in farmed salmon muscle harvested at different times of the year. *Aquaculture* **2004**, *240*, 489−504.

(10) Hagen, Ø.; Johnsen, C. A. Flesh quality and biochemistry of light-manipulated Atlantic cod (*Gadus morhua*) and the significance of collagen cross-links on fillet firmness and gaping. *Food Chem.* **2016**, *190*, 786−792.

(11) Kim, K.; Haard, N. F. Degradation of proteoglycans in the skeletal muscle of pacific rockfish (*Sebastes* Sp.) during ice storage. *J. Muscle Foods* **1992**, *3*, 103−121.

(12) Billinghurst, R. C.; Dahlberg, L.; Ionescu, M.; Reiner, A.; Bourne, R.; Rorabeck, C.; Mitchell, P.; Hambor, J.; Diekmann, O.; Tschesche, H.; Chen, J.; Van Wart, H.; Poole, A. R. Enhanced cleavage of type II collagen by collagenases in osteoarthritic articular cartilage. *J. Clin. Invest.* **1997**, *99*, 1534−1545.

(13) Danielson, K. G.; Baribault, H.; Holmes, D. F.; Graham, H.; Kadler, K. E.; Iozzo, R. V. Targeted disruption of decorin leads to abnormal collagen fibril morphology and skin fragility. *J. Cell Biol.* **1997**, *136*, 729−743.

(14) Costell, M.; Gustafsson, E.; Aszódi, A.; Mörgelin, M.; Bloch, W.; Hunziker, E.; Addicks, K.; Timpl, R.; Fässler, R. Perlecan maintains the integrity of cartilage and some basement membranes. *J. Cell Biol.* **1999**, *147*, 1109−1122.

(15) Ly, M.; Laremore, T. N.; Linhardt, R. J. Proteoglycomics: Recent Progress and Future Challenges. *OMICS* **2010**, *14*, 389−399.

(16) Farach-Carson, M. C.; Carson, D. D. Perlecan − a multifunctional extracellular proteoglycan scaffold. *Glycobiology* **2007**, *17*, 897−905.

(17) Prydz, K Determinants of glycosaminoglycan (GAG) structure. *Biomolecules* **2015**, *5*, 2003−2022.

(18) Bastiaansen-Jenniskens, Y. M.; Koevoet, W.; Jansen, K. M. B.; Verhaar, J. A. N.; DeGroot, J.; VanOsch, G. J. V. M. Inhibition of glycosaminoglycan incorporation influences collagen network formation during cartilage matrix production. *Biochem. Biophys. Res. Commun.* **2009**, *379*, 222−226.

(19) Chakravarti, S.; Magnuson, T.; Lass, J. H.; Jepsen, K. J.; LaMantia, C.; Carroll, H. Lumican regulates collagen fibril assembly: Skin fragility and corneal opacity in the absence of lumican. *J. Cell Biol.* **1998**, *141*, 1277−1286.

(20) al Jamal, R.; Roughley, P. J.; Ludwig, M. S. Effect of glycosaminoglycan degradation on lung tissue viscoelasticity. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **2001**, *280*, L306−L315.

(21) Tingbø, M. G.; Kolset, S. O.; Ofstad, R.; Enersen, G.; Hannesson, K. O. Identification and distribution of heparan sulfate proteoglycans in the white muscle of Atlantic cod (*Gadus morhua*) and spotted wolffish (*Anarhichas minor*). *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **2006**, *143*, 441−452.

(22) Kalamajski, S.; Oldberg, Å. The role of small leucine-rich proteoglycans in collagen fibrillogenesis. *Matrix Biol.* **2010**, *29*, 248−253.

(23) Ofstad, R.; Olsen, R. L.; Taylor, R.; Hannesson, K. O. Breakdown of intramuscular connective tissue in cod (Gadus morhua L.) and spotted wolffish (Anarhichas minor O.) related to gaping. *LWT−Food Sci. Technol.* **2006**, *39*, 1143−1154.

(24) Tingbø, M. G.; Kolset, S. O.; Ofstad, R.; Enersen, G.; Hannesson, K. O. Sulfated glycosaminoglycans in the extracellular matrix of muscle tissue in Atlantic cod (*Gadus morhua*) and spotted wolffish (*Anarhichas minor*). *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **2005**, *140*, 349−357.

(25) Hannessson, K. O.; Tingø, M. G.; Olsen, R. L.; Enersen, G.; Bævre, A. B.; Ofstad, R. An immunological study of glycosaminoglycans in the connective tissue of bovine and cod skeletal muscle. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **2007**, *146*, 512−520.

(26) Torgersen, J. S.; Koppang, E. O.; Stien, L. H.; Kohler, A.; Pedersen, M. E.; Mørkøre, T. Soft texture of Atlantic salmon fillets is associated with glycogen accumulation. *Plos One* **2014**, *9*, No. e85551.

(27) Shi, X.; Zaia, J. Organ-specific heparan sulfate structural phenotypes. *J. Biol. Chem.* **2009**, *284*, 11806−11814.

(28) Zaia, J. Glycosaminoglycan glycomics using mass spectrometry. *Mol. Cell. Proteomics* **2013**, *12*, 885−892.

(29) Ramachandra, R.; Namburi, R. B.; Ortega-Martinez, O.; Shi, X.; Zaia, J.; Dupont, S. T.; Thorndyke, M. C.; Lindahl, U.; Spillmann, D.

DOI: 10.1021/acsomega.9b01136
ACS Omega 2019, 4, 15337−15347

Brittlestars Contain Highly Sulfated Chondroitin Sulfates/Dermatan Sulfates that Promote fibroblast growth factor 2-induced Cell Signaling. *Glycobiology* **2014**, *24*, 195−207.
(30) Shao, C.; Shi, X.; White, M.; Huang, Y.; Hartshorn, K.; Zaia, J. Comparative glycomics of leukocyte glycosaminoglycans. *Rev. Geophys.* **2013**, *280*, 2447−2461.
(31) Mathias, D. K.; Pastrana-Mena, R.; Ranucci, E.; Tao, D.; Ferruti, P.; Ortega, C.; Staples, G. O.; Zaia, J.; Takashima, E.; Tsuboi, T.; Borg, N. A.; Verotta, L.; Dinglasan, R. R. A Small Molecule Glycosaminoglycan Mimetic Blocks *Plasmodium* Invasion of the Mosquito Midgut. *PLoS Pathog.* **2013**, *9*, No. e1003757.
(32) Thelin, M. A.; Svensson, K. J.; Shi, X.; Bagher, M.; Axelsson, J.; Isinger-Ekstrand, A.; van Kuppevelt, T. H.; Johansson, J.; Nilbert, M.; Zaia, J.; Belting, M.; Maccarana, M.; Malmström, A. Dermatan sulfate is involved in the tumorigenic properties of Esophagus Squamous Cell Carcinoma. *Cancer Res.* **2012**, *72*, 1943−1952.
(33) Staples, G. O.; Shi, X.; Zaia, J. Glycomics analysis of mammalian heparan sulfates modified by the human extracellular sulfatase HSulf2. *PLoS One* **2011**, *6*, No. e16689.
(34) Schumacher, V. A.; Schlötzer-Schrehardt, U.; Karumanchi, S. A.; Shi, X.; Zaia, J.; Jeruschke, S.; Zhang, D.; Pavenstädt, H.; Drenckhan, A.; Amann, K.; Ng, C.; Hartwig, S.; Ng, K.-H.; Ho, J.; Kreidberg, J. A.; Taglienti, M.; Royer-Pokora, B.; Ai, X. WT1-Dependent Sulfatase Expression Maintains the Normal Glomerular Filtration Barrier. *J. Am. Soc. Nephrol.* **2011**, *22*, 1286−1296.
(35) Langsdorf, A.; Schumacher, V.; Shi, X.; Tran, T.; Zaia, J.; Jain, S.; Taglienti, M.; Kreidberg, J. A.; Fine, A.; Ai, X. Expression regulation and function of heparan sulfate 6-O-endosulfatases in the spermatogonial stem cell niche. *Glycobiology* **2011**, *21*, 152−161.
(36) Stine, J. J.; Wu, T. H.; Oliveira, A. C. M.; Smiley, S.; Bechtel, P. J. Extraction and determination of chondroitin sulfate from fish processing byproducts. In *A sustainable future: Fish byprocessing byproducts*; Bechtel, P. J.; Smiley, S. Eds.; Alaska Sea Grant, University of Alaska Fairbanks: Fairbanks, Alaska, 2010; pp. 41−53. DOI: 10.4027/sffpb.2010.04.
(37) Maccari, F.; Galeotti, F.; Volpi, N. Isolation and structural characterization of chondroitin sulfate from bony fishes. *Carbohydr. Polym.* **2015**, *129*, 143−147.
(38) Tamura, J.-i.; Arima, K.; Imazu, A.; Tsutsumishita, N.; Fujita, H.; Yamane, M.; Matsumi, Y. Sulfation patterns and the amounts of chondroitin sulfate in the diamond squid, *Thysanoteuthis rhombus*. *Biosci., Biotechnol., Biochem.* **2009**, *73*, 1387−1391.
(39) Barbosa, I.; Garcia, S.; Barbier-Chassefière, V.; Caruelle, J. P.; Martelly, I.; Papy-García, D. Improved and simple micro assay for sulfated glycosaminoglycans quantification in biological extracts and its use in skin and muscle tissue studies. *Glycobiology* **2003**, *13*, 647−653.
(40) Stefansson, M.; Sjöberg, P. J. R.; Markides, K. E. Regulation of multimer formation in electrospray mass spectrometry. *Anal. Chem.* **1996**, *68*, 1792−1797.
(41) Domalain, V.; Tognetti, V.; Hubert-Roux, M.; Lange, C. M.; Joubert, L.; Baudoux, J.; Rouden, J.; Afonso, C. Role of cationization and multimers formation for diastereomers differentiation by ion mobility-mass spectrometry. *J. Am. Soc. Mass Spectrom.* **2013**, *24*, 1437−1445.
(42) Staples, G. O.; Shi, X.; Zaia, J. ExtendedN-Sulfated domains reside at the Nonreducing end of Heparan sulfate chains. *J. Biol. Chem.* **2010**, *285*, 18336−18343.
(43) Little, P. J.; Ballinger, M. L.; Burch, M. L.; Osman, N. Biosynthesis of natural and hyperelongated chondroitin sulfate glycosaminoglycans: New insights into an elusive process. *Open Biochem. J.* **2008**, *2*, 135−142.
(44) Igarashi, N.; Takeguchi, A.; Sakai, S.; Akiyama, H.; Hagashi, K.; Toida, T. Effect of molecular sizes of chondroitin sulfate on interaction with L-selectin. *Int. J. Carbohydr. Chem.* **2013**, 856142.
(45) Sugahara, K.; Takemura, Y.; Sugiura, M.; Kohno, Y.; Yoshida, K.; Takeda, K.; Khoo, K. H.; Morris, H. R.; Dell, A. Chondroitinase ABC-resistant sulfated trisaccharides isolated from digests of chondroitin/dermatan sulfate chains. *Carbohydr. Res.* **1994**, *255*, 165−182.
(46) Lauder, R. M.; Huckerby, T. N.; Nieduszynski, I. A. A fingerprinting method for chondroitin/dermatan sulfate and hyaluronan oligosaccharides. *Glycobiology* **2000**, *10*, 393−401.
(47) Zhang, F.; Zhang, Z.; Thistle, R.; McKeen, L.; Hosoyama, S.; Toida, T.; Linhardt, R. J.; Page-McCaw, P. Structural characterization of glycosaminoglycans from zebrafish in different ages. *Glycoconj. J.* **2009**, *26*, 211−218.
(48) Higashi, K.; Takeuchi, Y.; Mukuno, A.; Tomitori, H.; Miya, M.; Linhardt, R. J.; Toida, T. Composition of glycosaminoglycans in Elasmobranchs including several deep-sea sharks: Identification of chondroitin/dermatan sulfate from the dried fins of *Isurus oxyrinchus* and *Prionace glauca*. *PLoS One* **2015**, *10*, No. e0120860.
(49) Pan, Y.; Wang, P.; Zhang, F.; Yu, Y.; Zhang, X.; Lin, L.; Linhardt, R. J. Glycosaminoglycans from fish swim bladder: isolation, structural characterization and bioactive potential. *Glycoconj. J.* **2018**, *35*, 87−94.
(50) Vázguez, J. A.; Fraguas, J.; Novosa-Carvallal, R.; Reis, R. L.; Antelo, L. T.; Pérez-Martín, R. I.; Valcarcel, J. Isolation and chemical characterization of chondroitin sulfate from cartilage by-products of blackmouth catshark (*Galeus* melastomus). *Mar. Drugs* **2018**, *16*, 344.
(51) Kaneiwa, T.; Mizumoto, S.; Sugahara, K.; Yamada, S. Identification of human hyaluronidase-4 as a novel chondroitin sulfate hydrolase that preferentially cleaves the galactosaminidic linkage in the trisulfated tetrasaccharide sequence. *Glycobiology* **2010**, *20*, 300−309.
(52) Bhattacharyya, S.; Kotlo, K.; Shukla, S.; Danziger, R. S.; Tobacman, J. K. Distinct effects ofN-Acetylgalactosamine-4-sulfatase and galactose-6-sulfatase expression on chondroitin sulfates. *J. Biol. Chem.* **2008**, *283*, 9523−9530.
(53) Ulmer, J. E.; Vilén, E. M.; Namburi, R. B.; Benjdia, A.; Beneteau, J.; Malleron, A; Bonaffé, D.; Driguez, P.-A.; Descroix, K.; Lassalle, G.; Le Narvor, C.; Sandström, C.; Spillmann, D.; Berteau, O. Characterization of glycosaminoglycan (GAG) sulfatases from the human gut SymbiontBacteroides thetaiotaomicronReveals the first GAG-specific bacterial endosulfatase. *J. Biol. Chem.* **2014**, *289*, 24289−24303.
(54) Kawai, K.; Kamochi, R.; Oiki, S.; Murata, K.; Hashimoto, W. Probiotics in human gut microbiota can degrade host glycosaminoglycans. *Sci. Rep.* **2018**, *8*, 10674.
(55) Wang, W.; Han, W.; Cai, X.; Zheng, X.; Sugahara, K.; Li, F. Cloning and characterization of a novel chondroitin sulfate/dermatan sulfate 4-*O*-endosulfatase from a marine bacterium. *J. Biol. Chem.* **2015**, *290*, 7823−7832.
(56) Han, W.; Wang, W.; Zhao, M.; Sugahara, K.; Li, F. A novel eliminase from a marine bacterium that degrades hyaluronan and chondroitin sulfate. *J. Biol. Chem.* **2014**, *289*, 27886−27898.
(57) Warda, M.; Toida, T.; Zhang, F.; Sun, P.; Munoz, E.; Xie, J.; Linhardt, R. J. Isolation and characterization of heparan sulfate from various murine tissues. *Glycoconj. J.* **2006**, *23*, 555−563.
(58) Kreuger, J.; Spillman, D.; Li, J.-p.; Lindahl, U. Interactions between heparan sulfate and proteins: the concept of specificity. *J. Cell Biol.* **2006**, *174*, 323−327.
(59) Lindahl, U.; Kjellén, L. Pathophysiology of heparan sulphate: many diseases, few drugs. *J. Intern. Med.* **2013**, *273*, 555−571.
(60) Peterson, S.; Liu, J. Deciphering mode of action of heparanase using structurally defined oligosaccharides. *J. Biol. Chem.* **2012**, *287*, 34836−34843.
(61) Lawrence, R.; Lu, H.; Rosenberg, R. D.; Esko, J. D.; Zhang, L. Disaccharide structure code for the easy representation of constituent oligosaccharides from glycosaminoglycans. *Nat. Methods* **2008**, *5*, 291−292.
(62) Nugent, M. A.; Zaia, J.; Spencer, J. L. Heparan sulfate-protein binding specificity. *Biochemistry (Moscow)* **2013**, *78*, 726−735.
(63) Naimy, H.; Buczek-Thomas, J. A.; Nugent, M. A.; Leymarie, N.; Zaia, J. Highly sulfated nonreducing end-derived heparan sulfate domains bind fibroblast growth factor-2 with high affinity and are

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enriched in biologically active fractions. *J. Biol. Chem.* **2011**, *286*, 19311−19319.
(64) Jacobsen, Á.; Joensen, H.; Eysturskarδ, J. Gaping and loss of fillet firmness in farmed salmon (*Salmo* salarL.) closely correlated with post-slaughter cleaning of the abdominal cavity. *Aquac. Res.* **2017**, *48*, 321−331.
(65) Moser, B. K.; Stevens, G. R. Homogeneity of variance in the two-sample means test. *Am. Stat.* **1992**, *46*, 19−21.
(66) Altman, D. G. *Practical statistics for medical research*, 1st edition; Chapman and Hall/CRC: London, England, 1991.
(67) Wilcoxon, F Individual comparisons by ranking methods. *Biom. Bull.* **1945**, *1*, 80−83.

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Composition and dynamics of the bacterial communities present in the post-slaughter environment of farmed Atlantic salmon (*Salmo salar* L.) and correlations to gelatin degrading activity

Ása Jacobsen[1], Svein-Ole Mikalsen[2], Hóraldur Joensen[2] and Jonhard Eysturskarð[1]

[1] Fiskaaling, Aquaculture Research Station of the Faroes, Við Áir, Hvalvík, The Faroe Islands
[2] Department of Science and Technology, University of the Faroe Islands, Tórshavn, The Faroe Islands

ABSTRACT

Background. Microbial analyses performed in connection with the post-slaughter environment of farmed Atlantic salmon (*Salmo salar* L.) have mostly focused on specific bacteria that may have negative effects on the health of consumers. However, bacteria may also affect other quality variables. The objective of this study was to provide general knowledge about composition and dynamics of the bacterial communities present at slaughter and cold storage of farmed Atlantic salmon, as well as reveal any possible correlations to gelatinase activity, which may affect fillet quality. Thus, these data may provide a basis for optimization opportunities in the aquaculture industry.

Methods. Samples were taken from the digestive system harvested from 15 salmon immediately after slaughter. Another 17 salmon were taken from the processing line just before the final cleaning stage; of these eight were distributed in three iced storage boxes while the other nine were rinsed an extra time with industrial water before being distributed into another three storage boxes. In the following 6 days, samples were taken of skin mucus, liquids in the abdominal cavity and the storage ice. The compositions of the bacterial communities were analyzed by next-generation sequencing and gelatinase activity was measured in all samples except the storage ice.

Results. The bacterial communities in the digestive tract samples were dominated by the family *Mycoplasmataceae*. The genus *Aliivibrio* was also relatively abundant. Bacterial communities in the abdominal cavity were generally more diverse than the intestinal samples. However, all of the abdominal samples from storage box no. 3 had a high relative abundance of *Mycoplasmataceae*, and could not be distinguished from the intestinal samples ($Q = 1.27$, $p = 0.633$) while being significantly different from the other abdominal samples ($Q = 9.02$, $p = 0.01$). In addition, the abdominal samples from storage box no. 3 had a significantly higher gelatin degrading activity ($Q = 9.43$, $p = 0.001$) than those from the other storage boxes and similar to the high gelatinase activity in the intestinal samples. This indicated that in storage box no. 3 there was a transfer of intestinal fluids to the abdominal cavities, which was not removed by the cleaning procedure. There was a significant difference of the major phyla detected in the skin mucus of salmon rinsed an additional time, as these salmon had a higher

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Corresponding author
Ása Jacobsen, asa@fiskaaling.fo

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relative amount of *Firmicutes* ($F = 4.76$, $p = 0.04$) and lower amount of *Proteobacteria* ($F = 4.41$, $p = 0.047$).

Conclusions. The study showed a correlation between intestinal fluids and bacteria left in the abdominal cavity and gelatinase activity. This suggested that intestinal fluids and/or bacteria could enhance the degradation of connective tissue in the abdominal cavity and hence negatively affect the fillet quality. In addition, the study provided general knowledge of the composition and dynamics of bacterial communities present.

Subjects Aquaculture, Fisheries and Fish Science, Bioinformatics

Keywords Aquaculture, Atlantic salmon, Bacterial communities, Gelatinase activity, Post-slaughter

INTRODUCTION

The post-mortem degradation of connective tissue in Atlantic salmon (*Salmo salar* L.) fillets leading to lower quality has mainly been attributed to the enzymatic activity of matrix metalloproteinases (MMPs) (*Pedersen et al., 2015*). MMPs are excreted by various cells in the soft and hard connective tissues (*Verma & Hansch, 2007*), and are therefore present in the tissue of slaughtered fish in cold storage even after having been bled out. However, a recent study (*Jacobsen, Joensen & Eysturskarð, 2017*) found that blood and other bodily fluids or remains left in the abdominal cavity during cold storage had a significant effect on the degree of gaping and soft fillets in Atlantic salmon. This suggests that MMPs and enzymes other than those inherently present in the muscle tissue can be damaging to the connective tissue during cold storage of the salmon. High concentrations of several MMPs have been measured in salmon blood (*Eysturskarð et al., 2017*) and MMPs have also been reported in bile of other fish species (*Hauser-Davis, Lima & Campos, 2012*). In addition to MMPs produced by the salmon itself, many bacteria also produce MMPs and other collagenolytic proteases (*Zhang et al., 2015*; *Duarte, Correia & Esteves, 2016*) as well as proteolytic enzymes that can activate host proMMPs (*Okamoto et al., 1997*). Bacterial collagenases or gelatinases (MMP subfamilies) not directly associated with pathogenic activity have also been isolated from various fish species and their surroundings after slaughter (*Sai-Ut, Benjakul & Sumpavapol, 2013*). Previous analysis of bacteria present in the slaughtering and processing environment of farmed salmon or other farmed fish species have mostly focused on specific spoilage bacteria and the methods have often been culture dependent (*Morey, Himmelbloom & Olivieira, 2014*; *Langsrud et al., 2015*). The general composition and dynamics of the bacterial community present on or in the salmon and its cold-storage environment are more seldomly reported, although some recent analyses have been made (*Reynisson et al., 2010*; *Møretrøet al., 2016*). Here we have made concurrent analyses of the gelatin degrading potential and the bacterial community in the digestive system at slaughter and in the skin mucus, and fluids from the abdominal cavity over a period of seven days. In addition, the bacterial community composition in the storage ice was also investigated. This has resulted in an improved understanding of the potential correlations between external fluids and connective tissue degradation in

the fillets. Furthermore, the information gained about the bacterial compositions in the post-slaughter environment of salmon is a valuable addition to the basic knowledge of the bacterial communities on and in salmon.

MATERIALS & METHODS

Samples

The entire digestive system was harvested from 15 salmon at slaughter in a processing facility owned by the local farming company P/F Bakkafrost (Glyvrar, The Faroe Islands). Whole intestines were taken from the abdominal cavity of the salmon when gutted and put in sterile plastic bags and immediately stored in dry ice in closed containers. The containers were transported to the laboratory within a few hours and the bags with digestive systems stored at −80 °C until the experimental setup was completed approximately a week later. Prior to sampling, the bags containing the digestive systems were taken out of the freezer and left in a refrigerator (4 °C) to slowly thaw overnight. At sampling the digestive systems were still chill and frozen but manageable. Samples were taken from the distal intestine (DI), mid intestine (MI), pyloric caeca (Py), stomach (St) and oesophagus (Oe). Sterile scalpels were used to open the organs while using another sterile scalpel to carefully scrape out both content and wall mucus without scraping off organ material. Because the salmon, according to standard procedure, were starved for a few days before slaughter, limited amount of material was expected in the digestive tract. Where possible, 1 mL of material per sample was used from each salmon and materials from two salmon were since pooled into one 2 mL sample. If one of the salmon did not contain enough material a third salmon was used as supplement. In total there were six pooled samples per digestive tract location. See Fig. 1 for illustration of the experimental setup and sampling procedure. DNA extraction was performed immediately following sampling. Overall, the sampling procedure of the digestive system samples were designed to eliminate possible DNA degradation or alteration of the microbial composition (*Tedjo et al., 2015*).

In order to simulate standard storage and transport conditions for the slaughtered salmon, another group of 17 salmon were distributed into six storage boxes and sampled several times during cold storage (Fig. 1). These salmon were removed from the standard processing line just before the final cleaning operation in order to investigate the bacterial communities present with two different cleaning conditions. Two or three salmon were stored in each standard storage box and covered with ice of industrial (filtered and UV treated) water. The salmon in replicate storage boxes no. 1, 2, and 3 were manually rinsed once more with the same filtered and UV treated water (also used in the previous cleaning operations in the processing line) before being stored, while the salmon in replicate storage boxes no. 4, 5, and 6 did not go through the extra rinse. The storage boxes were thereafter placed in the cooling facility with a temperature of approximately 2.0 °C according to standard storage procedure. All sampling was performed in the cooling facility without taking the salmon out of the boxes.

Samples of skin mucus (samples abbreviated "S") were taken on day 1, 2, 3, 4 and 7. Samples of mucus and liquid from the abdominal cavity (samples abbreviated "B") were

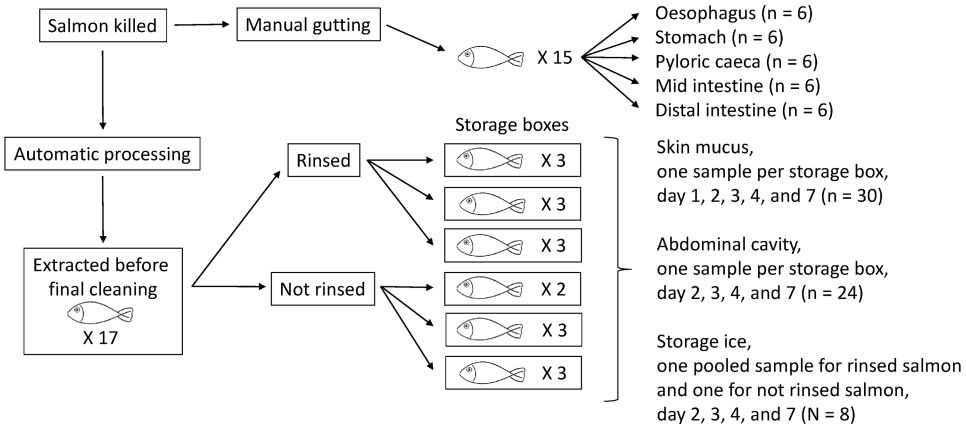

Figure 1 Illustration of experimental setup. Fifteen salmon were gutted manually immediately after being killed following standard procedure. Intestines were harvested and frozen for sampling at a later stage. The same day another 17 salmon were taken of the proceesing table just prior to the final cleaning stage. These salmon were distributed into six storage boxes with storage ice as standard procedure. Salmon in three of the storage boxes were rinsed manually with industrial water before being stored while salmon in the other three storage boxes were not. All storage boxes were placed in the cooling facility (∼2.0 °C) as standard storage procedure and all sampling was performed there without taking the salmon out of the storage boxes.

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taken on same occasions, except on day 1. Sterile scalpels were used to scrape of mucus and other liquids and remains from the two sampling sites. Care was taken not to puncture or otherwise damage the skin or inner lining of the salmon during sampling . The skin mucus sampled from all salmon in a storage box were pooled resulting in one sample per storage box per sampling day. Likewise for the abdominal samples. At the same time as abdominal samples were taken, 100 mL of slush ice (samples "K") was sampled from the bottom of each storage box. The ice from storage boxes no. 1, 2, and 3 were pooled into one sample for each sampling day and the same with the ice from storage boxes no. 4, 5, and 6 (Fig. 1).

Because conditions at the cooling facility were not appropriate for performing homogenization and aliquoting, all samples were put on dry ice immediately at sampling and since stored at −80 °C until further processing and analyses were performed. Deep freeze storage for a short period has been shown to have only minor effect on enzyme activity measurements and bacterial community analysis (*Wallenius et al., 2010*). Even two freeze thaw cycles prior to enzymatic measurements was estimated to have minor influence based on published experiments (*Murias, Rachtan & Jodynis-Liebert, 2005*; *Cuhadar et al., 2013*). When the experimental setup was finished, samples were thawed and homogenized by pipetting such that analyses of both bacterial community and enymatic activity could be performed of the same sample. Subsamples from the homogenized samples were taken for performing the sequencing protocol while the remainder of the sample was used for measurements of gelatinase activity. If analysis of the gelatinase activity could not be done in parallel to the bacterial community analyses the samples were re-frozen until those analyses could be performed later, within a few days.

DNA extraction

There were 96 samples in total (Fig. 1) that were subjected to DNA extraction. Samples from the digestive system were processed immediately after sampling. Samples were mixed until homogenous, and from these, subsamples of 220 mg were taken for DNA extraction using the QIAamp Stool mini kit (Qiagen, Hilden, Germany) while the remainings of the samples were frozen and stored at −80 °C until further analysis. The DNA extraction was performed according to the extraction kit protocol.

The ice from the storage boxes was thawed at room temperature and filtered using 0.22 μm filters. The 100 mL of ice from each of the storage boxes no. 1–3 from the same sampling days were filtered together and thus pooled into one sample while the storage ice from boxes no. 4–6 were pooled into one sample for each sampling day. DNA was then extracted from the filters using the PowerWater DNA extraction kit following the supplier's instructions (Qiagen).

Skin mucus and abdominal samples from all sampling days except day 3 were extracted using the PowerSoil DNA extraction kit (Qiagen) following the manufacturer's protocol. The PowerSoil DNA extraction kit is extensively used in metagenomics although it does not give high yield in comparison with other methods (*Vishnivetskaya et al., 2013*; *Rubin et al., 2014*). However, in a comparison of extraction methods and the subsequent results of sequencing on the Illumina MiSeq platform, the relatively low DNA concentration achieved in the initial extraction did not seem to have any substantial negative effect on the number of OTUs achieved and diversity measurements of the bacterial community (*Burbach et al., 2016*). For comparison, samples from day 3 were extracted using the DNeasy Blood and Tissue kit (Qiagen), which also has been used extensively for 16S rRNA sequence analysis, by following the protocol for pretreatment of gram negative and gram positive bacteria for two subsamples and combining the subsamples in step 4 in the supplier's protocol "Purification of total DNA from Animal Tissues". The DNA concentration was measured in all samples using the Quant-iT PicoGreen dsDNA Assay kit (Thermo Fisher Scientific, Waltham, MA, USA) and a Glomax Multi+ Detection System (Promega Biotech AB, Nacka, Sweden).

Sequencing

Library preparation was performed according to the Illumina "16S Metagenomic Sequencing Library Preparation" document (Part # 15044223 Rev. B) with minor modifications using the recommended universal amplicon primers (selected from *Klindworth et al., 2013*) covering the V3 and V4 regions for the first round PCR. The primer sequences including the Illumina overhang adapters were: 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′ and 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′ for the forward and reverse primers, respectively. The PCR mix contained 5 μl of 1 μM forward and reverse primers each, 12.5 μl of KAPA HiFi HotStart Ready Mix (Roche Diagnostics, Rotkreuz, Switzerland), and 2.5 μl sample DNA. The DNA concentration used for the amplicon PCR was approximately 6 ng/μl instead of the recommended 5 ng/μl. The concentration was increased due to the probability of host DNA presence.

The thermocycling conditions were: 95 °C for 3 min, then 26 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, followed by 72 °C for 5 min and final hold at 4 °C. Number of cycles in the amplicon PCR was increased from the recommended 25 to 26 because the DNA concentration was estimated to otherwise be too low. PCR products from representative samples for each sample types were run on a BioAnalyzer using the High Sensitivity DNA kit (Agilent, Santa Clara, CA, USA) to verify the size and purity before continuing analysis. After verification, the index PCR was performed using primers from the Illumina Nextera XT Index kit. The thermocycling conditions were the same as with the initial amplicon PCR, but with only 10 cycles this time. Representative samples were then run on the BioAnalyzer to verify the size and purity of the libraries. The libraries were measured for DNA concentration using the Quant-iT PicoGreen dsDNA Assay kit (Thermo Fisher Scientific). The final pooled library loaded into the MiSeq instrument for sequencing had a concentration of 5 pM containing 6.67% PhiX control.

Data analysis

Fastq files were downloaded from the BaseSpace Sequence Hub and analysed in QIIME (*Caporaso et al., 2010a*). Quality score plots of assembled and unassembled R1 and R2 reads after joining the paired end reads using the SeqPrep (https://github.com/jstjohn/SeqPrep) and fastq-join (*Aronesty, 2013*) methods were compared. SeqPrep performed better than fastq-join in this instance and was used for assembling all reads. The assembled reads with a minimum average quality score of Q30 were further quality filtered and sorted into samples by the *split_libraries-fastq.py* command (*Caporaso et al., 2012*) using the default values for quality thresholds. This resulted in the removal of single end reads with less than 75% consecutive high quality base calls and unassigned reads, as well as the truncation of reads with more than three consecutive low quality base calls. ChimeraSlayer was applied before OTU picking and did not detect any chimeras. The workflow command *pick_de_novo_otus.py* was applied to cluster the reads into OTUs with 97% similarity by the *de novo* method, representative reads were aligned with PyNAST (*Caporaso et al., 2010b*), and taxonomy was assigned using the UCLUST method (*Edgar, 2010*). A phylogenetic tree was also constructed with the program FastTree (*Price, Dehal & Arkin, 2009*) and finally an OTU table was produced. All OTUs with less than five reads were removed. Removal of low abundance OTUs has also been shown to reduce the content of chimeras substantially (*Majaneva et al., 2015*; *Auer et al., 2017*) compensating for possible failure of ChimeraSlayer to detect chimeras (*Majaneva et al., 2015*). The within sample diversity was analyzed using the *alpha_rarefaction.py* command, calculating the alpha diversity metrics Chao1 (*Chao, 1984*), observed OTUs, and PD whole tree (*Faith, 1992*). Calculations of the between samples diversity was made using the *beta_diversity_through_plots.py* including the phylogenetic tree and 4,000 reads per sample. The command produced a weighted UniFrac (*Lozupone & Knight, 2005*) distance matrix and a principle coordinates file that was visualised using the *make_emperor.py* command (*Vázquez-Baeza et al., 2013*). Bacterial communities were reported at phylum level and at the most specific taxonomic rank achieved from the analysis.

Multivariate analysis

In order to get a comprehensive evaluation of the sequencing data, a data matrix was subjected to principal component analysis (PCA) (*Wold, 1979*) using the software package SIRIUS (*Kvalheim & Karstang, 1987*). The objects were all successfully sequenced samples ($n = 73$) and the variables were all the different bacteria taxa detected and presented in the OTU table ($n = 365$). Before PCA, the variables were centered by subtracting their means and the objects were block normalized and log-transformed. These transformations warrant proper comparison of the objects and ensure appropriate influence of the variables, large or small. During PCA, the objects were placed in a multi-dimensional vector space, one coordinate for each variable. New orthogonal coordinates, the principal components (PCs) were then generated through the centroid of all samples in the multidimensional space in the direction of the largest and second largest and the third largest dispersion of the objects. In this way, the relationship among the objects could be depicted in only two and three dimensions without substantial loss of the total variance.

Measurements of gelatinase activity

The EnzChek® Gelatinase/Collagenase Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to screen the various samples for potential gelatinase/collagenase activity. DQ gelatin (20 µl of 100 µg/mL) was used as substrate and 50µL of homogenized sample was mixed with 50 µL of 1x buffer and added to each well. A negative control containing only substrate and 1x buffer and a positive control containing the *Clostridium* collagenase supplied with the kit were run with every plate measured. All samples were run in duplicates. The samples were measured in a Glomax Multi+ Detection System at ex/em = 490/510−570 nm every 10 min over a period of 15 h. The background fluorescence measured in the negative controls was subtracted from all samples to achieve the Relative Fluorescence Unit (RFU).

Statistics

Significant differences in the alpha diversity estimates and relative content of specific OTUs between two sample groups were tested with ANOVA (F-statistic). Comparisons between three or more groups was in addition analysed by the Tukey HSD (Q-statistic) for groups with unequal number of replicates (*Kramer, 1956*) as implemented in the online calculator (http://astatsa.com/OneWay_Anova_with_TukeyHSD/). Significance of beta diversity between sample types was tested with PERMANOVA performed in QIIME. Significance was accepted at p-values < 0.05.

Ethics statement

This study complied with the boundaries of EU legal frameworks relating to the protection of animals used for scientific purposes (i.e., Directive 2010/63/EU). No specific permit was needed since the industrial procedures in capture and slaughter were followed, and none of these parts were initiated or altered due to this study. Tissue sampling took place post-mortem following standard procedures performed by the local aquaculture industry, authorized by the Faroese Ministry of Foreign Affairs and Trade.

RESULTS

16S rRNA sequencing

The sequencing process resulted in 22,231,798 paired end reads in total. After paired end sequences were joined and quality filtered, a total of 4,869,297 high quality reads were available for analysis. After removal of the OTUs supported by less than five reads in total, the number of reads was 4,814,980. Seventy three samples were successfully sequenced. Most of the samples with insufficient number of reads for analysis (<1,000 reads) were from the upper gastrointestinal area. In addition five of the skin mucus samples taken on day 1 and 2 were not successfully sequenced. The 73 samples successfully sequenced are listed in Table 1 with description of sample type, sampling day, number of reads and OTUs.

The minimum and maximum number of reads per samples was 5,155 and 343,692, respectively. The average and median number of reads per sample was 65,959 and 53,371, respectively. The average number of OTUs per sample was 798.5. The individual rarefaction curves (Fig. S1) indicated sufficient, but not saturating, sequencing depths for most samples while a few of the samples with number of reads below approximately 25,000 would benefit from a higher number of reads. Group-wise rarefaction curves based on sample types are illustrated in Fig. S2.

Alpha diversity

OTU richness was estimated by several alpha diversity metrics: (i) observed OTUs, which is the number of OTUs detected by subsampling every sample several times at a standardised sequencing depth; (ii) Chao1, which adds a correction factor taking into account the low abundance OTUs, and (iii) the phylogenetic diversity estimate PD whole tree, which calculates the branch lengths in the phylogenetic tree constructed from each sample. Within the salmon digestive tract samples, the pyloric caeca had the highest OTU richness estimates (Figs. 2A and 2B) and phylogenetic diversity (Fig. 2C), followed by the mid intestine and then the distal intestine, but these differences were not statistically significant (Obs. OTUs: $F = 1.22$, $p = 0.33$; Chao1: $F = 1.3$, $p = 0.31$, PD wt: $F = 1.2$, $p = 0.335$). The alpha diversity estimates for the single stomach sample were within the range of the estimates for the other digestive tract samples. However, because only one sample was available for the stomach, it was not included in this or other statistical analyses.

OTU richness and phylogenetic diversity in the skin mucus and abdominal fluids were at a similar level and there was a great deal of variation between individual samples for both sample types (Fig. 2). The skin mucus and abdominal fluids had a significantly higher observed OTU estimate than the digestive tract samples from pyloric caeca ($Q = 45.46$, $p = 0.024$; $Q = 49.87$, $p = 0.01$), mid intestine ($Q = 46.19$, $p = 0.021$; $Q = 50.30$, $p = 0.009$), and distal intestine ($Q = 45.78$, $p = 0.022$; $Q = 49.52$, $p = 0.01$) respectively. For Chao1, the distal intestine had significantly lower values than the abdominal samples ($Q = 42.13$, $p = 0.045$) while the storage ice samples had significantly lower values than both skin mucus ($Q = 42.29$, $p = 0.044$) and abdominal samples ($Q = 50.67$, $p = 0.008$). On the other hand, the storage ice had significantly higher phylogenetic diversity values than all

Table 1 Sample information and number of reads and OTUs obtained. All organic samples were pooled from 2–3 individuals. Ice from storage boxes no. 1, 2, and 3 was pooled and ice from storage boxes no. 4, 5, and 6 was pooled. Alpha diversity estimates (displayed in Fig. 2) were based on 10 iterations using 16,018 rarefied reads for most samples, 10,682 reads † or 5,346 reads †† for a few samples and one sample was excluded †††.

Sample type—day	Sample name	No. of reads	No. of OTUs	Sample type—day	Sample name	No. of reads	No. of OTUs
Stomach—1	ST-5	96,776	851	Abd. Fluids—7	B4-day 7	40,291	960
Pyloric caeca—1	PY-1	53,549	629	Abd. Fluids—7	B5-day7	53,371	1,210
Pyloric caeca—1	PY-2	31,223	417	Abd. Fluids—7	B6-day7	46,045	712
Pyloric caeca—1	PY-3	87,905	812	Skin mucus—1	S3-day1	36,139	469
Pyloric caeca—1	PY-4	113,453	923	Skin mucus—1	S4-day1	93,522	1,278
Pyloric caeca—1	PY-5	81,104	750	Skin mucus—1	S5-day1	62,329	973
Pyloric caeca—1	PY-6	95,580	843	Skin mucus—1	S6-day1	77,240	932
Mid intestine—1	MI-2	122,253	723	Skin mucus—2	S3-day2	5,155	526†††
Mid intestine—1	MI-3	42,600	546	Skin mucus—2	S4-day2	5,925	514††
Mid intestine—1	MI-4	117,836	904	Skin mucus—2	S5-day2	63,979	1,148
Mid intestine—1	MI-5	101,842	840	Skin mucus—2	S6-day2	119,630	1,343
Mid intestine—1	MI-6	129,901	928	Skin mucus—3	S1-day3	49,433	664
Distal intestine—1	DI-1	240,851	1,407	Skin mucus—3	S3-day3	55,544	706
Distal intestine—1	DI-3	84,371	399	Skin mucus—3	S4-day3	63,941	880
Distal intestine—1	DI-4	132,766	826	Skin mucus—3	S5-day3	50,891	805
Distal intestine—1	DI-6	86,550	789	Skin mucus—3	S6-day3	81,530	870
Abd. Fluids—2	B1-day2	6,300	508††	Skin mucus—4	S1-day4	17,318	972
Abd. Fluids—2	B2-day2	26,565	772	Skin mucus—4	S2-day4	5,859	417††
Abd. Fluids—2	B3-day2	237,438	893	Skin mucus—4	S3-day4	16,106	933
Abd. Fluids—2	B4-day2	41,085	775	Skin mucus—4	S4-day4	11,235	527†
Abd. Fluids—2	B5-day2	21,318	790	Skin mucus—4	S5-day4	26,064	753
Abd. Fluids—2	B6-day2	22,222	326	Skin mucus—4	S6-day4	14,008	452†
Abd. Fluids—3	B1-day3	28,587	806	Skin mucus—7	S1-day7	42,759	733
Abd. Fluids—3	B2-day3	45,885	908	Skin mucus—7	S2-day7	34,291	857
Abd. Fluids—3	B3-day3	85,806	672	Skin mucus—7	S3-day7	24,385	906
Abd. Fluids—3	B4-day3	21,631	767	Skin mucus—7	S4-day7	20,006	388
Abd. Fluids—3	B5-day3	20,031	1,080	Skin mucus—7	S5-day7	62,777	798
Abd. Fluids—3	B6-day3	41,540	820	Skin mucus—7	S6-day7	21,771	734
Abd. Fluids—4	B1-day4	62,987	985	Storage ice—2	K123-day2	54,001	690
Abd. Fluids - 4	B2-day4	14,790	600†	Storage ice—2	K456-day2	34,806	558
Abd. Fluids—4	B3-day4	343,692	1,145	Storage ice—3	K123-day3	95,613	795
Abd. Fluids—4	B4-day4	34,765	764	Storage ice—3	K456-day3	69,222	803
Abd. Fluids—4	B5-day4	22,145	974	Storage ice—4	K123-day4	79,536	756
Abd. Fluids—4	B6-day4	17,807	875	Storage ice—4	K456-day4	20,499	451
Abd. Fluids—7	B1-day7	66,712	1,193	Storage ice—7	K123-day7	91,708	784
Abd. Fluids—7	B2-day7	90,507	1,139	Storage ice—7	K456-day7	90,332	598
Abd. Fluids—7	B3-day7	207,346	1,016				

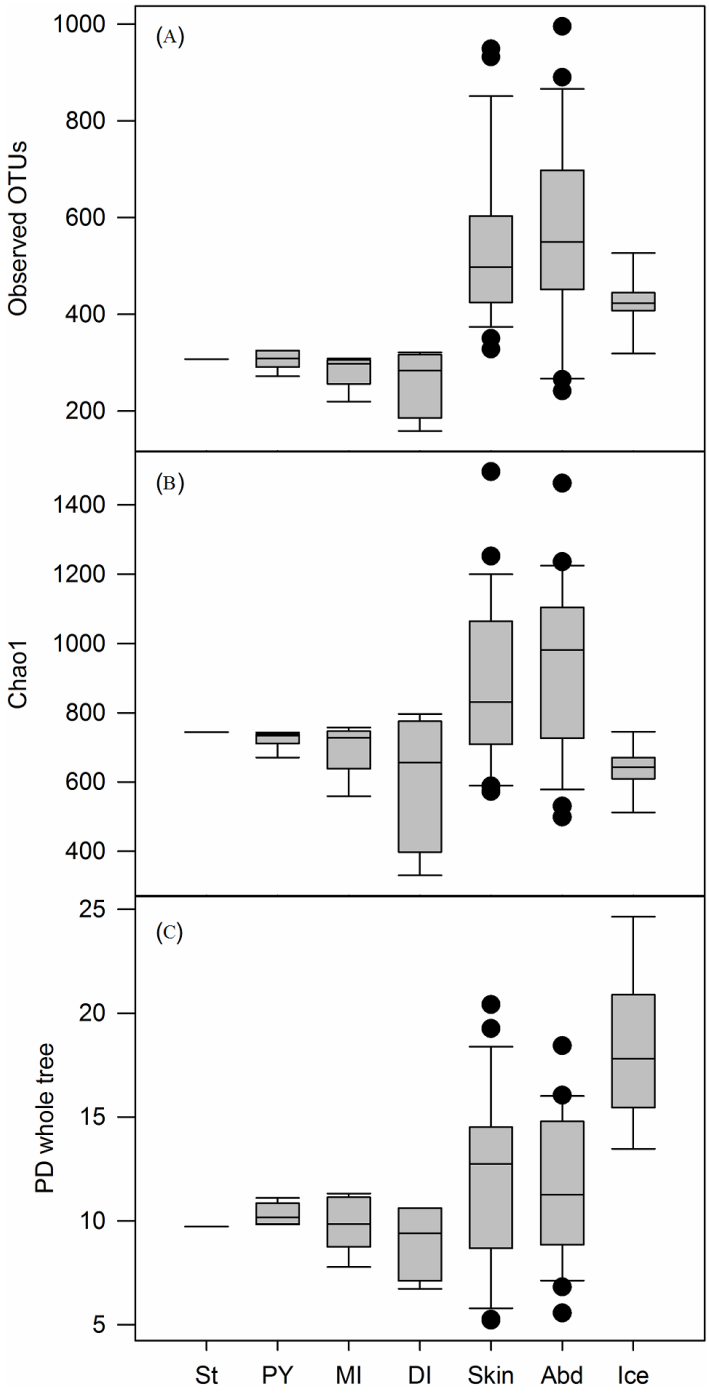

Figure 2 **Alpha diversity estimates for all samples types.** (A) Observed OTUs, (B) Chao1, and (C) PD whole tree estimates for stomach (St, $n = 1$), pyloric caeca (PY, $n = 6$), mid intestine (MI, $n = 5$), distal intestine (DI, $n = 4$), skin mucus (S, $n = 25$), abdominal fluids (B, $n = 24$) and storage ice (K, $n = 8$). Boxes indicate median and 1st and 3rd quartile. Whiskers indicate standard deviations and dots represent outliers. Samples and iterations for alpha diversity estimates are described in Table 1.

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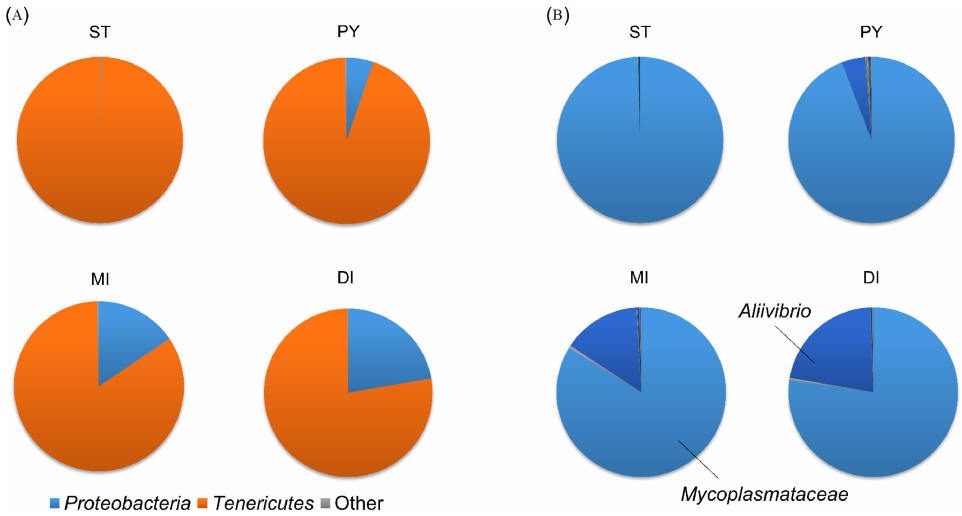

Figure 3 Composition of the bacterial community in the digestive system. Composition of the bacterial community at (A) phylum and (B) genus level. Each sample was pooled from 2–3 salmon. The graphs represent the averages of the various sample types. ST, Stomach ($n = 1$), PY, Pyloric caeca ($n = 6$), MI, Mid intestine ($n = 5$), DI, Distal intestine ($n = 4$). Main phyla and genera are indicated.

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the other sample types (PY: $Q = 59.08$, $p = 0.001$; MI: $Q = 58.75$, $p = 0.001$; DI: $Q = 60.58$, $p = 0.001$; S: $Q = 59.74$, $p = 0.001$; B: $Q = 65.96$, $p = 0.001$).

Bacterial community compositions

The digestive tract

The phylum *Tenericutes* was very dominating in the digestive tract, representing between 77.6% and 99.8% of the OTUs detected (Fig. 3A).

The phylum *Proteobacteria*, which is often detected in the intestines of salmon (*Gajardo et al., 2016*; *Dehler, Secombes & Martin, 2017*), was nearly absent in the single stomach sample with only 0.2% but was in average increasingly more abundant further down the digestive system and represented 22.3% of the OTUs detected in the distal intestines. However, the ANOVA/Tukey HSD Statistical test comparing the three sample groups (excluding the stomach sample) detected no significant difference ($F = 1.0078$, $p = 0.39$). The salmon digestive tract samples contained a bacterial community structure highly dominated by one single or two OTUs. *Mycoplasmataceae* of the phylum *Tenericutes* was the overall most dominant bacterial family represented in the digestive tract samples with between 77.3% and 99.4% (Fig. 3B). The genus *Aliivibrio* belonging to the family *Vibrionaceae* of the phylum *Proteobacteria* was also well represented in the samples, especially from the distal intestines where it represented 21.6% of the OTUs.

The abdominal fluids

The compositions of the bacterial communities in the abdominal samples taken from salmon in all storage boxes except no. 3 were relatively similar (Fig. 4).

In the abdominal samples from these five storage boxes, *Proteobacteria* was the dominating phylum. There was also a high representation of *Bacteroidetes*, and on some

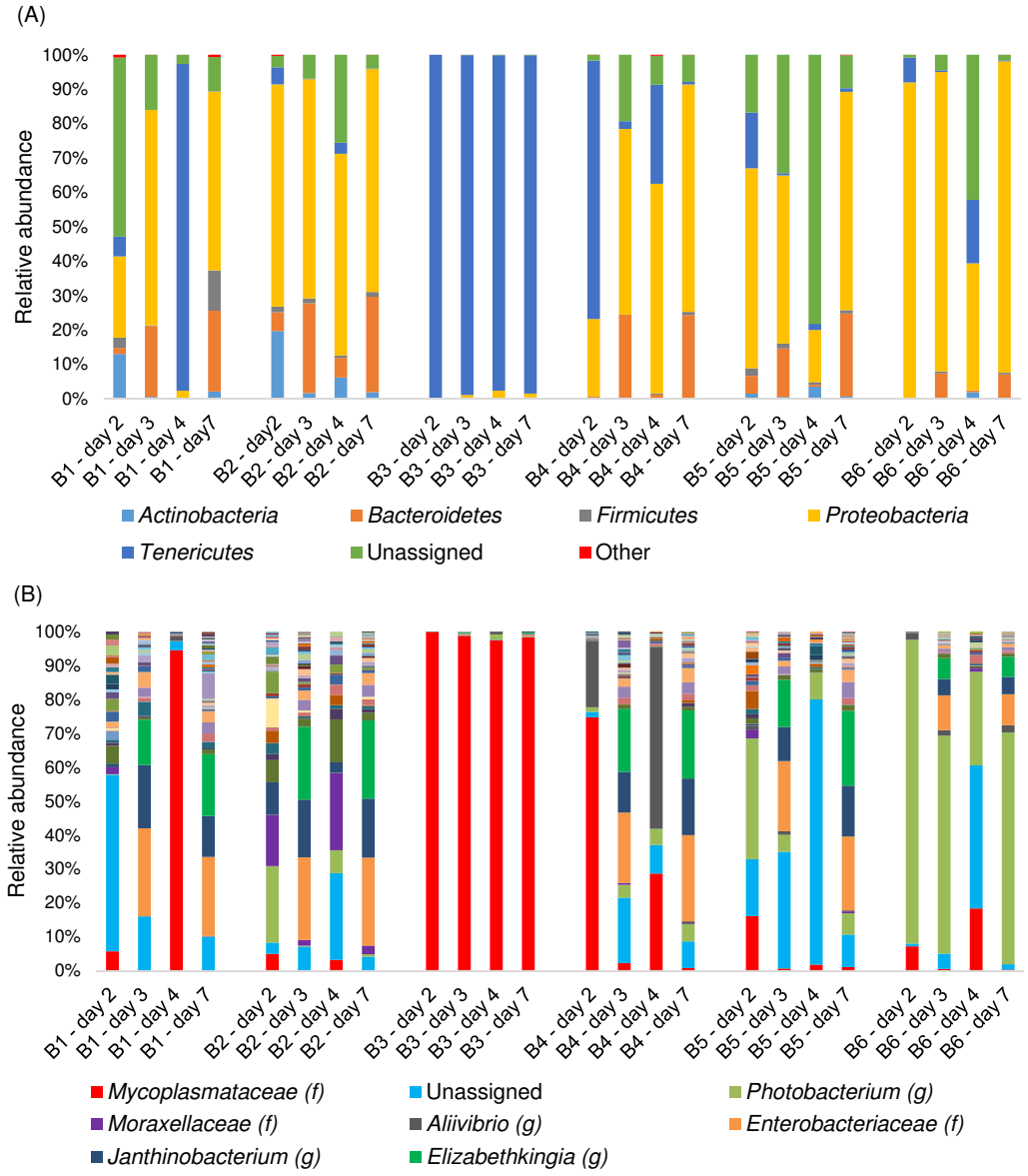

Figure 4 Composition of the bacterial community in samples from the abdominal cavity. Composition of the bacterial community at (A) phylum and (B) genus level. The six groups of columns represent the four sampling days from storage boxes no. 1–6. Each column is a pooled sample from the 2–3 salmon in each storage box. f, family, g, genus.

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occasions *Tenericutes* (Fig. 4A). In contrast, the abdominal samples taken from salmon in storage box no. 3 were highly dominated by the phylum *Tenericutes* with between 97.5% and 99.9% of the reads. The Tukey HSD test showed that the abdominal samples from storage box no. 3 had a significantly higher relative content of *Tenericutes* than the abdominal samples from the other storage boxes (B1: $Q = 6.07$, $p = 0.005$; B2: $Q = 7.98$, $p = 0.001$; B4: $Q = 5.94$, $p = 0.006$; B5: 7.75, $p = 0.001$; B6: $Q = 7.61$, $p = 0.001$). All abdominal samples from storage box no. 3 consisted almost entirely of the family *Mycoplasmataceae* (Fig. 4B),

in contrast to the other abdominal samples ($Q = 9.02$, $p = 0.01$) which generally had a higher bacterial diversity and had similar relative contents of *Mycoplasmataceae* ($F = 0.80$, $p = 0.544$). On the other hand, there was no significant difference detected between the relative content of *Mycoplasmataceae* in the abdominal samples from storage box no. 3 and the digestive tract samples ($Q = 1.27$, $p = 0.633$). In storage box no. 6 there was a relatively high representation of the genus *Photobacterium* (fam. *Vibrionaceae*) ranging between 27.6% and 89.6% of the reads obtained the various sampling days (Fig. 4B). This genus was detected at significantly lower abundances in some of the other samples from the other storage boxes ($F = 13.93$, $p < 0.001$). Members of the family *Enterobacteriaceae* were detected in all storage boxes, particularly on sampling days 3 and 7, with up to 25.9%. *Janthinobacterium* (fam. *Oxalobacteriaceae*) and *Elizabethkingia* (fam. *Flavobacteriaceae*) were also detected at highest relative abundance on these two sampling days. No significant differences were detected between the storage boxes for these three bacteria ($F = 0.78$, $p = 0.578$; $F = 1.57$, $p = 0.219$; $F = 0.86$, $p = 0.525$).

The skin mucus

The skin mucus microbiota in salmon from storage boxes no. 1, 2, and 3, which were rinsed an additional time compared to the other salmon, was dominated by *Bacteroidetes* with 10.9 - 16.2%, *Firmicutes* with 12.1–27.1% and *Proteobacteria* with 22.7–40.0% as well as containing a relatively large proportion of unassigned bacteria (Fig. 5A).

In comparison the skin mucus microbiota from the other three storage boxes contained a significantly higher proportion of *Proteobacteria*, 51.8–56.1% ($F = 4.41$, $p = 0.047$) and a significantly lower proportion of *Firmicutes* ($F = 4.76$, $p = 0.04$). On average, storage boxes no. 4, 5, and 6 also had a higher occurrence of *Tenericutes* with up to 23.8% compared to maximum 6.6% in the samples from storage boxes no. 1, 2, and 3, although the difference was not statistically significant ($F = 3.61$, $p = 0.07$). There were several relatively abundant OTUs in the skin mucus samples from all storage boxes. *Janthinobacterium, Chryseobacterium* and *Elizabethkingia* (both belonging to fam. *Flavobacteriaceae*) and *Enterobacteriaceae* were all relatively abundant in most storage boxes (Fig. 5B). *Staphylococcus* (fam. *Staphylococcaceae*) and Lactobacillales were only relatively abundant in skin mucus samples from a few of the storage boxes. *Mycoplasmataceae, Moraxellaceae,* and *Aliivibrio* were mainly detected in the storage boxes containing salmon not rinsed an extra time and the relative content of *Photobacterium* was significantly higher in these samples than in those from salmon rinsed an extra time ($F = 5.46$, $p = 0.03$). The salmon rinsed an extra time on the other hand had a larger proportion of unknown bacteria.

The storage ice

Proteobacteria and *Bacteroidetes* were the most abundant phyla detected in the storage ice represented with between 55.9–90.9% and 6.2–39.0%, respectively (Fig. 6A), while *Actinobacteria* and *Firmicutes* were detected at lower levels. *Tenericutes* was detected at low levels the first sampling day, and further diminished in abundance over time.

On the first sampling day, there was a relatively even OTU abundance distribution, but over time the tendency was that a few OTUs became dominating while the low

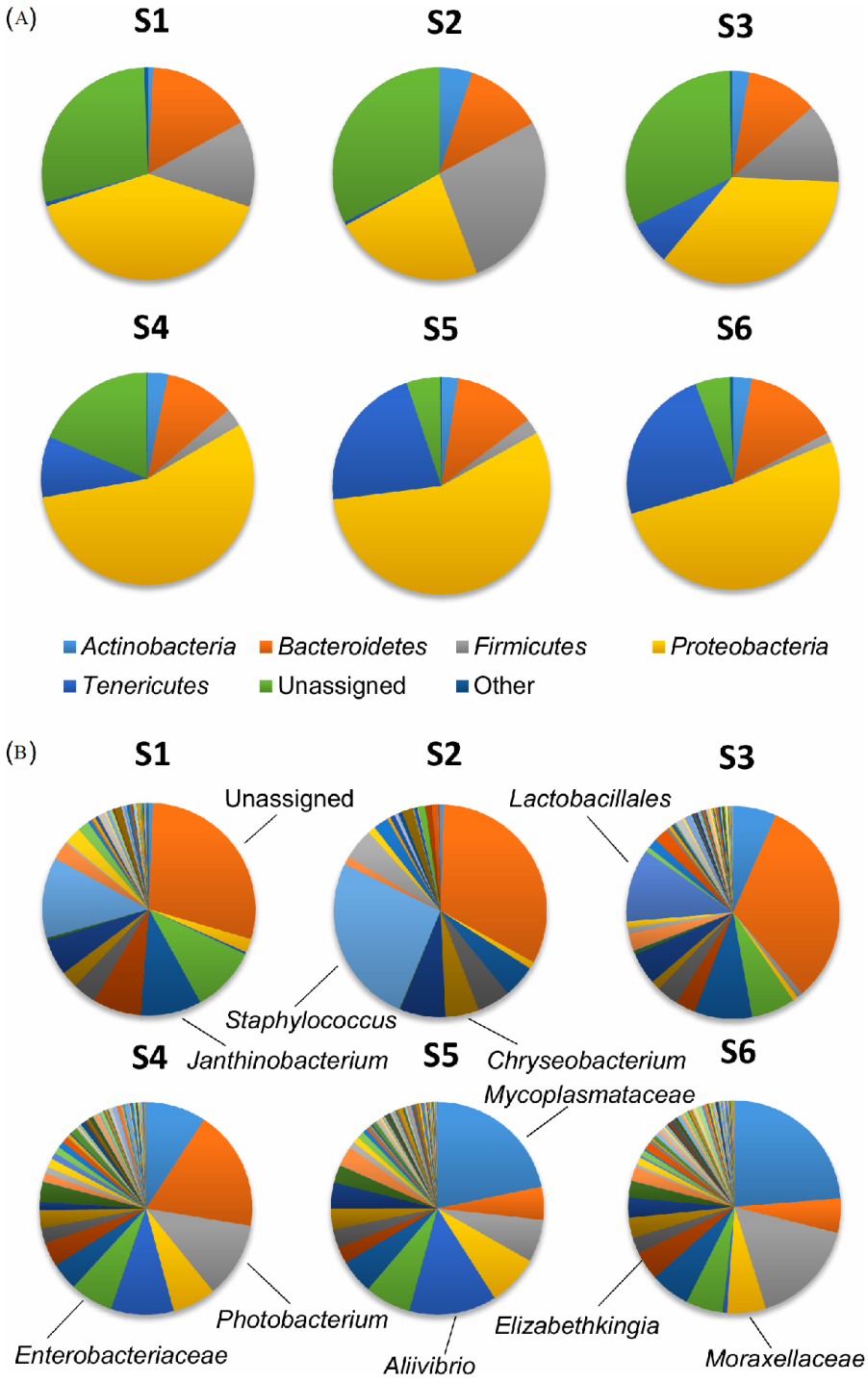

Figure 5 Composition of the bacterial community in the skin mucus. Composition of the bacterial community at (A) phylum and (B) genus level. Each pie represents the average of all sampling days from a storage box. S1 ($n = 3$), S2 ($n = 2$), S3 ($n = 5$), S4 ($n = 5$), S5 ($n = 5$), S6 ($n = 5$).

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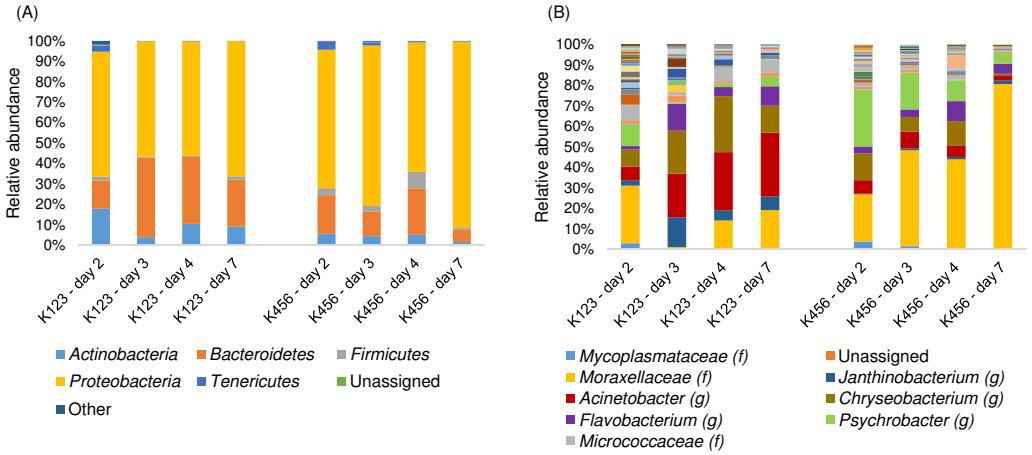

Figure 6 Composition of the bacterial community in samples from the storage ice. Composition of the bacterial community at (A) phylum and (B) genus level. The four bars on the left of both figures are the pooled samples from storage boxes 1–3 and the bars on the right represent the pooled samples from storage boxes 4–6. (A) The dominating phyla are illustrated in separate legends while those of very low abundance are combined in "Other" (B) The most abundant families (f) or genera (g) are illustrated in the legends.

Full-size DOI: 10.7717/peerj.7040/fig-6

level OTUs represented a consistently decreasing part of the community (Fig. 6B). Ice from storage boxes no. 1–3, containing salmon that were rinsed an extra time, and ice from storage boxes no. 4–6 had a relatively similar bacterial community composition the first sampling day. However, over time the bacterial compositions seemed to change in two different ways. In storage boxes no. 4–6, the relative content of an unidentified bacterium in the family *Moraxellaceae* increased over the sampling period from 22.9% until it was a very dominant part of the bacterial community at 80.4%, while there was no increase on the relative content of *Moraxellaceae* in the storage ice from boxes no. 1–3. An ANOVA statistical comparison revealed a significant difference ($F = 6.25$, $p = 0.047$) in the content of *Moraxellaceae* between the storage ice from boxes no. 1–3 and boxes no. 4–6. *Acinetobacter*, another genus from the *Moraxellaceae* family increased from 6.7% to 31.2% in the ice from storage boxes no. 1–3 during the sampling period, which was significantly different ($F = 8.39$, $p = 0.028$) than the more constant relatively low abundance detected in the ice from storage boxes no. 4–6.

Beta diversity

All of the 73 samples and 10 of the most discriminating bacteria were displayed as objects and variables, respectively, in a PC1 versus PC2 coordinate system, resulting in a 2D plot (Fig. 7). Closely situated variables are positively correlated, while variables on either side of the origo are negatively correlated.

Variables positioned far away from the origo, marked as a cross in the 2D plot, had the largest influence on the placements of the samples in the plot. The variables with highest discriminating power were *Mycoplasmataceae* (A) and *Janthinobacterium* (H) for PC1 and *Moraxellaceae* (C) and *Enterobacteriaceae* (J) for PC2 (Fig. 7). The ten variables could

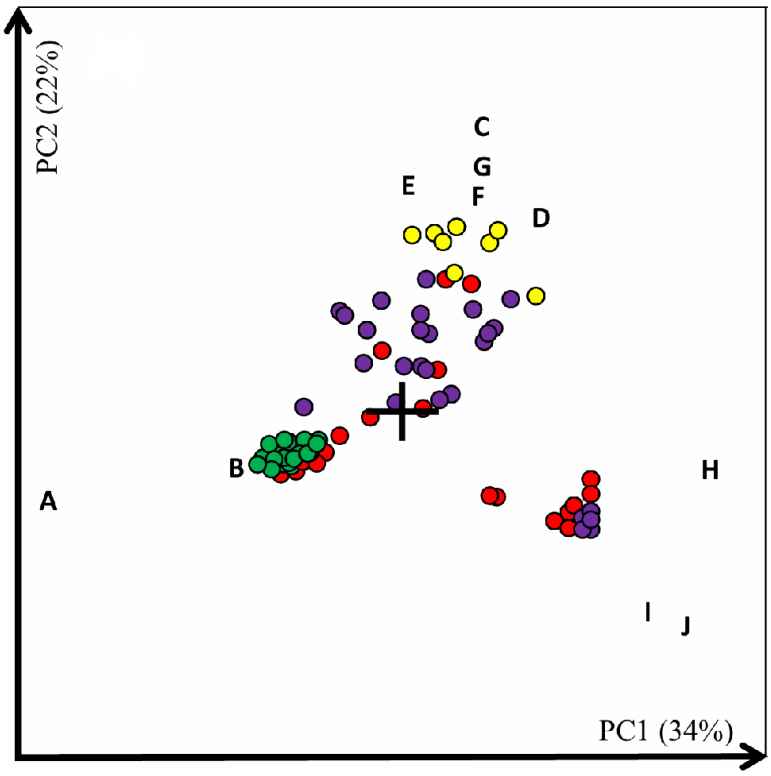

Figure 7 PC-plot of all samples based on variables comprising bacterial taxa. 2D PC-plot. Each circle represents one sample. Green, combined visceral samples including stomach, pyloric caeca, midgut and hindgut. Red, abdominal cavities. Violet, skin mucus. Yellow, storage ice. The first and second principal components (PC1 and PC2) describe 34% and 22%, respectively, of the total variance. The ten most discriminatory variables are illustrated. A, *Mycoplasmataceae* (f), B, *Aliivibrio* (g), C, *Moraxellaceae* (f), D, *Acinetobacter* (g), E, *Psychrobacter* (g), F, *Flavobacterium* (g), G, *Chryseobacterium* (g), H, *Janthinobacterium* (g), I, *Elizabethkingia* (g) and J, *Enterobacteriaceae* (f). f, family, g, genus.

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effectively be sorted into four groups. Variables *Mycoplasmataceae* (A) and *Aliivibrio* (B), composing one such group, had a positive correlation with the grouped intestinal samples, marked in green, as would be expected by the dominating presence of these bacteria in those samples. Variables *Mycoplasmataceae* (A) and *Aliivibrio* (B) were also positively associated with seven samples from the abdominal cavity, which were inter-twined with the 15 samples from the digestive tract (Fig. 7). These abdominal samples included all four samples from storage box no. 3 as well as three others from various storage boxes. The second group with the positively correlated variables *Elizabethkingia* (I) and *Enterobacteriaceae* (J) were associated with some of the abdominal cavity and skin mucus samples which were drawn toward them in the right lower corner (Fig. 7). The variable *Janthinobacterium* (H), sole member of the third group, also influenced the positioning of these samples further to the right along the PC1 axis. However, the association between these two sample types was not as strong as between the intestinal samples and their related abdominal samples. The last group containing the variables *Moraxellaceae* (C), *Acinetobacter* (D), *Psychrobacter* (E), *Flavobacterium* (F) and *Chryseobacterium* (G) were revealed as the distinguishing features

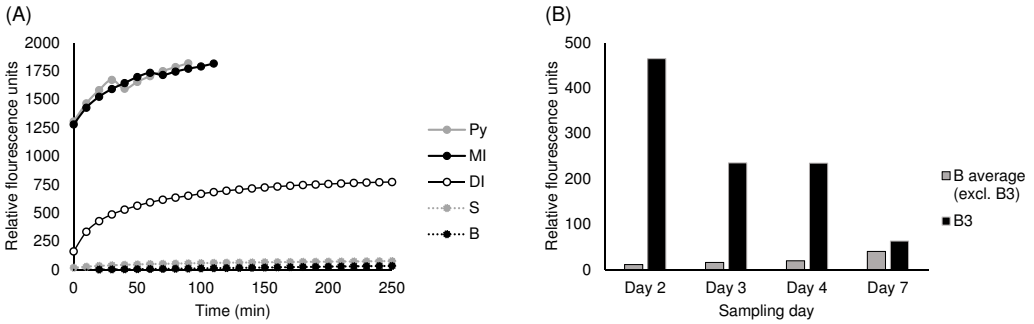

Figure 8 Gelatinase activity in (A) various sample types and in (B) abdominal samples. (A) Gelatinase activity in various representative samples from salmon taken on day 2. DI, distal intestine, MI, mid intestine, PY, pyloric caeca, Oe, Oesophagus, S, skin mucus, B, abdominal cavity. (B) Gelatinase activity in all samples taken from the abdominal cavity over a seven days period post slaughter. B3, abdominal cavity samples from storage box no. 3, B average, abdominal cavity samples from all storage boxes other than no. 3.

Full-size DOI: 10.7717/peerj.7040/fig-8

of the storage ice (Fig. 7). Several of the skin mucus and abdominal samples were also drawn towards these variables, but they were not as tightly associated with the variables as the ice samples. There was no association between the storage ice and the intestinal samples. The green digestive tract samples were clustered tightly as they were dominated by only two variables. The yellow ice samples were also grouped, albeit more dispersed as their position in the plot was influenced by several variables. On the other hand, the purple skin mucus samples and the red abdominal samples were more scattered along both PC1 and PC2.

Weighted (Fig. S3A) and unweighted (Fig. S3B) UniFrac beta diversity calculations were also made for comparison and were in agreement with the principal component analysis. They also showed that the abdominal samples from storage box no. 3 grouped together with the digestive tract samples at one end of the PC1 axis, explaining ~72% of the variation in the samples (Fig. S3A). The other abdominal samples were more mingled together with the skin mucus samples further along the PC1 axis and the ice samples were situated furthest away from the digestive tract samples. A PERMANOVA test of the significance of sample groupings according to sample type using the weighted UniFrac distance matrix and based on 999 permutations proved significant ($F = 13.82$, $p = 0.001$).

Gelatinase activity

The initial screening comparing the various sample types showed that gelatinase activity (Fig. 8A) in the skin mucus and abdominal cavity was low and had a slow linear growth similar to the negative control and was thus consistent with the absence or near absence of enzyme activity.

On the other hand, samples from the pyloric caeca and mid intestine had a very high activity of gelatin degrading enzymes as the flourescence was high already after a few minutes of incubation. The distal intestine also showed gelatinase activity, although lower than the pyloric caeca and the mid intestine. The further analysis of all samples revealed that there was high enzymatic activity in the abdominal samples taken from the salmon in

storage box no. 3. This was significantly different from the abdominal samples from the salmon in the other five storage boxes (Fig. 8B) ($Q = 9.43$, $p = 0.001$), where the measured gelatin degradation was consistent with the findings in the initial screening (Fig. 8A). The high enzyme activity in storage box no. 3 decreased over time and on day 7 was almost as low as in the other storage boxes. The skin mucus samples had very low gelatinase activity consistent with the initial screening.

DISCUSSION

The alpha diversity values for the digestive tract samples were consistent with previous studies on salmon intestinal microbiota (*Gajardo et al., 2016*; *Dehler, Secombes & Martin, 2017*). The high relative content of *Mycoplasmateaceae* detected in the samples from the digestive tract (Fig. 3B) is also consistent with other studies also based on salmon living in seawater (*Llewellyn et al., 2016*; *Karlsen et al., 2017*) although previous studies on the salmon intestinal microbiota have reported *Proteobacteria*, *Bacteriodetes*, *Firmicutes* and *Tenericutes* as the main phyla detected (*Gajardo et al., 2016*; *Llewellyn et al., 2016*; *Dehler, Secombes & Martin, 2017*). *Mycoplasmataceae* is a heterogeneous group of small bacteria lacking a cell wall and inhabiting a wide range of hosts as part of a parasitic lifestyle. Although *Mycoplasmataceae* includes many pathogens, they seem to be a commensal part of the intestinal microbiota of salmon (*Llewellyn et al., 2016*). The genus *Aliivibrio,* which was relatively abundant in the samples from the digestive tract, was also previously detected at relatively high levels in the digestive tract of sea-farmed salmon (*Green, Smullen & Barnes, 2013*; *Karlsen et al., 2017*). *Aliivibrio* consists of marine bacteria some of which are mutualistic, symbionts or pathogens in a range of marine animals including salmon (*Beaz-Hidalgo et al., 2010*). The similarity in bacterial community composition detected in this study is similar to that reported by some studies while others have found more diversification along the digestive tract (*Gajardo et al., 2016*; *Egerton et al., 2018*).

To our knowledge no previous studies have included samples from the abdominal cavity and direct comparison is therefore not possible. The clear contrast between the bacterial composition in abdominal fluids taken from salmon in storage box no. 3 and the other storage boxes indicated that the bacterial communities had different origins. The dominating *Mycoplasmataceae* likely originated from the digestive system as the relative content of *Mycoplasmatacea* in the abdominal samples from storage box no. 3 was no different than that of the digestive tract samples. The beta diversity analysis (Fig. 7) also indicated that *Mycoplasmataceae* and *Aliivibrio* originated from the digestive system and supported the suggestion of transfer of bacterial communities from the gastrointestinal tract to some of the abdominal samples, mainly those from storage box no. 3. The presence of *Enterobacteriaceae, Elizabethkingia,* and other marine and environmental bacteria, such as the genus *Janthinobacterium* and the family *Moraxellaceae*, in the abdominal samples from salmon in the other five storage boxes, indicated that these bacterial communities more likely originated from the seawater and the exterior surfaces of the salmon, or from the industrial water used in processing the salmon. *Photobacterium*, which was relatively abundant in some of these five storage boxes, consists mainly of marine bacteria that

can inhabit both outer surfaces as well as the intestines of various marine fish species, including Atlantic salmon (*Urbanczyk, Ast & Dunlap, 2011*). The differences detected between sampling days in samples from the same storage box might be a reflection of shifts in the compositions of the bacterial communities, which is natural especially for perturbed environments (*Gerber, 2014*) such as the abdominal cavity of newly slaughtered salmon. The co-occurrence of *Enterobacteriaceae, Janthinobacterium,* and *Elizabethkingia* at their highest relative abundances on day 3 and 7 while all were detected at low relative abundances on day 2 and 4 might suggest a a mutualistic relationship between them and/or a common intolerance of certain other bacteria.

The microbial community in the skin mucus had a relatively even OTU abundance distribution (Fig. 5B) and relatively high Chao1 and observed OTU estimates. Other studies have reported a lower level of Chao1 estimates but higher phylogenetic diversity in skin microbiota of Atlantic salmon (*Lokesh & Kiron, 2016*). Analysis of skin microbiota in other fish species have shown lower levels of observed OTUs and lower or similar levels of phylogenetic diversity (*Chiarello et al., 2015*; *Lowrey et al., 2015*), although with fewer reads. However, the different sampling and storage conditions have to be taken into account when comparing the skin mucus microbiota detected in this study with that found in other studies. We have in this study not investigated to what degree the slaughtering and cleaning procedure has affected the bacterial composition detected, but others have also reported high diversity and even distribution of OTU abundances in skin mucus of various fish species (*Chiarello et al., 2015*; *Lowrey et al., 2015*). In general, the bacteria presently detected at high abundances did not correlate well with previous studies of the skin mucus of salmon (*Lokesh & Kiron, 2016*; *Minniti et al., 2017*), but this might also be explained by the different experimental setups and sampling conditions in the different studies, in particular by the industrial rinsing of the fish in our study and the potential mixture of bacterial communities originating from the intestines.

The bacterial community structure in the skin mucus from salmon in storage boxes no. 1, 2, and 3 had similar features, while the bacterial community in skin mucus from salmon in the other three boxes seemed to have other characteristics. This suggested an effect of the cleaning procedure. *Proteobacteria* has been reported as the overall dominant phylum present in the skin microbiota of salmon living in a marine environment (*Minniti et al., 2017*) and one possible explanation for the lower content of *Proteobacteria* in the skin mucus of salmon in storage boxes no. 1, 2, and 3 might be that more of the *Proteobacteria* has been washed away or further diluted with presumably dead bacteria by the additional industrial fresh water rinsing. In addition, the relative content of *Mycoplasmataceae* varied a great deal between the samples and ranged from less than 0.01% to 76.29%. *Mycoplasmataceae* has not previously been mentioned as a normal part of the skin mucus microbiota (*Chiarello et al., 2015*; *Lowrey et al., 2015*). It is possible that the *Mycoplasmataceae* detected in the skin mucus in this study might again be due to transfer from the intestines during slaughter. Therefore, in contrast to the abdominal fluids, the extra rinsing seemed to reduce the potential presence of intestinal fluids on the skin mucus. In addition, the predominantly marine bacterial genus *Photobacterium,* containing several species associated with fish, was also detected at higher abundances in the skin mucus of salmon not rinsed an extra

time. Most of the remaining relatively abundant bacteria found in the skin mucus, like *Janthinobacterium*, *Chryseobacterium* and *Elizabethkingia*, and *Moraxellaceae* are common in freshwater as well as in the marine environment. In addition, *Staphylococcus*, which on average represented 6.4% of the bacterial community detected in the skin mucus, is commonly detected on skin and mucous membranes of various organisms. Also *Enterobacteriaceae*, which represented on average 6.0% of the skin mucus microbiota, and the order *Lactobacillales* contain numerous species of bacteria found widespread in nature. The multivariate analysis (Fig. 7) indicated that *Elizabethkingia, Enterobacteraceae*, and *Janthinobacterium* mainly originated from the marine environment or salmon exterior as they were not associated with intestinal or storage ice samples but with some of the skin mucus and abdominal samples. The PC plot also indicated that the skin mucus and abdominal samples had varying degrees of correlations with a multitude of bacteria, including the ten variables shown as well as the other 355 bacterial groups used in the multivariate analysis.

Although the relative content of *Moraxellaceae* in the skin mucus of salmon from storage boxes no. 1, 2, and 3 and storage boxes no. 4, 5, and 6, was not statistically different ($F = 3.84$, $p = 0.062$), 40% of the skin mucus samples from storage boxes no. 4, 5, and 6 contained >5% of *Moraxellaceae* (5.3% –27.4%) while only 10% of the skin mucus samples from storage boxes no. 1, 2, and 3 contained >5% (6.2%). Therefore, the difference in *Moraxellaceae* content in the storage ice might be caused by the skin mucus bacterial composition. On the other hand, the relative content of the known psychrotrophic genus in *Moraxellaceae, Psychrobacter,* did not increase. The reason for this counter-intuitive pattern might be that *Psychrobacter* was detected at relatively low levels only in the skin mucus. Therefore, the source of these bacteria might have been the water and/or storage ice. Because the water and ice used in the slaughtering facility is UV-treated the bacteria were likely dead and therefore either remained the same or diminished in comparative abundance while other living bacteria were transferred to the ice and could grow in abundance. This would further suggest that the unidentified *Moraxellaceae* in the slush ice originated from elsewhere than the industrial water, and most likely from the skin mucus.

Because *Acinetobacter* was detected at relatively low levels in the skin mucus of all salmon, the reason for the increase in relative abundance in the ice of storage boxes no. 1, 2, and 3 only is uncertain, but might be because the increase of the unidentified *Moraxellaceae* in the ice in storage boxes no. 4, 5, and 6 either hampered or camouflaged any increase in *Acinetobacter*. Four other bacterial genera also detected at relatively high abundances, *Janthinobacterium*, *Micrococcaceae, Chryseobacterium*, and *Flavobacterium* (fam. *Flavobacteriaceae*), are widespread in nature and could originate from the freshwater used during processing or the storage ice as well as be transferred from the salmon skin mucus, where they also were detected. Overall, the bacterial composition in the storage ice changed more than the other sample types during the sampling period, and seemed partly influenced by the skin mucus microbiota of the salmon. The PC plot (Fig. 7) also suggested that *Moraxellaceae, Acinetobacter, Psychrobacter, Flavobacterium*, and *Chryseobacterium* either originated from the storage ice or were transferred from the exterior of the salmon. In addition, the high phylogenetic diversity values of the storage ice samples might be

due to a mixture of microbiota originating from the fish and microbiota killed by UV treatment but still present in the industrial water that the storage ice was made from. *Mycoplasmataceae* was detected only at low levels in the storage ice at the first sampling day with a maximum of 3.9% and further decreased the following days, indicating that the transfer of intestinal material to the storage ice was minor or that their survival in the ice slush without the immediate contact with the fish was minimal. This was supported by the multivariate analysis which showed no correlation between *Mycoplasmataceae* and the storage ice samples.

The measurements of gelatinase activity clearly demonstrated that the digestive tract samples contained enzymes capable of degrading gelatin. The DQ gelatin can be degraded by several enzymes including gelatinases such as MMP 2 and MMP 9 (*Gill & Parks, 2011*), and therefore this was not a measurement of a specific enzyme but rather the collective gelatin degrading activity in the samples. The results indicated a source of gelatinase activity in the abdominal cavity of the salmon in storage box no. 3 not present in the other boxes. The previous finding that all the abdominal samples from storage box no. 3 also contained a bacterial community structure highly similar to those in the digestive tract samples suggests that the high gelatinase activity may be due to enzymes originating from the intestinal fluids. The bacterial family *Mycoplasmataceae,* which was the dominating OTU detected in both intestinal samples and abdominal samples from storage box no. 3, contains several gelatinase producing bacteria in the genus *Mycoplasma* (*Czekalowski, Hall & Woolcock, 1973*). Therefore, the high gelatinase activity detected might be due to bacterial activity. However, a few other abdominal samples also had high relative abundances of *Mycoplasmataceae* without showing high gelatinase activity. The absolute amount of bacteria present was not estimated in this study, but might of course be of importance in relation to the gelatinase activity measurements. Because *Mycoplasma* can grow in intestinal fluids and blood, the presence of these in the abdominal cavity post-slaughter might also be a contributing factor. In addition, blood and intestinal fluids can contain gelatinases and other MMPs produced by the salmon (*Hauser-Davis, Lima & Campos, 2012*; *Eysturskarð et al., 2017*). The decreasing activity in the abdominal samples from storage box no. 3 may be due to the gradual inactivation of enzymes introduced at slaughter from either blood, intestinal fluids, intestinal bacteria, or a mixture thereof. In contrast, the slow increase in gelatinolytic activity detected in the other five storage boxes could possibly suggest a growth of other bacteria capable of degrading gelatin. Other genera detected at low relative abundance in most abdominal samples, such as *Staphylococcus, Bacillus* (fam. *Bacillaceae*), *Pseudomonas* (fam. Pseudomonadaceae), and *Clostridium* (fam. Clostridiaceae) contain species with gelatinolytic capabilities (*Whaley et al., 1982*; *Chakraborty, Mahapatra & Roy, 2011*; *Balan et al., 2012*; *Zhang et al., 2015*; *Abed et al., 2016*).

CONCLUSIONS

A correlation was detected between the bacterial community composition and the gelatinase activity in the abdominal cavity of the salmon during cold storage. The bacterial composition in the intestines was highly dominated by *Mycoplasmataceae* and to a lesser

degree *Aliivibrio*. The same dominance of *Mycoplasmataceae* was detected in the abdominal samples from storage box no. 3, while the abdominal samples from the other five storage boxes had a significantly different and more diverse bacterial community structures. The multivariate analysis grouped the abdominal samples from storage box no. 3 together with the intestinal samples. In addition, the gelatinase activity in the abdominal samples from storage box no. 3 was significantly higher than in the abdominal samples from the other storage boxes. At the same time the gelatinase activity was highest in the intestinal samples. This indicated the presence of intestinal fluids and bacteria in the abdominal cavity of salmon in storage box no. 3 and a possibility of connective tissue degradation as a consequence. This knowledge provides the industry with an incentive to be meticulous with the cleaning procedure and potential methods to use in quality control thereof.

The gelatinase activity in the skin mucus was low throughout. The relative content of *Mycoplasmataceae* varied but was generally low in the skin mucus and storage ice samples. The microbiota in the skin mucus was highly diverse and contained a mixture of bacteria likely stemming from both the marine environment and the industrial water used in the slaughtering facility. The relative content of *Firmicutes* was significantly higher in the skin mucus samples from salmon rinsed an extra time while *Proteobacteria* was significantly lower in these samples. The microbial community in the storage ice had significantly higher phylogenetic diversity than the other sample types. Potentially, the storage ice samples might have contained various bacteria common in freshwater as well as bacteria originating from the skin mucus.

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The authors declare there are no competing interests.

Author Contributions

- Ása Jacobsen conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.
- Svein-Ole Mikalsen and Jonhard Eysturskarð conceived and designed the experiments, contributed reagents/materials/analysis tools, authored or reviewed drafts of the paper, approved the final draft.
- Hóraldur Joensen analyzed the data, contributed reagents/materials/analysis tools, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.

DNA Deposition

The following information was supplied regarding the deposition of DNA sequences:

The sequence data supporting the conclusions of this article is available in the NCBI Sequence Read Archive database, study accession no.: SRP149649.

Data Availability

The following information was supplied regarding data availability:

A complete OTU table is available in Table S1. The gelatin degrading activity measurements are available in Table S2.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.7040#supplemental-information.

REFERENCES

Abed H, Rouag N, Mouatassem D, Rouabhi A. 2016. Screening for *Pseudomonas* and *Bacillus* antagonistic rhizobacteria strains for the biocontrol of Fusarium wilt of chickpea. *Eurasian Journal of Soil Science* **5(3)**:182–191 DOI 10.18393/ejss.2016.3.182-191.

Aronesty E. 2013. Comparison of sequencing utility programs. *Open Bioinformatics Journal* **7**:1–8 DOI 10.2174/1875036201307010001.

Auer L, Mariadassou M, O'Donohue M, Klopp C, Hernandez-Raquet G. 2017. Analysis of large 16S rRNA Illumina data sets: impact of singleton read filtering on microbial community description. *Molecular Ecology Resources* **17(6)**:e122–e132 DOI 10.1111/1755-0998.12700.

Balan SS, Nethaji R, Sankar S, Jayalakshmi S. 2012. Production of gelatinase enzyme from *Bacillus* spp isolated from the sediment sample of Porto Novo Coastal sites. *Asian Pacific Journal of Tropical Biomedicine* **2(3)**:S1811–S1816 DOI 10.1016/S2221-1691(12)60500-0.

Beaz-Hidalgo R, Doce A, Balboa S, Barja JL, Romalde JL. 2010. *Aliivibrio finisterrensis* sp. Nov. isolated from Manila clam, *Ruditapes philippinarum* and emended description of the genus *Aliivibrio*. *International Journal of Systematic and Evolutionary Microbiology* **60**:223–228 DOI 10.1099/ijs.0.010710-0.

Burbach K, Seifert J, Pieper DH, Camarinha-Silva A. 2016. Evaluation of DNA extraction kits and phylogenetic diversity of the porcine gastrointestinal tract based on Illumina sequencing of two hypervariable regions. *MicrobiologyOpen* **5(1)**:70–82 DOI 10.1002/mbo3.312.

Caporaso JG, Bittinger K, Bushman FD, DeSantis TZ, Andersen GL, Knight R. 2010b. PyNAST: a flexible tool for aligning sequences to a template alignment. *Bioinformatics* **26(2)**:266–267 DOI 10.1093/bioinformatics/btp636.

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Peña AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knight SD, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widman J, Yatsunenko T, Zaneveld J, Knight R. 2010a. QIIME allows analysis of high-throughput community sequencing data. *Nature Methods* **7(5)**:335–336 DOI 10.1038/nmeth.f.303.

Caporaso JG, Lauber CL, Walter WA, Berg-Lyons D, Huntley J, Fierer N, Owens SM, Betley J, Fraser L, Bauer M, Niall G, Gilbert JA, Smith G, Knight R. 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *The ISME Journal* **6**:1621–1624 DOI 10.1038/ismej.2012.8.

Chakraborty SP, Mahapatra SK, Roy S. 2011. Biochemical characters and antibiotic susceptibility of *Staphylococcus aureus* isolates. *Asian Pacific Journal of Tropical Biomedicine* **1(3)**:212–216 DOI 10.1016/S2221-1691(11)60029-4.

Chao A. 1984. Non-parametric estimation of the number of classes in a population. *Scandinavian Journal of Statistics* **11**:265–270 DOI 10.2307/4615964.

Chiarello M, Villéger S, Bouvier C, Bettarel Y, Bouvier T. 2015. High diversity of skin-associated bacterial communities of marine fishes is promoted by their high variability among body parts, individuals and species. *FEMS Microbiology Ecology* **91(7)**:Article fiv061 DOI 10.1093/femsec/fiv061.

Cuhadar S, Koseoglu M, Atay A, Dirican A. 2013. The effect of storage time and freeze-thaw cycles on the stability of serum samples. *Biochemia Medica* **23(1)**:70–77 DOI 10.11613/BM.2013.009.

Czekalowski JW, Hall DA, Woolcock PR. 1973. Studies on proteolytic activity of Mycoplasmas: gelatinolytic property. *Journal of General Microbiology* **75**:125–133 DOI 10.1099/00221287-75-1-125.

Dehler CE, Secombes CJ, Martin SAM. 2017. Seawater transfer alters the intestinal microbiota profiles of Atlantic salmon (*Salmo salar* L.). *Science Reports* **7**:13877 DOI 10.1038/S41598-017-13249-8DO.

Duarte AS, Correia A, Esteves AC. 2016. Bacterial collagenases—a review. *Critical Reviews in Microbiology* **42(1)**:106–126 DOI 10.3109/1040841X.2014.904270.

Edgar RC. 2010. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* **26**:2460–2461 DOI 10.1093/bioinformatics/btq461.

Egerton S, Culloty S, Whooley J, Stanton C, Ross RP. 2018. The gut microbiota of marine fish. *Frontiers in Microbiology* **9**:Article 873 DOI 10.3389/fmicb.2018.00873.

Eysturskarð J, Kongsstovu Sí, Færø D, Jacobsen Á, Joensen H. 2017. *Fucus vesiculosus* extract inhibits the proteolytic activity and gene expression of matrix metalloproteinases in Atlantic salmon (*Salmo salar* L.). *Aquaculture International* **25**:1813–1819 DOI 10.1007/S10499-017-0157-7.

Faith DP. 1992. Conservation evaluation and phylogenetic diversity. *Biological Conservation* **61**:1–10 DOI 10.1013/0006-3207(92)91201-3.

Gajardo K, Rodiles A, Kortner TM, Krogdahl Å, Bakke AM, Merrifield DL, Sørum H. 2016. A high-resolution map of the gut microbiome in Atlantic salmon (*Salmo salar*): a basis for comparative gut microbial research. *Science Reports* **6**:30893 DOI 10.1038/srep30893.

Gerber GK. 2014. The dynamic microbiome. *FEBS Letters* **588**:4131–4139 DOI 10.1016/j.febslet.2014.02.037.

Gill SE, Parks WC. 2011. Matrix Metalloproteinases and their inhibitors in turnover and degradation of extracellular matrix. In: Parks WC, Mecham RP, eds. *Extracellular matrix degradation*. Heidelberg: Springer, 1–22 DOI 10.1007/978-3-642-16861-1.

Green TJ, Smullen R, Barnes AC. 2013. Dietary soybean protein concentrate-induced intestinal disorder in marine farmed Atlantic salmon, *Salmo salar* is associated with alterations in gut microbiota. *Veterinary Microbiology* **166**:286–292 DOI 10.1016/j.vetmic.2013.05.009.

Hauser-Davis RA, Lima AA, Campos RC. 2012. First-time report of metalloproteinases in fish bile and their potential as bioindicators regarding environmental contamination. *Aquatic Toxicology* **110-111**:99–106 DOI 10.1016/j.aquatox.2011.12.04.

Jacobsen Á, Joensen H, Eysturskarð J. 2017. Gaping and loss of fillet firmness in farmed salmon (*Salmo salar* L.) closely correlated with post-slaughter cleaning of the abdominal cavity. *Aquaculture Research* **48(1)**:321–331 DOI 10.1111/are.12884.

Karlsen C, Ottem KF, Brevik ØJ, Davey M, Sørum H, Winther-Larsen HC. 2017. The environmental and host-associated bacterial microbiota of Arctic seawater-farmed Atlantic salmon with ulcerative disorders. *Journal of Fish Diseases* **40**:1645–1663 DOI 10.1111/jfd.12632.

Klindworth A, Pruesse E, Schweer T, Peplies J, Quast C, Horn M, Glöckner FO. 2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Research* **21(1)**:e1 DOI 10.1093/nar/gks808.

Kramer CY. 1956. Extension of multiple range tests to group means with unequal numbers of replications. *Biometrics* **12**:307–310 DOI 10.2307/3001469.

Kvalheim OM, Karstang TV. 1987. A general-purpose program for multivariate data analysis. *Chemometrics and Intelligent Laboratory Systems* **2**:235–237 DOI 10.1016/0169-7439(87)80101-6.

Langsrud S, Moen B, Møretrø T, Løype M, Heir E. 2015. Microbial dynamics in mixed culture biofilms of bacteria surviving sanitation of conveyor belts in salmon-processing plants. *Journal of Applied Microbiology* **120**:366–378 DOI 10.1111/jam.13013.

Llewellyn MS, McGinnity P, Dionne M, Letourneau J, Thonier F, Carvalho GR, Creer S, Derome N. 2016. The biogeography of the Atlantic salmon (*Salmo salar*) gut microbiome. *The ISME Journal* **10(15)**:1280–1284 DOI 10.1038/ismej.2015.189.

Lokesh J, Kiron V. 2016. Transition from freshwater to seawater reshapes the skin-associated microbiota of Atlantic salmon. *Science Reports* **6**:19707 DOI 10.1038/srep19707.

Lowrey L, Woodhams DC, Tacchi L, Salinas I. 2015. Topographical mapping of the rainbow trout (*Oncorhynchus mykiss*) microbiome reveals a diverse bacterial community with antifungal properties in the skin. *Applied Environmental Microbiology* **81**:6915–6925 DOI 10.1128/AEM.01826-15.

Lozupone C, Knight R. 2005. UniFrac: a new phylogenetic method for comparing microbial communities. *Applied Environmental Microbiology* **71(12)**:8228–8235 DOI 10.1128/AEM.71.12.8228-8235.2005.

Majaneva M, Hyytiänen K, Varvio SL, Nagai S, Blomster J. 2015. Bioinformatic amplicon read processing strategies strongly affect eukaryotic diversity and the taxonomic composition of communities. *PLOS ONE* **10(6)**:e013003 DOI 10.1371/journal.pone.0130035.

Minniti G, Hagen LH, Porcellato D, Jørgensen SM, Pope PB, Vaaje-Kolstad G. 2017. The skin-mucus microbial community of farmed Atlantic salmon (*Salmo salar* L.). *Frontiers in Microbiology* **8**:Article 2043 DOI 10.3389/fmicb.2017.02043.

Møretrø T, Moen B, Heir E, Hansen AÅ, Langsrud S. 2016. Contamination of salmon fillets and processing plants with spoilage bacteria. *International Journal of Food Microbiology* **237**:98–108 DOI 10.1016/j.ijfoodmicro.2016.08.016.

Morey A, Himmelbloom BH, Olivieira ACM. 2014. Bacterial diversity and changes towards spoilage microflora of iced Alaska pink salmon. *Journal of Nutritional Health & Food Engineering* **1(1)**:25–29 DOI 10.15406/jnhfe.2014.01.00005.

Murias M, Rachtan M, Jodynis-Liebert J. 2005. Effect of multiple freeze-thaw cycles of cytoplasm samples on the activity of antioxidant enzymes. *Journal of Pharmacological and Toxicological Methods* **52(2)**:302–305 DOI 10.1016/j.vascn.2005.03.002.

Okamoto T, Akaike T, Suga M, Tanase S, Horie H, Miyajima S, Ando M, Ichinose Y, Meada H. 1997. Activation of human matrix metalloproteinases by various bacterial proteinases. *Journal of Biological Chemistry* **272(9)**:6059–6066 DOI 10.1074/jbc.272.9.6059.

Pedersen M, Vuong TT, Rønning SB, Kolset SO. 2015. Matrix metalloproteinases in fish biology and matrix turnover. *Matrix Biology* **44-46**:86–93 DOI 10.1016/j.matbio.2015.01.009.

Price MN, Dehal PS, Arkin AP. 2009. FastTree: computing large minimum evolution trees with profiles instead of a distance matrix. *Molecular Biology and Evolution* **26(7)**:1641–1650 DOI 10.1093/molbev/msp077.

Reynisson E, Magnússon SH, Rúnarsson ÁR, Marteinsson Vþ. 2010. Bacterial diversity in the processing environment of fish products, Skýrsla Matís. *Available at http://www.matis.is/media/matis/utgafa/11-10-Lokaskyrsla-1790.pdf* .

Rubin BER, Sanders JG, Hampton-Marcell J, Owens SM, Gilbert JA, Moreau CS. 2014. DNA extraction protocols cause differences in 16S rRNA amplicon sequencing efficiency but not in community profile composition or structure. *MicrobiologyOpen* **3(6)**:910–921 DOI 10.1002/mbo3.216.

Sai-Ut S, Benjakul S, Sumpavapol P. 2013. Gelatinolytic enzymes from *Bacillus amyloliquefaciens* isolated from fish docks: characteristics and hydrolytic activity. *Food Science and Biotechnology* **22(4)**:1015–1021 DOI 10.1007/s10068-013-0178-6.

Tedjo DI, Jonkers DMAE, Savelkoul PH, Masclee AA, Van Best N, Pierik MJ, Penders J. 2015. The effect of sampling and storage on the fecal microbiota composition in healthy and diseased subjects. *PLOS ONE* **10(5)**:e0126685 DOI 10.1371/journal.pone.0126685.

Urbanczyk H, Ast JC, Dunlap PV. 2011. Phylogeny, genomics and symbiosis of Photobacterium. *FEMS Microbiology Reviews* **35**:324–342 DOI 10.1111/j.1574-6976.2010.00250.x.

Vázquez-Baeza Y, Pirrung M, Gonzales A, Knight R. 2013. EMPeror: a tool for visualizing high-throughput microbial community data. *Gigascience* **2(1)**:Article 16 DOI 10.1186/2047-217X-2-16.

Verma RP, Hansch C. 2007. Matrix metalloproteinases (MMPs): chemical-biological functions and (Q)SARs. *Bioorganic & Medical Chemistry* **15**:2223–2268 DOI 10.1016/j.bmc.2007.01.011.

Vishnivetskaya TA, Layton AC, Lau MCY, Chauhan A, Cheng KR, Meyers AJ, Murphy JR, Rogers AW, Saarunya GS, Williams DE, Pfiffner SM, Biggerstaff JP, Stackhouse BT, Phelps TJ, Whyte L, Sayler GS, Onstott TC. 2013. Commercial DNA extraction kits impact observed microbial community composition in permafrost samples. *FEMS Microbiology Ecology* **87**:217–230 DOI 10.1111/1574-6941.12219.

Wallenius K, Rita H, Simpanen S, Mikkonen A, Niemi RM. 2010. Sample storage for soil enzyme activity and bacterial community profiles. *Journal of Microbiological Methods* **81**:48–55 DOI 10.1016/j.mimet.2010.01.021.

Whaley DN, Dowell Jr VR, Wanderlinder LM, Lombard GL. 1982. Gelatin agar medium for detecting gelatinase production by anaerobic bacteria. *Journal of Clinical Microbiology* **16(2)**:224–229.

Wold S. 1979. Pattern recognition by means of disjoint principal component models. *Pattern Recognition* **8**:127–139 DOI 10.1016/0031-3203(76)90014-5.

Zhang Y, Ran L, Li C, Chen X. 2015. Diversity, structures, and collagen-degrading mechanisms of bacterial collagenolytic proteases. *Applied and Environmental Microbiology* **81(18)**:6098–6107 DOI 10.1128/AEM.00883-15.

4.4 Jacobsen Á, Eysturskarð J, Mikalsen S-O. Initial metagenomic screening of microbial communities in stomach and intestines of four fish species inhabiting coastal waters. *In prep.*

Á. Jacobsen[1§], J. Eysturskarð[1], S.-O. Mikalsen[2]

[1]Fiskaaling, Aquaculture Research Station of the Faroes, við Áir 11, Hvalvík, Faroe Islands

[2]Department of Science and Technology, University of the Faroe Islands, Vestara Bryggja 15, Tórshavn, Faroe Islands

[§]Corresponding author. Tel.:+298 774748; e-mail: asa@fiskaaling.fo

Abstract

This study is an initial screening of the microbiome present in samples from the stomach and intestines of farmed Atlantic salmon *Salmo salar* L. and three wild fish species: cod *Gadus morhua* L., haddock *Melanogrammus aeglefinus* L., and ling *Molva molva* L.. All sampled fish inhabited Faroese coastal waters in near proximity of each other. Stomach and intestinal samples were analysed by 16S rRNA Next Generation sequencing (454) and after quality filtration 141 OTUs were detected based on 127,187 sequences. The most abundant phyla were Firmicutes and Tenericutes, together representing 80.4 % of the reads, while the occurrence of Proteobacteria was low, representing between 0.0 % and 3.2 % of the reads in the various samples. Several bacteria capable of degrading connective tissue as well as potential pathogens and indicators of pollution were detected. *Clostridium perfringens* was highly abundant in all *G. morhua* samples, with 57.4 % to 87.9% of the reads, and the *M. aeglefinus* stomach samples, with 49.5 % to 91.3 % of the reads, but detected at low abundances in all other samples, with 1.7 % at the most. The family *Mycoplasmataceae* was exceedingly abundant in the *S. salar*, representing between 68.5 % and 97.9 % of the reads, while representing between 0.0 % and 27.3 % in the other samples. All OTUs dominating the bacterial community in any sample, were differently represented in the various individuals, species, and sample types. A principal coordinate analysis separated the *S. salar* samples and the *M. molva* samples from the others while the *G. morhua* and *M. aeglefinus* samples overlapped due to similarities in their microbial compositions. A few OTUs were detected in all fish species; 10 and 14 in intestinal and stomach samples, respectively. Amongst these were potentially harmful bacteria such as the species *Micrococcus luteus* and the genus *Renibacterium.*

Keywords: *Gadus morhua; Melanogrammus aeglefinus;* microbiome; *Molva molva; Salmo salar*.

Significance statement

The impact of the gastrointestinal microbiome on its host has recently become ever more apparent as the microbial community seems to affect a wide range of physiological mechanisms. This manuscript contributes to the basic knowledge of the composition of the gastrointestinal microbiome in four fish species of commercial importance, and two of which have not been

analysed before. Such knowledge is important for potential screening of health status and/or intentional alterations of the microbiome.

Introduction

In recent years, there has been a growing understanding of the importance of the gastrointestinal microbiome to its host. The composition of the bacterial community appears to have a major impact on various physiological functions in the host. The microbiome obviously affects the functionality of the digestive system, but it also seems to be an integral part of the development and effectiveness of the host's immune system (Ventura *et al.*, 2007; Nayak, 2010; Hanning & Dias-Sanchez, 2015). Broad and non-selective identification methods, in particular next generation sequencing (NGS), have led to a substantial increase of research in this area and thereby been essential in gaining this knowledge. Microbiome studies of various fish species of commercial value are becoming more frequent (Llewellyn *et al.*, 2014). In aquaculture, the practical goal of research in the fish microbiome is often to see if some form of intended alteration in the microbial composition can improve certain production parameters (e.g., Abid *et al.*, 2013; Ni *et al.*, 2014; Reveco *et al.*, 2014; Xia *et al.*, 2014). The results from these studies are promising, though many uncertainties must be addressed before microbiome manipulation can become standard aquaculture production procedure (Llewellyn *et al.*, 2014; Ringø *et al.*, 2016). In addition, the possibility of intestinal bacteria left in the abdominal cavity of post-slaughter Atlantic salmon *Salmo salar* (L. 1758) affecting fillet quality has been suggested (Jacobsen *et al*., 2017; Jacobsen *et al*., submitted). This issue might also concern other commercially exploited fish species. Less focus has been on the microbiome of wild fish species, but knowledge about these species provides crucial information for answering fundamental questions, such as which factors form and influence the bacterial community, its functions in the fish gastrointestinal system as well as microbial interactions (Sullam *et al.*, 2012; Hanning & Dias-Sanchez, 2015; Rakoff-Nahoum *et al.*, 2016). This is important for improved interpretation of microbiome analyses, for instance the significance of potentially pathogenic bacteria present in the intestine.

This initial screening of four different fish species inhabiting the same coastal waters had the objective to detect if bacteria with the potential to affect fish fillet quality or fish health were present in the aquaculture species of choice in the Faroe Islands, *S. salar*, and three other commercially important fish species inhabiting the same coastal areas, namely wild cod *Gadus*

morhua (L. 1758), haddock *Melanogrammus aeglefinus* (L. 1758), and ling *Molva molva* (L. 1758). At the present, no studies of this kind have been conducted on fish inhabiting the Faroese coastal waters. However, farmed *S. salar* located elsewhere (e.g. Holben *et al*. 2002; Hovda *et al.*, 2007; Dehler *et al.*, 2017) and wild *S. salar* (Llewellyn *et al.*, 2016) have been studied to some degree. The microbial community detected in these studies provides a basis for comparison of our data. Although various wild marine fish species have been analyzed, few studies are available on *G. morhua* (Star *et al.*, 2013) for comparison, and to our knowledge, no microbiome studies have been conducted on *M. molva* and *M. aeglefinus*. Many of the previous microbiome studies were also based on techniques other than next generation sequencing. Therefore, we considered it important to contribute to a baseline for future more focused research of these commercially important fish species and a potential development of health status checks of wild fish species in the form of screening the intestinal microbiome. Additionally, these topics are of current interest for fish biology research regarding both aquaculture and wild fish (Jacobs *et al*., 2018).

Materials and Methods

Samples

Samples were collected from four finfish species, two individuals of each species: *S. salar*, *M. aeglefinus*, *G. morhua,* and *M. molva*. All fish species were caught or harvested in January 2012. Hiddenfjord aquaculture farming company (Sørvágur, Faroe Islands) kindly provided the *S. salar*. The *S. salar* were taken from a pen located in one of the fjords of the western island, Vágar. The *S. salar* were slaughtered under standard procedure and the intestines harvested. The intestines were thereafter contained in separate sterile bags and frozen at -18 °C until further processing within the next two weeks. The *M. aeglefinus*, *G. morhua*, and *M. molva* were caught by a small fishing vessel approximately 45 km northeast of the pen location, by the coast of one of the northern islands. The fish were stored in chilled seawater onboard the fishing vessel until sampling in the laboratory later the same day. The stomach and intestines were put in separate sterile bags and frozen at -18 °C until further processing within the next two weeks. At sampling the stomachs and intestines were thawed and samples taken from the stomach and mid intestines, using sterile scalpels, of all species while still chilled. The samples comprised both content and inner lining mucus layer from the stomach and intestines. The samples were immediately stored at -18 °C until processing.

DNA extraction

Samples were homogenized while frozen using sterilized mortars and pestles. DNA was extracted immediately from 200 mg of each sample using the QIAamp DNA Stool Mini Kit (Qiagen, Sollentuna, Sweden) following the supplier's instructions. DNA concentration was measured by NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

NGS primer design

A primer pair amplifying the V5/V6 regions of the 16s rRNA gene was used in this study. The primers were U786F (5'GATTAGATACCCBGGTAGTC) and A1067R (5'TGRCGRCGGCCATGCAC) corresponding to E. coli positions 839-858 and 1120-1104, respectively. Due to the known problem with claimed universal primers not being able to cover all microbial genera (Horz *et al.*, 2005; Mao *et al*., 2012; Fredriksson *et al.*, 2013), the selection of primer design was based on a trial run where fourteen primers targeting the 16s rRNA gene were tested for amplification succes. The fourteen primers were universal primers previously used for amplification of the variable regions of 16s rRNA (Nadkarni *et al.*, 2002; Maeda *et al.*, 2003) or slightly modified versions thereof. Modifications of primers were based on a comparison of selected bacterial sequences, covering a wide range of bacterial phyla. Amplicon fusion primers were designed for 454 sequencing (Roche, Branford, CT, USA) adding directional GS FLX Titanium primers A and B with a four base library key and MID (multiplex identifier) sequences at both ends for barcoding of the various samples to the selected template specific primers. The primers were synthesized by DNA Technology (now LGC; Risskov, Denmark).

16s rRNA amplification and sequencing

PCR amplification was performed using the Qiagen Multiplex PCR kit (Qiagen), containing a HotStarTaq DNA Polymerase. Reaction solutions for amplification of the V5/V6 region contained 8.8 µl Master Mix, 1.7 µl Q-solution, 1.0 µM of the appropriate barcoded primers and 20 ng corresponding DNA template. Total reaction volume was 25 µl and thermocycling conditions were 95 °C for 15 min, 94 °C for 4 min, 38 cycles of 95 °C for 30 s, 53 °C for 90 s and 72 °C for 60 s, followed by 72 °C for 10 min. All PCR products were run on agarose gels for verification of amplification success. The V5/V6 PCR products were since purified with the Agencourt AMPure XP PCR purification system to eliminate any primer dimers. DNA concentration of all PCR products were measured in a Glomax Multi Detection System (Promega, Nacka, Sweden) using the

Quant-iT^{TM} PicoGreen dsDNA Assay kit (LifeTechnologies, Nærum, Denmark). The PCR products were thereafter pooled in equal amounts according to specifications provided by the Norwegian High-Throughput Sequencing Centre (NSC; University of Oslo, Norway) where the 454 bidirectional sequencing was performed.

Real-time PCR

The NGS primers failed to amplify the Bacteroidetes phylum. Therefore, real-time PCR was applied in order to establish the relative content of this phylum in the samples. Because the phylum Firmicutes was detected in all samples by NGS, it was also analysed by real-time PCR for comparison together with a primer pair covering all bacteria. The primer pairs used were Bact934F and Bact1060R, and Firm934F and Firm1060R (Guo *et al.*, 2008), which anneal specifically to Bacteroidetes and Firmicutes, respectively. The primer pair Eub338F and Eub518R (Fierer *et al.*, 2005), also used, covers all bacteria. These primers were purchased from LifeTechnologies. The amplification and detection was performed in a StepOnePlus Real-Time PCR system (Fisher Scientific, Slangerup, Denmark) using the SYBR Green PCR Master Mix (LifeTechnologies). The PCR reaction was performed with a total volume of 10 µl containing 100 nMol/L primer concentrations and 1 ng of DNA. The amplification conditions were 50 °C for 2 min, 95 °C for 10 min followed by 40 cycles of 95 °C for 15 sec and 60 °C for 1 min. All samples were amplified twice with each of the three primers.

Data analysis

All handling of the NGS raw data was performed in QIIME (Caporaso *et al.*, 2010a; Kuczynski *et al.*, 2011). In the PCR process, all sequences were tagged with barcodes on both sides for identification. The first step in the data analysis was to name and split the sequences into separate files, according to sample ID, and remove primer sequences. In this process, all sequences less than sixty bases in length were also discarded as well as those with an average base calling quality estimate below twenty-five. Three samples (the stomach and intestinal samples from one of the *M. molva* individuals as well as the stomach sample from one of the *S. salar*) failed to give adequate amount of sequencing data. Therefore, they were eliminated from further analyses. Reads were clustered into OTUs (operational taxonomic units) with at least 97 % sequence similarity by the de novo method and representative reads were aligned using PyNAST (Caporaso *et al.*, 2010b) and taxonomy was assigned using the UCLUST method (Edgar, 2010). Ninety-seven percent sequence identity is the current widely used threshold for determining separate microbial species (Gevers *et*

al., 2005; Morales *et al.*, 2011). In order to make a more robust dataset, OTUs with less than three reads were removed from further analysis, a typical method for removing many of the artefacts produced in the sequencing process. The risk of an artificially inflated estimate of species richness in the samples is also reduced. However, it will undoubtedly also exclude many legitimate, but rare, microbes (Zhan *et al.*, 2014), which might be confirmed with deeper sequencing. A phylogenetic tree was constructing using the program FastTree (Price *et al.*, 2009). The alpha diversity estimate Chao1 (Chao, 1984) and phylogenetic diversity estimate PD whole tree (Faith, 1992) were also calculated in QIIME. In addition, weighted UniFrac beta diversity (Lozupone & Knight, 2005) was calculated, including the phylogenetic tree. This produced a distance matrix and a principal coordinates file, which was visualized with the program EMPeror (Vasquez-Baeza *et al.*, 2013). Real-time PCR data were analyzed according to the comparative CT method ($\Delta\Delta C_T$) (Livak & Schmittgen, 2001). Relative content of Bacteroidetes bacteria in each sample was calculated using the signal covering all bacteria as reference and the Firmicutes signal as calibrator.

Results

Reads and operational taxonomic units

After quality filtration the total number of reads obtained was 127,187, and 141 OTUs were detected when disregarding the OTUs supported by less than three reads. The number of reads acquired from each of the thirteen samples successfully analyzed was between 5,702 and 16,346 (Table 1), comparable to other studies of the same nature (Wu *et al.*, 2012; Ye *et al.*, 2014). Number of OTUs detected from each sample was between 15 and 61 (Table 1), which compared to some publications on fish microbiomes is relatively low (Wu *et al.*, 2012; Star *et al.*, 2013; Ye *et al.*, 2014). However, the comparatively low number of OTUs detected might partly be due to our removal of the low abundance OTUs, which these other studies did not do. Also, we did not detect Bacteroidetes by NGS, as the primers did not have sufficient specificity for this phylum (see real-time PCR results below). This has caused a reduction in the number of OTUs recovered by NGS. The rarefaction curves (Figure 1S) showed that the sequencing depth was satisfactory and suggested that the bulk of the high abundance OTUs were detected already at about four thousand sequenced reads. The values for the within-sample diversity estimate, Chao1, were between 20.2 and 151.2 (Table 1).

Sample	Reads	OTUs	Chao1	PD wt
Sal1Int	7,215	15	20.2	2.03
Sal2Sto	13,131	32	117.3	4.25
Sal2Int	12,570	29	58.2	2.98
Cod1Sto	5,702	44	63.9	4.65
Cod1Int	5,997	15	25.5	2.29
Cod2Sto	16,346	47	81.0	5.38
Cod2Int	16,211	56	151.2	6.01
Had1Sto	6,649	29	55.8	3.12
Had1Int	6,167	31	53.3	3.30
Had2Sto	6,224	32	58.5	4.69
Had2Int	7,666	29	46.2	4.16
Lin1Sto	10,751	61	139.2	7.77
Lin1Int	12,558	37	50.2	3.99

Table 1. Sample data and alpha diversity estimates. Number of reads obtained and OTUs detected for each sample obtained from *Salmo salar* (Sal), *Gadus morhua* (Cod), *Melanogrammus aeglefinus* (Had), and *Molva molva* (Lin). Sample name is composed of the fish species abbreviations, individual number and the sample type abbreviations "Sto" for stomach and "Int" for intestine. In addition, the alpha diversity estimate, or within-sample diversity estimate, Chao1 (Chao, 1984) and the phylogenetic diversity estimate, PD whole tree (Faith, 1992), were calculated for each sample. The diversity estimates were based on iterations using 5,372 reads. Chao1 is a measure of species richness. It estimates the total number of OTUs present in the sample based on the number of OTUs detected in a subsample and the number of singletons and doubletons. PD whole tree is a measure of the branch length in the phylogenetic tree.

In comparison, a study of farmed channel catfish *Ictalurus punctatus* (R. 1818), largemouth bass *Micropterus salmoides* (L. 1802), and bluegill *Lepomis machrochirus* (R. 1810) showed Chao1 estimates between 153 and 218 (Larsen *et al.* 2014) while another study of farmed turbot *Scophtalmus maximus* (L. 1758) had values between 23 and 44 (Xing *et al.* 2013). The phylogenetic diversity estimates ranged from 2.03 to 7.77 (Table 1).

Microbiome composition

The OTUs detected from the 454 sequencing belonged to seventeen phyla, the dominating phyla being Tenericutes and Firmicutes (Fig. 1a), together representing 80.4 % of all reads. Tenericutes dominated the *S. salar* microbiome, representing 84.5 % and 97.9 % of the sequences in the two intestinal samples. Firmicutes was most prominent in all *G. morhua* samples as well as in *M. aeglefinus* stomach samples. The following phyla were Actinobacteria and Fusobacteria, which accounted for 5.5 % and 4.6 %, respectively. Phyla found in all species and sample types were Tenericutes, Firmicutes and Actinobacteria. Fusobacteria, the fourth most abundant phylum with regard to total number of reads, was mainly detected in *M. aeglefinus*. Proteobacteria were detected in low abundances only, if at all, and represented between 0.0 % and 3.2 % of the reads in the various samples. Between 0.2 % and 12.4 % of the reads in a sample could not be assigned to any phyla.

The phylum Bacteroidetes, which is commonly found in fish digestive systems (Givens, 2012; Xia *et al.*, 2014), was detected in all species by real-time PCR (data not shown). However, it was not found in either the *M. molva* stomach sample or the *G. morhua* intestinal samples. On the other hand, Bacteroidetes were relatively abundant in the *M. molva* intestinal sample with 12.7 % of total microbial presence in the sample, which means that the microbial community in the *M. molva* intestinal sample was more diverse than illustrated in Figure 1. In the *G. morhua* stomach samples, Bacteroidetes constituted on average a moderate 4.7 % of the microbial community and therefore the influence on phyla diversity and composition was minor. The amount of Bacteroidetes in *S. salar* was minor, only 0.2 % and 0.7 % on average of all bacteria in the stomach and intestinal samples, respectively, while the presence in the *M. aeglefinus* stomach and intestinal samples was 11.4 % and 1.2 %, respectively. This means that Firmicutes was slightly less dominating in the *M. aeglefinus* stomach samples than illustrated. Overall, the Bacteroidetes was not a dominating phylum in any of the samples. At lowest possible defined taxonomical levels (Fig. 1b) all the *S. salar* samples were highly dominated by a bacterium from the family *Mycoplasmataceae* with between 68.5 % and 97.9 % of the reads.

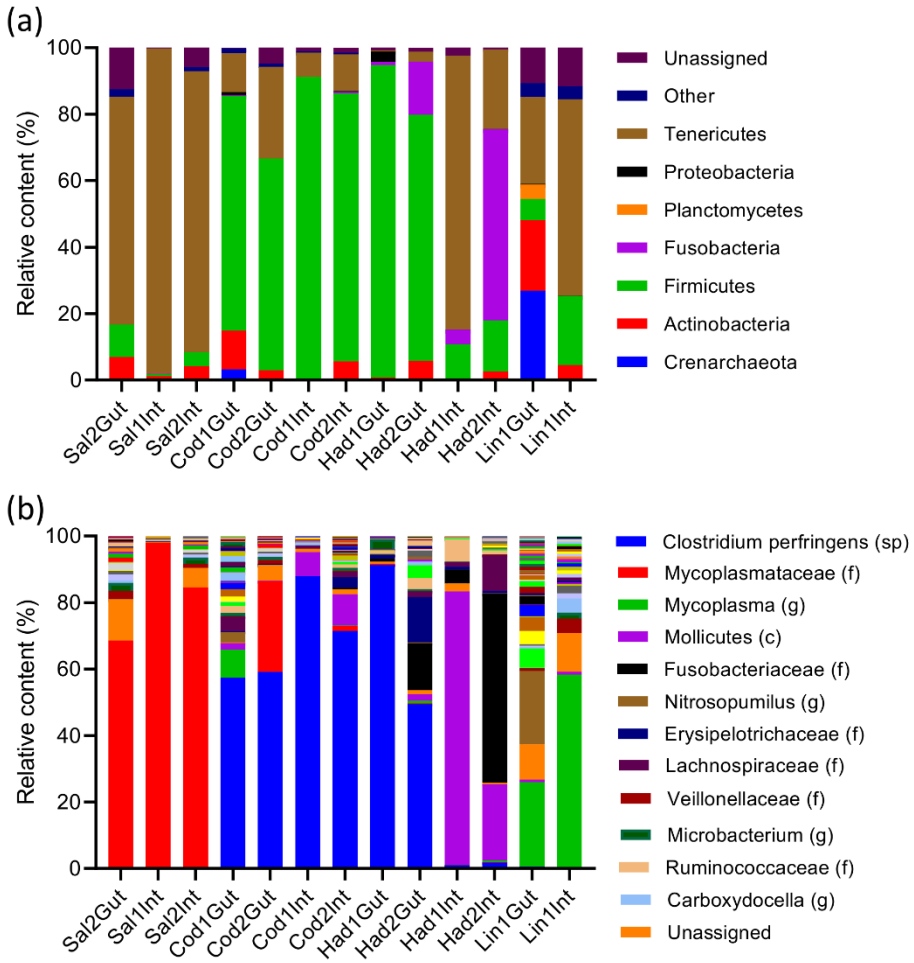

Figure 1. Composition of the bacterial community at phylum (a) and lowest defined (b) level in stomach and intestinal samples from *Salmo salar, Gadus morhua, Melanogrammus aeglefinus,* and *Molva molva.* Major phyla are indicated (a). Phylogenetic identification and level is indicated (b) for each OTU: class (c), family (f), genus (g), species (sp). Sample names are abbreviations of fish species (sal = *S. salar*, cod = *G. morhua*, Had = *M. aeglefinus*, Lin = *M. molva*), individual number, and sampling site (Sto = stomach, Int = intestine).

In the *S. salar* samples, only three OTUs comprised 1.0 % or more of the microbial community. Apart from the dominating *Mycoplasmataceae*, a bacterium from the *Veillonellaceae* family and another from the genus *Microbacterium* (family *Microbacteriaceae*) were present at lower abundance, 2.4 % and 2.5 % at the most, respectively. Both the stomach and the intestinal *M. molva* samples had a relatively high proportion of the genus *Mycoplasma*, also from the family *Mycoplasmataceae*, with 58.3 % of the reads in the intestinal samples. On the other hand, the

stomach sample contained 25.4 % *Mycoplasma* and in addition had a nearly as high abundance of *Nitrosopumilus* (22.1 %), an ammonia-oxidizing archaeae common in both coastal marine environments and sediment (DeLong, 1992; Francis *et al*., 2005). All the *G. morhua* samples along with the *M. aeglefinus* stomach samples contained relatively high abundances of *Clostridium perfringens* with between 49.5 % and 91.3 %. In comparison, the two *M. aeglefinus* intestinal samples contained only low levels of *C. perfringens* (0.9 % and 1.7 %). One of the intestinal samples was instead dominated by bacteria from the class Mollicutes at 82.2 % while the other was dominated by bacteria from the family *Fusobacteriaceae* with 56.8 %.

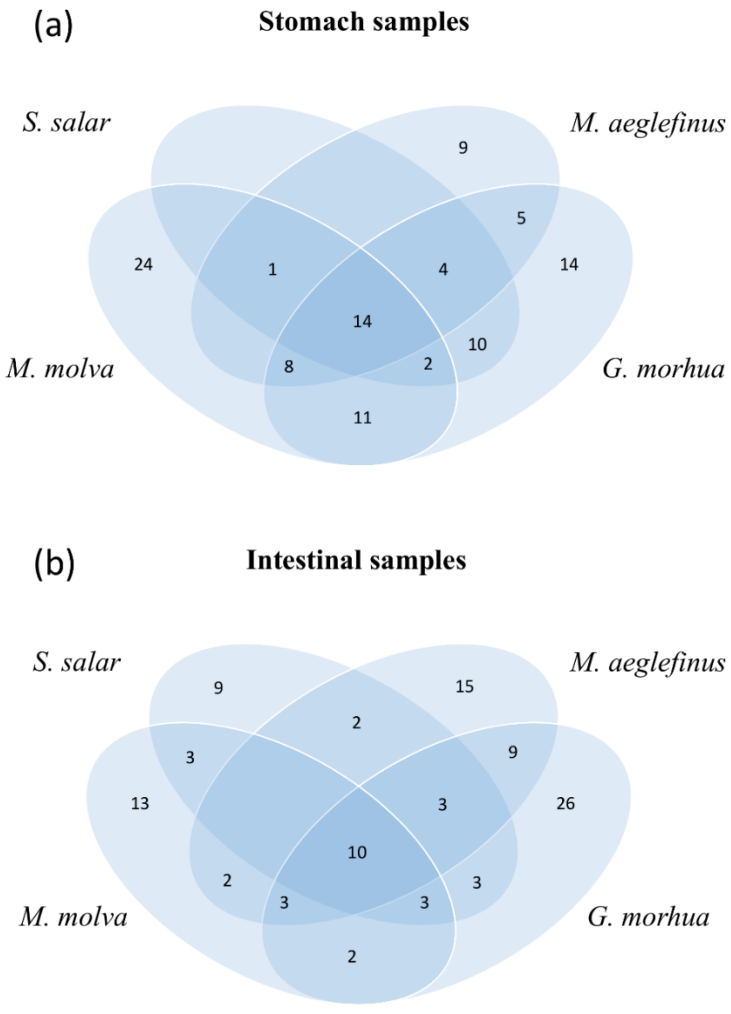

Figure 2. Shared and exclusive OTUs in stomach and intestinal samples from *Salmo salar, Gadus morhua, Melanogrammus aeglefinus,* and *Molva molva.* Venn diagrams of OTUs detected in stomach (a) and intestinal (b) samples.

Members of the family *Fusobacteriaceae* are normal constituents of the oral and intestinal mucosa of animals, including fish (Olsen, 2014), and are regularly detected by NGS analysis of the digestive systems of various fish species (e.g. Dulski *et al.*, 2017; Parris *et al.*, 2016). However, *Fusobacteriaceae* does not seem to be a part of the core microbiota of marine fish (Egerton *et al.* 2018). The number of OTUs shared between or exclusive to the various fish species was illustrated in Venn diagrams for stomach samples (Fig. 2a) and for intestinal samples (Fig. 2b).

Closest defined Identification (Taxonomical level†)	**Phylum**	**Reads in total**	**Stomach**	**Intestine**
Clostridium perfringens (sp)	Firmicutes	39,187	X	
Mycoplasmataceae (f)	Tenericutes	31,438		X
Mycoplasma (g)	Tenericutes	10,658	X	X
Nitrosopumilus (g)	Thaumarchaeota	2,589	X	
Veillonellaceae (f)	Firmicutes	1,357	X	X
Microbacterium (g)	Actinobacteria	1,252	X	X
Carboxydocella (g)	Firmicutes	894	X	X
Micrococcus luteus (sp)	Actinobacteria	781	X	X
Streptococcus (sp)	Firmicutes	670	X	X
Acidimicrobiales (o)	Actinobacteria	635	X	
Propionibacterium acnes (sp)	Actinobacteria	471	X	X
Renibacterium (g)	Actinobacteria	308	X	X
Clostridiceae (f)	Firmicutes	224	X	
Caldinitratiruptor microaerophilus (sp)	Firmicutes	220	X	X
Thermoanaerobacterales (o)	Firmicutes	135	X	

Table 2. **OTUs shared between all fish species**. The OTUs shared between all fish species in either stomach samples, intestinal samples or combined. The OTUs are listed according to abundance rank with information about number of reads supporting the OTU and shared sampling location. The OTUs are described with closest defined taxonomical identification and to which phyla they belong. †c: class, o: order, f: family, g: genus, sp: species.

In the stomach samples 46.1 % of the OTUs were deteced in one fish species only (Fig. 2a), while 13.7 % of the OTUs were shared between all species. In the intestinal samples (Fig. 2b) 61.2 % of the OTUs were exclusive to one fish species, while 9.7 % were shared between all species.

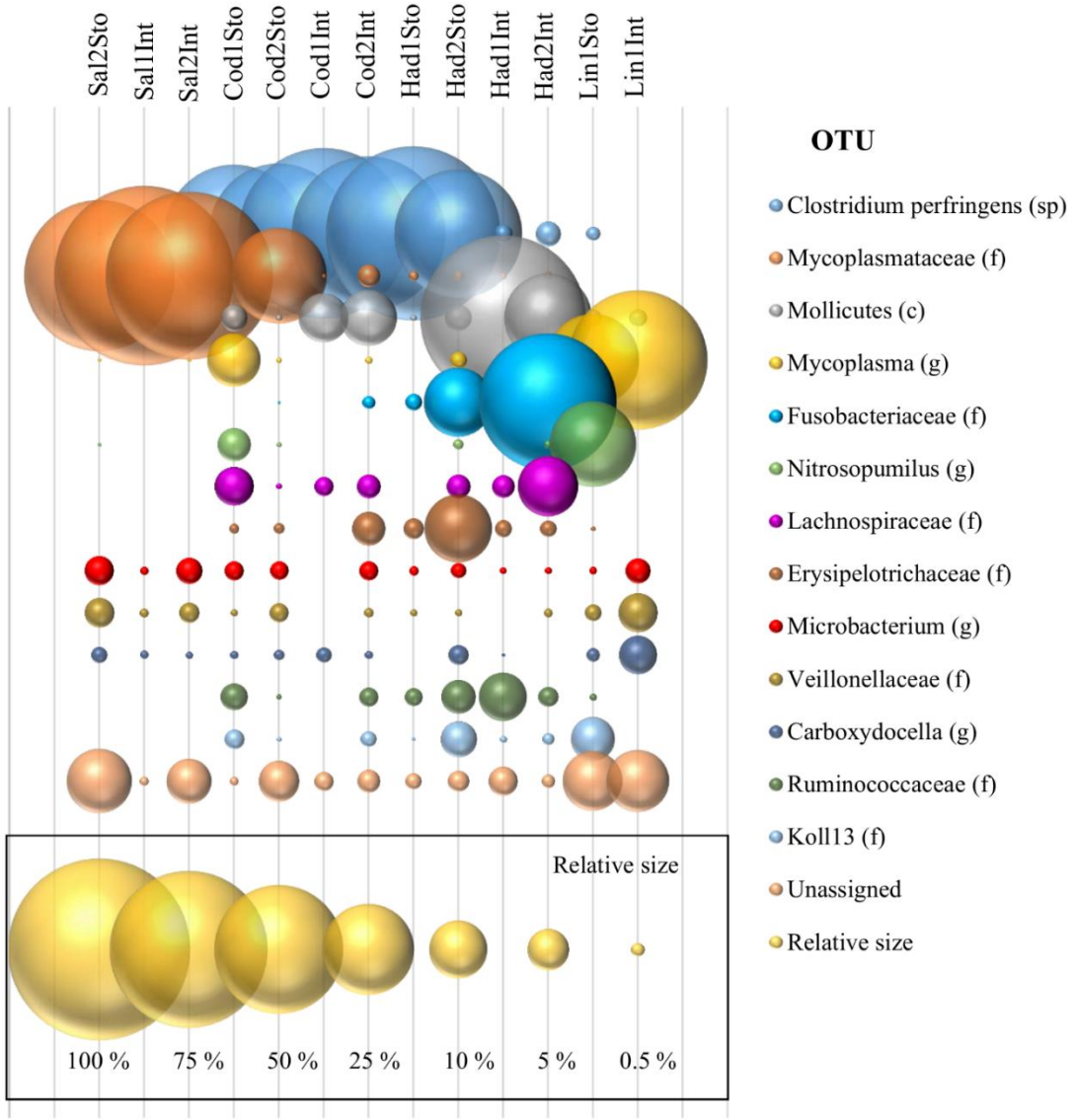

Figure 3. OTU bubble heatmap of stomach and intestinal samples from *Salmo salar, Gadus morhua, Melanogrammus aeglefinus,* and *Molva molva.* Bubble heatmap of OTUs with a minimum of 0.7 % of the total reads. Phylogenetic level of the OTUs are indicated: class (c), family (f), genus (g), species (sp). Relative size bubbles are placed at the bottom for comparison. Sample names are abbreviations of fish species (sal = *S. salar*, cod = *G. morhua*, Had = *M. aeglefinus*, Lin = *M. molva*), individual number, and sampling site (Sto = stomach, Int = intestine).

In comparison Larsen *et al.* (2014) found ~ 31 % of the OTUs were exclusive and ~ 38 % were shared when comparing the microbial communities in the intestines of three freshwater fish species. The OTUs shared between all fish species' stomach and intestinal samples are listed in Table 2. Some of the shared microbes are part of the commensal or symbiotic intestinal microbiome e.g. *Mycoplasmataceae* (Llewellyn *et al.*, 2016), but some are potential pathogens: *Clostridium perfringens* (Uzal *et al*., 2015), *Micrococcus luteus* (Pękala *et al.*, 2018), and *Renibacterium* (Wiens, 2011). Nearly all of the top abundance OTUs, including all those dominating the bacterial community in any sample, were differently represented in the various individuals, species, and sample types (Fig. 3). The most abundant OTU in *G. morhua*, *C. perfringens*, was completely absent from all *S. salar* samples as well as the *M. molva* intestinal sample. Conversely, *Mycoplasmataceae*, the most abundant OTU in *S. salar*, was absent or detected at very low abundances in several other samples (Fig. 3).

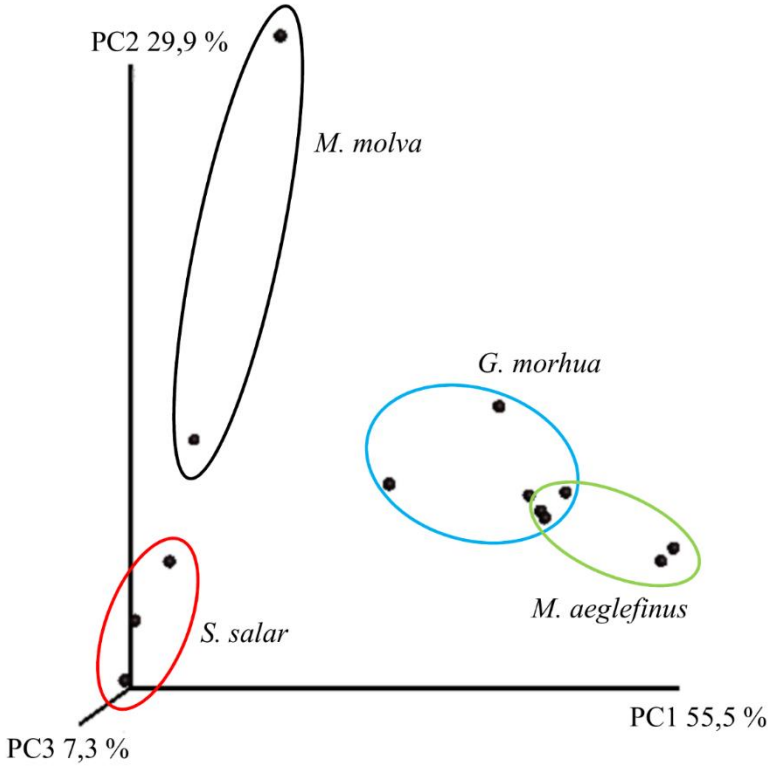

Figure 4. Principal Coordinate analysis of the microbial communities in stomach and intestinal samples from *Salmo salar, Gadus morhua, Melanogrammus aeglefinus,* and *Molva molva.* All samples were distributed in a 3-dimensional PC plot. The analysis was based on a weighted UniFrac distance matrix produced from the phylogenetic tree of each microbial community (Lozupone & Knight, 2005). Fish species are enclosed in circles; red: *S. salar*, blue: *G. morhua*, green: *M. aeglefinus*, black: *M. molva*.

Overall, of the top abundance OTUs only *Veillonellaceae, Microbacterium,* and *Carboxydocella*, which ranked as the 9th, 10th and 13th most abundant OTUs, were relatively evenly distributed among most samples.A multivariate analysis including all samples, illustrated in a 3-dimensional principal coordinate (PC) plot (Fig. 4), showed that the *S. salar* samples were clearly separated from the others along the PC1 axis, which explained 55.5 % of the variation. The two *M. molva* samples were similarly disconnected from the other samples. The *M. molva* stomach sample was located away from the other samples along the PC2 axis, explaining 29.9 % of the variation, while the intestinal *M. molva* sample was mainly separated from the rest along the PC3 axis, explaining 7.3 % of the variation (Fig. 4). The *G. morhua* and *M. aeglefinus* samples seemed more similar and slightly overlapped in the PC plot.

Discussion

The study revealed that the bacterial communities in the stomach and intestines of the four fish species examined contained several bacteria capable of gelatinase and collagenase activity, potentially affecting fillet quality if left in the abdominal cavity post-slaughter. There was a high relative content of *Clostridium perfringens* in several samples. *C. perfringens* is highly capable of producing collagenases (Harrington, 1996; Zhang *et al.*, 2015). Another bacterial genus, *Streptococcus*, found in stomach and intestines of all fish species investigated, is also capable of producing collagenases (Harrington, 1996). In addition, the genus *Mycoplasma* within the family *Mycoplasmataceae*, is capable of gelatinolytic activity (Chekalowski *et al.*, 1973). *Mycoplasma* was mainly detected in the *M. molva* samples and *Mycoplasmataceae* in the *S. salar* samples. A recent study by Medvecky *et al.* (2018) also revealed the high potential of *Bacteroidetes*, which in our study was detected in all fish by qPCR, to produce collagenases, hyaluronidases, heparinases, and chondroitinases. Thus, it seems that fish intestines commonly contain bacteria capable of producing enzymes that degrade extracellular matrix components, and could affect the fillet quality in case of inadvertent contamination.

To our knowledge, no previous analyses have been made of the microbiome of *M. aeglefinus* and *M. molva* and therefore no comparisons with historical data were possible for these two fish species. However, the distribution of phyla detected in samples from all four fish species (Fig. 1) had certain interesting features, which were a bit different from what is usually reported for fish microbiomes. Firstly, the abundance of Proteobacteria detected in this study was surprisingly low. Proteobacteria

are very often the most prominent phylum in fish microbiome (Givens, 2012; Xing *et al.*, 2013; Xia *et al.*, 2014; Llewellyn *et al.*, 2016). In this study, Proteobacteria were detected in all four fish species and sample types but at a low level throughout. The abundance of Firmicutes in both *G. morhua* individuals and one of the *M. aeglefinus* individuals was unusually high (Fig. 4), even though Firmicutes often is a prominent phylum also in other fish microbiome studies (Givens, 2012; Xia *et al.*, 2014; Ye *et al.*, 2014). The overwhelming presence of the Firmicutes species *C. perfringens* in *G. morhua* and partly *M. aeglefinus* is surprising and interesting because this bacterial species is often used as an indicator of sewage pollution in environmental samples (Edwards *et al.*, 1998; Hughes & Thompson, 2004) and is a potential pathogen (Uzal *et al.*, 2015). As the wild fish were caught close to shore, they might have been feeding on the bottom close to an outlet of some kind, which has caused an enrichment of *C. perfringens*. *C. perfringens* has previously been detected, but not quantified, by PCR methods in fecal samples from *G. morhua* caught along the northern Norwegian coast (Aschfalk & Müller, 2002). In that study, *C. perfringens* was detected in nearly 40 % of the sampled individuals, and all of these bacteria were alpha toxin positive (Aschfalk & Müller, 2002), indicating the potential for pathogenic activity. *C. perfringens* has also been determined by Next Generation Sequencing of intestinal samples of *G. morhua* from the Oslo fjord, Norway (Star *et al.*, 2013). Star *et al.* (2013) determined lower relative amounts compared with the present data. However, while we analyzed samples directly from the catch, Star *et al*. (2013) kept the fish in controlled conditions without feed for seven to twelve days before sampling. *C. perfringens* has also been detected in several Eastern African fish-scale eating cichlid fish species (Baldo *et al.*, 2015). It was suggested that the clostridial enzyme activities, including the collagenases, was important for the digestion of the fish scales (Baldo *et al.*, 2015). *C. perfringens* is otherwise seldom reported as part of the intestinal microbiome of marine fishes. *G. morhua* and *M. aeglefinus* both partly feed on benthic crustaceans (Du Buit, 1982; Jiang & Jørgensen, 1996; Magnussen, 2011). As *C. perfringens* is commonly present and thrives in marine sediment (Davies *et al.*, 1995; Lisle *et al.*, 2004), these fish species are more likely to ingest significant amounts of *C. perfringens* compared to *M. molva*, which feeds only on fish [Faroe Marine Research Institute, unpublished data, P. Steingrund, personal communication, May 2016] and thus does not ingest the same amount of sediment even though it also is a bottom dweller. The farmed *S. salar* was nearly devoid of these bacteria, possibly because they live closer to the surface and feed on manufactured pellets. It is also interesting that the very high representation of *C. perfringens* in the *M. aeglefinus* stomach (71 %) was not reflected in the intestines (1.3 %). One

possible explanation could be that the relative amount of ingested bacteria detected in the stomach and intestines depends on the time passed between feeding and sampling because these high concentrations of ingested bacteria may not persist in the digestive system of some fish (Schmidt *et al.,* 2015; Yan *et al.,* 2016) due to species-specific differences in the intestinal environment (Sullam *et al.,* 2015). *Lachnospiraceae* was also detected in *G. morhua* and *M. aeglefinus* only (Fig. 2), and can also be used as an indicator for fecal contamination of water (McLellan *et al.,* 2013; Newton *et al.,* 2011) as it is a normal microbiome constituent in humans (García-Aljaro *et al.,* 2018) as well as some other mammals and is found at comparatively low concentrations in seawater (Meehan & Beiko, 2014). Future studies designed to determine the content of *C. perfringens* and other potential pollutants at similar coastal areas as well as the content in marine fish living there would be of great interest.

Several potential pathogens were detected in addition to *C. perfringens*, some shared between all species and others not. *Erysipelotrichaceae*, a family containing several opportunistic pathogens of fish (Verbarg *et al.,* 2013) was detected in both *G. morhua* and *M. aeglefinus*, while either absent or nearly so in the samples from the other two fish species (Fig. 2). The most notable of the shared potential pathogens detected was the Actinobacteria genus *Renibacterium*. The only known species is *Renibacterium salmoninarum*, which causes bacterial kidney disease, otherwise known as BKD, in Salmonidae species (Sanders & Fryer, 1980). It is interesting that this bacterium, or a very closely related one, was present in both stomach and intestines of all four fish species, but in modest levels. It might therefore be assumed that it was a harmless part of the intestinal microbiome. This is consistent with the findings of others (Llewellyn *et al.,* 2014) that certain circumstances need to change before bacterial pathogens in the fish microbiome somehow develop and become a cause of disease. Learning more of the microbiome of both farmed fish and wild fish inhabiting the same area increases the possibility of distinguishing between a natural, healthy state and a microbiome in dysbiosis due to stress or disease as long-term stress is a well-known inducer of disease in aquaculture (Olsen & Hellberg, 2011). It is also known from the human microbiome that the distinction between commensal and pathogenic bacteria depends more on the host's ability to resist infection than on the bacteria itself (Cogen *et al.,* 2008; De Steenhuijsen Piters & Bogaert, 2016). The other shared Actinobacteria are regularly detected in various environmental samples (Jensen & Lauro, 2008).

Another interesting finding was that Tenericutes was detected at high relative abundances in the three fish species, *S. salar, M. molva,* and *M. aeglefinus*. Tenericutes has previously been detected

as a dominant phylum in *S. salar* intestines (Llewellyn *et al.*, 2016) but seldomly so in other fish species (Egerton *et al.*, 2018), and has not been identified as part of the intestinal core microbiota in other studies of fish microbiome (Givens, 2012; Llewellyn *et al.*, 2014). The abundant presence of a single bacterial species from the *Mycoplasmataceae* family in the *S. salar* samples could possibly be caused by the homogenous environment in which the farmed *S. salar* lives and their uniform diet in the form of manufactured feed pellets. Other studies have also detected high levels of microbial species from the family *Mycoplasmataceae* in both farmed and wild *S. salar* (Holben *et al.*, 2002; Llewellyn *et al.*, 2016), including our recent and independent study, where we used salmon from another source and partly also other laboratory protocols (Jacobsen *et al.*, submitted). It is also worth noticing that unique Tenericutes species dominated in each of *S. salar*, *M. aeglefinus*, and *M. molva* (Fig. 1). The Tenericutes detected in *M. molva* and *M. aeglefinus* were not defined as *Mycoplasmataceae* as in the *S. salar* samples, but as the genus *Mycoplasma* and class Mollicutes, respectively. This suggests that Tenericutes might be well represented with various bacterial species in the microbiome of marine fish in certain areas or surroundings. The fact that each individual fish or species was dominated by one of these three Tenericutes bacteria and not a mixture of them, might suggest a competitive element between the bacteria and/or an effect of different diets. The limitations of the sample sizes in this study certainly have to be taken into account along with the intra-species differences between individuals. However, it is interesting that there were such clear differences between species and further research could clarify if there truly are distinguishing features in the distribution of Tenericutes and if this can be related to species-specific and/or feed-related influence. It has previously been indicated that host phylogeny and genetics can affect the microbiome composition in fish (Sullam *et al.*, 2015).

The high abundance OTUs were in most cases unevenly distributed between individuals and species (Fig. 3). This is in contrast to the previously mentioned study of *I. punctatus*, *M. salmoides*, and *L. macrochirus* (Larsen *et al.* 2014), where all top abundance OTUs containing > 98 % of the reads were shared between all species. Another studies including Asian silver carp *Hypophthalmichthys molitrix* (V. 1844) and gizzard shad *Dorosoma cepedianum* (L. 1818) (Ye *et al.* 2014) had results more similar to this study and found numerous OTUs with large quantitative differences between the fish species. However, the differences found here seem comparatively large. Some OTUs present in huge numbers in some samples were completely absent in others. The large variation in the OTU distribution means that a diversity comparison between samples, illustrated in 3-dimensional PC plots, separated the different fish species fairly well (Fig. 4). The small number of

samples obviously limits the strength of the data, but the analysis never-the-less gives an interesting suggestion of the inter-species microbiome relationships, which could be further investigated. The fact that the farmed *S. salar* differentiates from the others might be expected due to the difference in feed and habitat, but the *G. morhua*, *M. aeglefinus* and *M. molva* individuals were caught simultaneously at the same locality, they are all bottom dwellers although they vary a bit in their presence further up in the water column, their prey species overlap even though they also feed on different prey [Faroe Marine Research Institute, unpublished data, P. Steingrund, personal communication, May 2016]. *G. morhua* and *M. aeglefinus* are also closely related species, belonging to the same family (*Gadidae*), while *M. molva* is a bit more distantly related to them, belonging to the same order (*Gadiformes*). Together with the high percentage (61.2 %) of detected bacteria from the intestinal samples that were exclusive to one fish species (Fig. 2), it suggests that the water environment itself has limited influence on the composition of the microbial community in the digestive system, also indicated by previous research focusing more in detail on these issues (Sullam *et al.,* 2012; Miyake *et al.,* 2015; Schmidt *et al.,* 2015). It is also possible that the composition is continuously fluctuating, perhaps differently for the various fish species, in response to the immediate feeding behavior. Investigating the microbiome of these fish species further might contribute to the discussion about the influence of phylogeny, feeding habits, and environment on the microbiome and also enhance the potential of screening these fish species' microbiome for obtaining an estimate of the stock's health status and/or response to environmental changes.

All in all, this initial screening of the microbiome of four marine fish species of economic importance has presented a microbial composition and diversification between species worth of further investigation. There were several potential pathogens and sewage indicators among the high abundance bacteria or those shared between all species. Among these were *Clostridium perfringens, Lachnospiraceae, Erysipelotrichaceae, Micrococcus luteus* and *Renibacterium*. Also bacteria potentially capable of affecting fillet quality if left in the abdominal cavity post-slaughter were detected. These include *C. perfringens*, Bacteroidetes and *Mycoplasma.* Larger screenings of these marine coastal fish species would be advantageous in order to establish a basic knowledge of their normal microbiome in relation to their environment and to estimate the consequences of potential bacterial pathogens and pollutants on fish health and quality and to what degree these bacteria are a commensal part of the fish microbiome.

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Availability of data and materials

The metagenomics sequence data supporting the conclusions of this article is available in the NCBI Sequence Read Archive database, study accession no.: SRP076157.

Supporting Information

Figure 1S. Rarefaction curves for all samples based on observed OTUs

Authors' contributions

ÁJ and SOM designed the study. ÁJ performed the laboratory work and metagenomics data analysis. SOM and JE supervised the study. ÁJ wrote the manuscript. SOM and JE edited the manuscript. All authors read and approved the final manuscript

Competing interests

The authors declare that they have no competing interests.

References

Abid, A., Davies, S. J., Waines, P., Emery, M., Castex, M., Gioacchini, G., Carnevali, O., Bickerdike, R., Romero, J. & Merrifield, D. L. (2013) Dietary symbiotic application modulates Atlantic salmon (*Salmo salar*) intestinal microbial communities and intestinal immunity. *Fish and Shellfish Immunology* **35**, 1948-1956. doi: 10.1016/j.fsi.2013.09.039

Aschfalk, A. & Müller, W. (2002) *Clostridium perfringens* toxin types from wild-caught Atlantic cod (*Gadus morhua L.*), determined by PCR and ELISA. *Canadian Journal of Microbiology* **48**, 365-368.

Baldo, L., Riera, J. L., Tooming-Klunderud, A., Albà, M. M. & Salzburg, W. (2015) Gut microbiota dynamics during dietary shift in Eastern Africa cichlid fishes. *PLoS ONE*. **10**, e0127462. doi: 10.1371/journal.pone.0127462.

Caporaso, J. G., Kuczynski, J., Stombauch, J., Bittinger, K., Bushman, F. D., Costello, E. K., Fierer, N., Gonzalez Pena, A., Goodrich, J. K., Gordon, J. I., Huttley, G. A., Kelley, S. T., Knights, D., Koenig, J. E., Ley, R. E., Lozupone, C. A., McDonald, D., Muegge, B. D., Pirrung, M., Reeder, J., Sevinsky, J. R., Turnbaugh, P.J., Walters, W. A., Widmann, J., Yatsunenko, T., Zaneveld, J. & Knight, R. (2010a) QIIME allows analysis of high-throughput community sequencing data. *Nature Methods* **7**(5), 335-336. doi: 10.1038/nmeth.f.303

Caporaso, J. G., Bittinger, K., Bushman, F. D., DeSantis, T. Z., Andersen, G. L. & Knight, R. (2010b) PyNAST: a flexible tool for aligning sequences to template alignment. *Bioinformatics* **26**(2), 266-267. doi: 10.1093/bioinformatics/btp636

Chao, A. (1984) Non-parametric estimation of the number of classes in a population. *Scandinavian Journal of Statistics* **11**, 265-270.

Cogen, A. L., Nizet, V. & Gallo, R. L. (2008) Skin microbiota: a source of disease or defence? *Yhe British Journal of Dermatology* **158**(3), 442-445. doi: 10.1111/j.1365-2133.2008.08437.x

Davies, C. M., Long, J. A. H., Donald, M. & Ashbolt, N. J. (1995) Survival of fecal microorganisms in marine and freshwater sediments. *Applied and Environmental Microbiology* **61**(5), 1888-1896.

De Steenhuijsen Piters, W. A. A. & Bogaert, D. (2016) Unraveling the molecular mechanisms underlying the nasopharyngeal bacterial community structure. *mBIO*. **7**(1), e00009-16. doi: 10.1128/mBio.00009-16

Dehler, C., Secombes, C. J. & Martin, S. A. M. (2017) Seawater transfer alters the intestinal microbiota profiles of Atlantic salmon (*Salmo salar* L.). *Scientific Reports* **7**: 13877. doi.org/10.1038/s41598-017-13249-8DO

DeLong, E. F. (1992) Archaea in coastal marine environments. *Proceeding of the National Academy of Sciences of the United States of Americs* **89**, 5685-5689.

Du Buit, M. H. (1982) Essai sur la predation la morue (*Gadus morhua*, L.), l'eglefin (*Melanogrammus aeglefinus* (L.)) et du lieu noir (*Pollachius virens* (L.)) aux Faeroe. *Cybium* **6**(3), 3-19.

Dulski, T., Zakęś, Z., Ciesielski, S. (2018) Characterization of the gut microbiota in early life stages of pikeperch *Sander lucioperca*. *Journal of Fish Biology* **92**(1), 94-104. doi: 10.1111/jfb.13496

Edgar, R. C. (2010) Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* **26**(19), 2460-2461. doi: 10.1093/bioinformatics/btq461

Edwards, D. E., McFeters, G. A. & Venkatesan, M. I. (1998) Distribution of *Clostridium perfringens* and fecal sterols in a benthic coastal marine environment influenced by the sewage outfall from McMurdo Station, Antarctica. *Applied and Environmental Microbiology* **64**(7), 2596-2600.

Egerton, S., Culloty, S., Whooley, J., Stanton, C., Ross, R. P. (2018) The gut microbiota of marine fish. *Frontiers in Microbiologt* 9:873. doi: 10.3389/fmicb.2018.00873.

Faith, D. P. (1992) Conservation evaluation and phylogenetic diversity. *Biological Conservation* **61**, 1-10. doi.org/10.1016/0006-3207(92)91201-3

Fierer, N., Jackson, J. A., Vilgalys, R. & Jackson, R. B. (2005) Assessment of soil microbial community structure by use of taxon-specific quantitative PCR assays. *Applied and Environmental Microbiology* **71**(7), 4117-4120. doi: 10.1128/AEM.71.7.4117-4120.2005

Francis, C. A., Roberts, K. J., Beman, J. M., Santoro, A. E., Oakley, B. B. (2005) Ubiquity and diversity of ammonia-oxidizing archaea in water columns and sediments of the ocean. *Proceedings of the National Academy of Sciences of the United States of America* **102**(41), 14683-14688. doi.org/10.1073//pnas.0506625102

Fredriksson, N. J., Hermansson, M. & Wilén, B.-M. (2013) The choice of PCR primers has great impact on assessments of bacterial community diversity and dynamics in a wastewater treatment plant. *PLoS ONE* **8**(10), e76431. doi: 10.1371/journal.pone.0076431

García-Aljaro, C., Blanch, A. R., Campos, C., Jofre, J., Lucena, F. (2018) Pathogens, fecal indicators and human-specific microbial source-tracking markers in seawage. *Journal of Applied Microbiology.* doi: 10.1111/jam.14112.

Gevers, D., Cohan, F. M., Lawrence, J. G., Spratt, B. G., Coenye, T., Feil, E. J., Stackebrandt, E., Van de Peer, Y., Vandamme, P., Thompson, F. L. & Swings, J. (2005) Re-evaluating prokaryotic species. *Nature Reviews. Microbiology* **3**, 733-739. doi: 10.1038/nrmicro1236

Givens, C. E. (2012) A fish tale: Comparison of the stomach microbiome of 15 fish species and the influence of diet and temperature on its composition. *Ph.D. thesis*, University of Georgia, Georgia, USA.

Guo, X., Xia, X., Tang, R., Zhou, J., Zhao, H. & Wang, K. (2008) Development of a real-time PCR method for Firmicutes and Bacteroidetes in faeces and its application to quantify intestinal population of obese and lean pigs. *Letters in Applied Microbiology* **47**(5), 367-373. doi: 10.1111/j.1472-765X.2008.02408.x

Hanning, I. & Dias-Sanchez, S. (2015) The functionality of the gastrointestinal microbiome in non-human animals. *Microbiome* **3**, 51. doi: 10.1186/s40168-015-0113-6

Harrington, D. J. (1996) Bacterial collagenases and collagen-degrading enzymes and their potential role in human disease. *Infection and Immunity* **64**(6), 1885-1891.

Holben, W. E., Williams, P., Gilbert, M., Saarinen, M., Särkilahti, L. K. & Apajalahti, J. H. (2002) Phylogenetic analysis of intestinal microflora indicates a novel Mycoplasma phylotype in farmed and wild salmon. *Microbial Ecology* **44**(2), 175-185. doi: 10.1007/s00248-002-1011-6

Horz, H. P., Vianna, M. E., Gomes, B. P. F. A. & Conrads, G. (2005) Evaluation of universal probes and primer sets for assessing total bacterial load in clinical samples: General implications and practical use in endodontic antimicrobial therapy. *Journal of Clinical Microbiology* **43**(10), 5332-5337. doi: 10.1128/JCM.43.10.5332-5337.2005

Hovda, M. B., Lunestad, B. T., Fontanillas, R. & Rosnes, J. T. (2007) Molecular characterisation of the intestinal microbiota of farmed Atlantic salmon (*Salmo salar L.*). *Aquaculture* **272**, 581-588. doi.org/10.1016/j.aquaculture.2007.08.045

Hughes, K. A. & Thompson, A. (2004) Distribution of sewage pollution around a maritime Antarctic research station indicated by faecal coliforms, *Clostridium perfringens* and faecal sterol markers. *Environmental Pollution* **127**, 315-321.

Jacobs, A., Doran, C., Murray, D. S., Duffill Telsnig, J., Laskowski, K. L., Jones, N. A. R., Auer, S. K. & Præbel, K. (2018) On the challenges and opportunities facing fish biology: a discussion of five key knowledge gaps. *Journal of Fish Biology* **92**, 690-698. doi.org/10.1111/jfb.13545

Jacobsen, Á., Joensen, H. & Eysturskarð, J. (2017) Gaping and loss of fillet firmness in farmed salmon (*Salmo salar* L.) closely correlated with post-slaughter cleaning of the abdominal cavity. *Aquaculture Research* **48**(1), 321-331. doi: 10.1111/are.12884

Jensen, P. R. & Lauro, F. M. (2008) An assessment of actinobacterial diversity in the marine environment. *Antonie Van Leeuwenhoek* **94**(1), 51-62. doi: 10.1007s10482-008-9239-x

Jiang, W. & Jørgensen , T. (1996) The diet of haddock (*Melanogrammus aeglefinus* L.) in the Barents Sea during the period 1984-1991. *ICES Journal of Marine Science* **53**, 11-21. doi.org/10.1006/jmsc.1996.0002

Kuczynski, J., Stombaugh, J., Walters, W. A., Gonzáles, A., Caporaso, G. & Knight, R. (2011) Using QIIME to analyze 16S rRNA gene sequences from microbial communities. *Current Protocols in Bioinformatics* **36**(1), 10.7.1-10.7.20. doi:10.1002/0471250953.bi1007s36

Larsen, A. M., Mohammed, H. H. & Arias, C. R. (2014) Characterization of the gut microbiota of three commercially valuable warmwater fish species. *Journal of Applied Microbiology* **116**, 1396-1404. doi: 10.1111/jam.12475

Lisle, J. T., Smith, J. J., Edwards, D. D. & McFeters, G. A. (2004) Occurrence of microbial indicators and *Clostridium perfringens* in wastewater, water column samples, sediments, drinking water, and Weddell seal feces collected at McMurdo Station, Antarctica. *Applied and Environmental Microbiology* **70**(12), 7269-7276. doi: 10.1128/AEM.70.12.7269-7276.2004

Livak, K. J. & Schmittgen, T. D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(Delta Delta C(T)) method. *Methods* **25**(4), 402-408. doi: 10.1006/meth.2001.1262

Llewellyn, M. S., Boutin, S., Hoseinifar, S. H. & Derome, N. (2014) Teleost microbiomes: the state of the art in their characterization, manipulation and importance in aquaculture and fisheries. *Frontiers in Microbiology* **5**, 207. doi.org/10.3389/fmicb.2014.00207

Llewellyn, M. S., McGinnity, P., Dionne, M., Letourneau, Thonier, F., Carvalho, G. R., Creer, S. & Derome, N. (2016) The biogeography of the Alantic salmon (*Salmo salar*) gut microbiome. *The ISME Journal* **6**, 1280-1284. doi: 10.1038/ismej.2015.189

Lozupone, C. & Knight, R. (2005) UniFrac: a new phylogenetic method for comparing microbial communities. *Applied and Environmental Microbiology* **71**(12), 8228-8235. doi: 10.1128/AEM.71.12.8228-8235.2005

Maeda, H., Fujimoto, C., Haruki, Y., Maeda, T., Kokeguchi, S., Petelin, M., Arai, H., Tanimoto, I., Nishimura, F. & Takashiba, S. (2003) Quantitative real-time PCR using TaqMan and SYBR Green for *Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia, tetQ* gene and total bacteria. *FEMS Imunnology and Medical Microbiology* **39**, 81-86. doi: 10.1016/S0928-8244(03)00224-4

Magnussen, E. (2011) Food and feeding habits of cod (*Gadus morhua*) on the Faroe Bank. *ICES Journal of Marine Science* **68**(9), 1909-1917. doi.org/10.1093/icesjms/fsr104

Mao, D.-P., Zhou, Q., Chen, C.-Y. & Quan, Z.-X. (2012) Coverage evaluation of universal bacterial primers using the metagenomics datasets. *BMC Microbiology* **12**, 66. doi: 10.1186/1471-2180-12-66

McLellan, S. L., Newton, R. J., Vandawalle, J. L., Shanks, O. C., Huse, S. M., Eren, A. M., Sogin, M. L. (2013) Sewage reflects the distribution of human faecal Lachnospraceae. *Environmental Microbiology* **15**(8), 2213-2228. doi: 10.1111/1462-2920.12092

Medvecky, M., Cejkova, D., Polansky, O., Karasova, D., Kubasova, T., Cizek, A. & Rychlik, I. (2018) Whole genome sequencing and function prediction of 133 gut anaerobes isolated from chicken caecum in pure cultures. *BMC Genomics* **19**, 561. doi.org/10.1186/s12864-018-4959-4

Meehan, C. J., Beiko, R. G. (2014) A phylogenomic view of ecological specialization in the Lachnospiraceae, a family of digestive tract-associated bacteria. *Genome Biology and Evolution* **6**(3), 703-713. doi: 10.1093/gbe/evu050

Miyake, S., Ngugi, D. K. & Stingl, U. (2015) Diet strongly influences the gut microbiota of surgeonfishes. *Molecular Ecology* **24**, 656-672. doi: 10.1111/mec.13050

Morales, S. E., Cosart, T. F., Johnson, J. V. & Holben, W. E. (2011) Extensive phylogenetic analysis of a soil bacterial community illustrates extreme taxon evenness and the effects of amplicon length, degree of coverage, and DNA fractionation on classification and ecological parameters. In *Handbook of Molecular Microbial Ecology II: Metagenomics in different habitats*, (de Bruijn FJ, editor), pp. 151-163. Hoboken, New Jersey: Wiley-Blackwell.

Nadkarni, M. A., Martin, F. E., Jacques, N. A. & Hunter, N. (2002) Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primer set. *Microbiology* **148**, 257-266. doi: 10.1099/00221287-148-1-257

Newton, R. J., Vandawalle, J. L., Borchardt, M. A., Gorelick, M. H., McLellan, S. L. (2011) Lachnospiraceae and Bacteroidales alternative fecal indicators reveal chronic human seawage contamition in urban harbour. *Applied and Environmental Microbiology* **77**, 6972-6981. doi: 10.1128/AEM.05480-11

Nayak, S. K. (2010) Probiotics and immunity: A fish perspective. *Fish & Shellfish Immunology* **29,** 2-14. doi: 10.1016/j.fsi.2010.02.017

Ni, J., Yan, Q., Yu, Y. & Zhang, T. (2014) Factors influencing the grass carp stomach microbiome and its effect on metabolism. *FEMS Microbiology Ecology* **87**, 704-714. doi: 10.1111/1574-6941.12256

Olsen, A. B. & Hellberg, H. (2011) The health situation in Norwegian aquaculture 2011. *Norwegian Veterinary Institute*. ISSN:1893-1480.

Olsen, I. (2014) The family *Fusobacteriaceae*. In *The Procaryotes*, (Rosenberg, E., DeLong, E. F., Lory, S., Stackebrandt, E., Thompson, F., editors), pp. 109-132. Heidelberg, Germany: Springer.

Parris, D. J., Brooker, R. M., Morgan, M. A., Dixson, D. L., Stewart, F. J. (2016) Whole gut microbiome composition of damselfish and cardinalfish before and after reef settlement. *PeerJ* 4:e2412. doi.org/1077.17/peerj.2412

Pękala, A., Paździor, E., Antychowicz, J., Bernad, A., Głowacka, H., Więcek, B. Niemczuk, W. (2018) *Kocuria rhizophila* and *Micrococcus luteus* as emerging oppertunist pathogens in brown trout (*Salmo trutta* Linnaeus, 1758) and rainbow trout (*Onchorhynchus mykiss* Walbaum, 1792). *Aquaculture* **486**, 285-289. doi: 10.1016/j.aquaculture.2017.12.028

Price, M. N., Dehal, P. S. & Arkin, A. P. (2009) FastTree: computing large minimum evolution trees with profiles instead of a disance matrix. *Molecular Biology and Evolution* **26**(7), 1641-1650. doi.org/10.1093/molbev/msp077

Rakoff-Nahoum, S., Foster, K. R. & Comstock, L. E. (2016) The evolution of cooperation within the gut microbiota. *Nature* **533**, 255-259. doi.org/10.1038/nature17626

Reveco, F. E., Øverland, M., Romarheim, O. H. & Mydland, L. T. (2014) Intestinal bacterial community structure differs between healthy and inflamed intestines in Atlantic salmon (*Salmo salar L.*). *Aquaculture* **420-421**, 262-269. doi.org/10.1016/j.aquaculture.2013.11.007

Ringø, E., Zhou, Z., Vecino, J. L. G., Wadsworth, S., Romero, J., Krogdahl, Å., Olsen, R. E., Dimitriglou, A., Foey, A., Davies, S., Owen, M., Lauzon, H. L., Martinsen, L. L., De Schryver, P., Bossier, P., Sperstad, S. & Merrifield, D. L. (2016) Effect of dietary components on the gut microbiota of aquatic animals. A never-ending story? *Aquaculture Nutrition* **22**, 219-282. doi.org/10.1111/anu.12346

Sanders, J. E., Fryer, J. L. (1980) *Renibacterium salmoninarum* gen. nov., sp. nov., the causative agent of bacterial kidney disease in salmonid fishes. *International Journal of Systematic Bacteriology* **30**(2), 496-502.

Schmidt, V. T., Smith, K. F., Melvin, D. W. & Amaral-Zettler, L. A. (2015) Community assembly of a euryhaline fish microbiome during salinity acclimation. *Molecular Ecology* **24**, 2537-2550. doi: 10.1111/mec.13177

Star, B., Haverkamp, T. H. A., Jentoft, S., Jakobsen, K. S. (2013) Next generation sequencing shows high variation of the intestinal microbial species composition in Atlantic cod caught at a single location. *BMC Microbiology* **13**, 248. doi.org/10.1186/1471-2180-13-248

Sullam, K. E., Essinger, S. D., Lozupone, C. A., O'Connor, M. P., Rosen, G. L., Knight, R., Kilham, S. S. & Russel, J. A. (2012) Environmental and ecological factors that shape the stomach

bacterial communities of fish: a meta-analysis. *Molecular Ecology* **21**, 3363-3378. doi: 10.1111/j.1365-294X.2012.05552.x

Sullam, K. E., Rubin, B. E. R., Dalton, C. M., Kilham, S. S., Flecker, A. S. & Russel, J. A. (2015) Divergence across diet, time and populations rules out parallel evolution in the gut microbiomes of Trinidadian guppies. *The ISME Journal* **9**, 1508-1522. doi: 10.1038/ismej.2014.231

Vázquez-Baeza, Y., Pirrung, M., Gonzales, A. & Knight, R. (2013) EMPeror: a tool for visualizing high-throughput microbial community data. *Gigascience* **2**(1), doi: 10.1186/2047-217X-2-16.

Ventura, M., Canchaya, C., Tauch, A., Chandra, G., Fitzgerald, G. F., Chater, K. F. & van Sinderen, D. (2007) Genomics of Actinobacteria: Tracing the evolutionary history of an ancient phylum. *Microbiology and Molecular Biology Reviews* **71**(3), 495–548. doi: 10.1128/MMBR.00005-07

Verbarg, S., Göker, M., Scheuner, C., Schumann, P., Stackebrandt, E. (2013) The Families Erysipelotrichaceae emend., Coprobacillaceae fam. Nov., and Turicibacteraceae fam. Nov.. In *The Procaryotes: Firmicutes and Tenericutes* (Rosenberg, E., DeLong, E. F., Lory, S., Stackebrandt, E., Thompson, F., editors), pp. 79-105. Springer.

Wiens, G. D. (2011) Bacterial kidney disease (*Renibacterium salmoninarum*). In *Fish Diseases and Disorders, Volume 3: Viral, Bacterial, and Fungal Infections, 2nd Edition,* (Woo, P. T. K., Bruno, D. V., editors), pp. 338-374. CABI.

Wu, S., Wang, G., Angert, E. R., Wang, W., Li, W. & Zou, H. (2012) Composition, diversity, and origin of the bacterial community in grass carp intestine. *PLoS ONE* **7**(2), e30440. doi.org/10.1371/journal.pone0030440

Uzal, F. A., McClane, B. A., Cheung, J. K., Theoret, J., Garcia, J. P., Moore, R. J. & Rood, J. I. (2015) Animal models to study the pathogenesis of human and animal *Clostridium perfringens* infections. *Veterinary Microbiology* **179**(1-2), 23-33. doi: 10.1016/j.vetmic.2015.02.013

Xia, J. H., Lin, G., Fu, G. H., Wan, Z. Y., Lee, M., Wang, L., Liu, X. J., Yue, G. H. (2014) The intestinal microbiome of fish under starvation. *BMC Genomics* **15,** 266. doi.org/10.1186/1471-2164-15-266

Xing, M., Hou, Z., Yuan, J., Liu, Y., Qu, Y. & Liu, B. (2013) Taxonomic and functional metagenomics profiling of gastrointestinal tract microbiome of the farmed adult turbot

(*Scophthalmus maximus)*. *FEMS Microbiology Ecology* **86**, 432-443. doi: 10.1111/1574-6941.12174

Yan, Q., Li, J., Yu, Y., Wang, J., He, Z., Van Nostrand, J. D., Kempher, M. L., Wu, L., Wang, Y., Liao, L., Li, X., Wu, S., Ni, J., Wang, C. & Zhou, J. (2016) Environmental filtering decreases with fish development for the assembly of gut microbiota. *Environmental Microbiology* **18**(12), 4739-4754. doi:10.1111/1462-2920.13365.

Ye, L., Amberg, J., Chapman, D., Gaikowski, M. & Liu, W.-T. (2014) Fish stomach microbiota analysis differentiates physiology and behavior of invasive Asian carp and indigenous American fish. *The ISME Journal* **8,** 541-551. doi: 10.1038/ismej.2013.181

Zhan, A., Xiong, W., He, S. & MacIsaac, H. J. (2014) Influence of artifact removal on rare species recovery in natural complex communities using high-throughput sequencing. *PLoS ONE* **9**(5), e96928. doi.org/10.1371/journal.pone.0096928

Zhang, Y., Ran, L., Li, C. & Chen, X. (2015) Diversity, structures, and collagen-degrading mechanisms of bacterial collagenolytic proteases. *Applied and Environmental Microbiology* **81**(18), 6098-6107. doi: 10.1128/AEM.00883-15

4.5 Jacobsen Á, Mikalsen S-O, Salter I. Comparison of custom made QIIME and standard pipeline SILVAngs bioinformatics for investigation of microbial communities in farmed salmon (*Salmo salar* L.) post-slaughter. *In prep.*

Jacobsen Á[1*], Mikalsen S-O[2], Salter I[3]

[1]Fiskaaling, Aquaculture Research Station of the Faroes, við Áir 11, Hvalvík, Faroe Islands

[2]Department of Science and Technology, University of the Faroe Islands, Vestara Bryggja 15, Tórshavn, Faroe Islands

[3]Faroe Marine Research Institute, Nóatún 1, Tórshavn, Faroe Islands

*Corresponding author

Abstract

The analysis of next-generation sequencing data for investigating microbial communities is complex. Decisions have to be made about a wide range of analysis options that all affect the results, such as which programs, settings, filters, and thresholds to use. In recent years standard bioinformatics pipelines have been introduced, which should make comparisons between studies easier. However, any one standard pipeline might not be optimal for all sample types and studies. Deciding which methods to use and estimating the implications of these choices can be complicated. This is especially true for studies of a relatively rare nature that have fewer similar studies for comparison. Here we report a comparison of the results from a microbiome study originally analyzed using a custom made method in QIIME with a subsequent analysis in the standard bioinformatics pipeline, SILVAngs. The comparison showed large differences in the alpha diversity estimates Chao1, as SILVAngs had much higher values and a different sample type pattern. The taxonomic assignments were similar at family level, but at higher resolution, there were differences.

Introduction

The application of next-generation sequencing techniques for metagenomics analyses of microbiomes has become standard in most research areas entailing investigation of bacterial community structure and diversity. The targeted 16S rRNA gene sequencing is a method often applied to investigations of bacterial communities, as it still has some advantages over other methods such as shotgun sequencing (Mukherjee *et al.* 2017). However, the continually growing list of options for analyzing the vast amount of data produced by next-generation sequencing has led to an increasing demand for clarification of how to relate to the large variation of bioinformatics pipelines (Siegwald et al. 2017; Angers-Loustau et al. 2018; D'Argenio 2018; Lambert et al. 2018). Some argue that clustering-first pipelines are more reliable than assignment-first pipelines for analyzing microbiomes that are not well described, because assignment-first pipelines are highly sensitive to the quality of databases and their annotations (Siegwald et al. 2017). Siegwald *et al.* (2017) estimate that the choice of reference database has a bigger impact on correct taxa identification for assignment-first pipelines while the richness estimation is more impacted for clustering-first pipelines. However, Edgar (2018) has demonstrated that the taxonomic annotation of sequences in two commonly used reference databases, SILVA (https://www.arb-silva.de/) and

Greengenes (http://greengenes.secondgenome.com), is inherently flawed (Edgar 2018). Edgar (2018) found large disagreements between taxonomic annotations by Greengenes and SILVA of the same sequences. Most disagreements were caused by unassigned sequences in one but not the other database and some were conflicting taxon names. However, other comparisons of microbiome analysis using different bioinformatics pipelines have shown that even though there are differences the same biological conclusion can be made (Allali et al. 2017).

The *in silico* and compiled dataset efficiency analyses of various pipelines and reference databases are valuable for identifying strengths and flaws in the various procedures (Almeida *et al.* 2018). However, the true implications on the analysis of environmental samples are still difficult to predict. Comparisons using real environmental samples of course have the downside of not having a correct answer for comparison. Such comparisons are nevertheless important for evaluating the true impact of using different bioinformatics pipelines on the biological meaning derived from the results, as manufactured samples never are as complex as the real thing. Recently, a comparison of bioinformatics pipelines applied to ruminal samples was performed (López-Garcia *et al*. 2018), which gained valuable knowledge to researchers in that area. In a recent study, we analyzed the microbial communities in the post-slaughter environment of farmed Atlantic salmon (*Salmo salar* L.) using next-generation sequencing (Jacobsen *et al.* 2019). The bioinformatic analysis in QIIME (Caporaso *et al.* 2010b) was custom made, using Greengenes as the reference database for the 16S rRNA sequences. Because of the current uncertainties in optimal bioinformatic analysis procedures, we compared our results with the results from the more standardized bioinformatics pipeline SILVA rRNA gene database project, SILVAngs 1.3 (Quast *et al.* 2013). Here we present some of the observations from that comparison, which we consider to be of value for researchers considering the best approach.

Materials and Methods

Details about samples, DNA extraction, sequencing protocol and data analysis from the original work are described in Jacobsen *et al.* (2019). In short, 96 samples from the post-slaughter environment of farmed Atlantic salmon were sequenced on an Illumina MiSeq instrument. The library preparation was largely performed according to the "16S Metagenomic Sequencing Library Preparation" document (Part # 15044223 Rev. B). The amplicon PCR was run using the recommended primers covering the V3 and V4 regions (selected from Klindworth *et al.* 2013). The

Index PCR was performed using primers from the Illumina Nextera XT Index kit. The sequencing kit was a MiSeq Reagent Kit v3 (600 cycles) producing paired-end reads. The samples were from the salmon intestines, skin mucus, abdominal cavity and storage ice. The SILVAngs analysis was based on the same paired-end reads as the original custom made analysis in QIIME.

Table 1. Main differences in analysis methods.

	QIIME	SILVAngs
Merging reads and aligning	SeqPrep	SINA,
	PyNAST, 75 % identity	50 % identity
Dereplication and clustering	De novo UCLUST,	De novo CD-HIT,
	97 % min identity	98 % min identity
Reference database	Greengenes	blastn, SILVA SSU
Unassigned threshold	90 %	93 %

For both pipelines, the minimum sequence quality was 30 and rejection rates due to ambiguities or homopolymers were similar (2 % for SILVAngs and 3 % for QIIME). However, in the SILVAngs pipeline, the SILVA Incremental Aligner (SINA v. 1.2.10) (Pruesse *et al.* 2012) was used for sequence alignment against the SILVA SSU rRNA SEED with a minimum of 50 % alignment identity. In contrast, SeqPrep (https://github.com/jstjohn/SeqPrep) was used in QIIME for merging paired end reads and alignment was performed by PyNAST (Caporaso *et al.* 2010) with a minimum of 75 % alignment identity. Dereplication and clustering was performed with CD-Hit (v. 3.1.2) (Li and Godzik 2006) in the SILVAngs pipeline with a minimum identity of 98% and with UCLUST (Edgar 2010) in QIIME using a minimum identity of 97%. The custom analysis used the Greengenes database (McDonald et al. 2012) as reference in a de novo OTU picking method. The SILVAngs protocol used BLAST (version 2.2.30+) with standard settings (Camacho et al. 2009) searching against the SILVA SSU ref dataset (release 128; Yilmaz et al. 2014). Minimum similarity required before rendering "unassigned" was 93% ((% sequence identity + % alignment coverage)/2) for the SILVAngs pipeline and 90% for the custom analysis. An overview of the main differences in the two bioinformatics analysis is displayed in Table 1. Further details on the original analysis can be found in Jacobsen *et al.* (2019).

Results and discussion

In samples with approximately 20,000 reads and higher the SILVAngs pipeline gave a higher number of reads per sample after joining of paired-end reads and quality filtration than the custom approach in QIIME. (Figure 1). In these samples, the SINA method in SILVAngs resulted in between approximately 26 % - 38 % more reads than the counterpart method in QIIME. The main reason for this might be the different percentage of 50% and 75% alignment identity required by SINA and PyNAST, respectively. On the other hand, samples with lower amount of reads more often retained a higher proportion of the reads with the custom analysis in QIIME. Total number of reads retained after removal of OTUs with less than five reads was 6.201.743 for the SILVAngs pipeline compared to 4.814.980 for the custom analysis in QIIME.

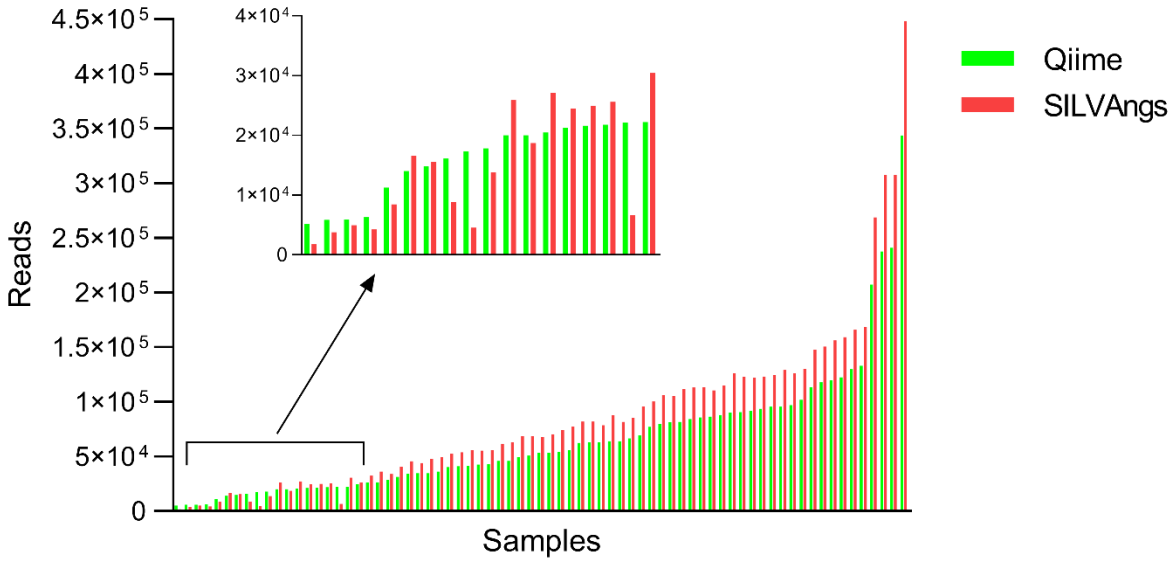

Figure 1. Reads per sample for QIIME and SILVAngs.

The CD-Hit method used in the SILVAngs approach for dereplication and clustering resulted in 484,761 OTUs. UCLUST, which was used in the custom analysis in QIIME, produced 8,464 OTUs. UCLUST and CD-Hit are both heuristic clustering methods using greedy list removal algorithms with defined thresholds. They compare sequences to the representative sequence of already established clusters. If the similarity is within the defined threshold of the OTU centroid the sequence is added to the cluster, otherwise the sequence will be the centroid of a new cluster. Both methods also apply a prefilter to reduce the amounts of pairwise sequence alignments. This speeds

up the process but increases the risk of false negatives, which results in a loss of clustering sensitivity. This means that there is a tendency for too many clusters to be formed. Comparisons often show the two methods have similar clustering sensitivity (Li *et al.* 2012; Steinegger and Söding 2018). However, others state that the methods differ in how the sequences are sorted and assigned to clusters (Chen *et al.* 2013). Edgar (2010) found that there are disagreements on determination of percentage identity between the methods and that CD-HIT produces more false negatives than UCLUST. In addition, a study by Chen *et al.* (2016) showed that the number of redundant sequences increases significantly when the identity threshold is over 90 % compared to lower thresholds, and that a tolerance level of 2 %, as applied in this study, resulted in a redundancy ratio of nearly 20 % for an identity threshold of 98 %. Based on their studies, others have also recommended relatively low clustering threshold (~97 %) in order to avoid gross overestimation of diversity (Huse *et al*. 2010; Kunin *et al.* 2010).

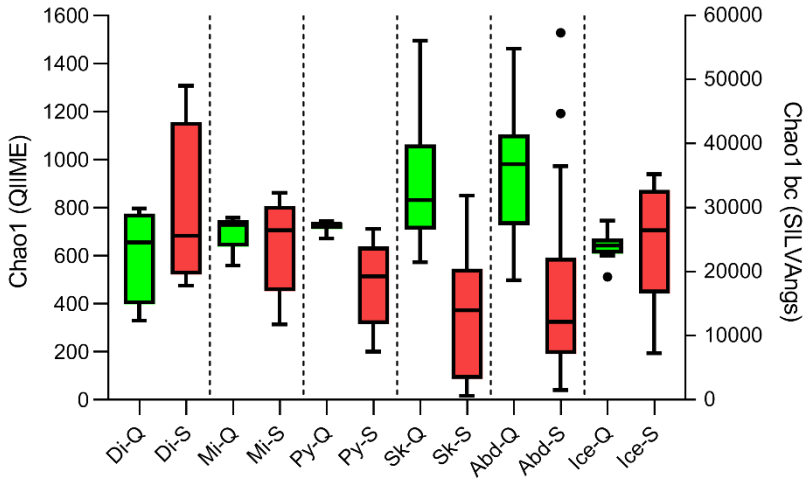

Figure 2. Chao1 estimates for the different sample types. Chao1 estimates from QIIME (Q) in green and SILVAngs (S) in red for different sample types from salmon post-slaughter environment; Di: distal intestine, Mi: mid intestine, Py: pyloric caeca, Sk: skin mucus, Abd: abdominal cavity, Ice: storage ice. Boxes show median line and 25th and 75th percentile. Whiskers show maximum and minimum values (Tukey) and dots represent outliers.

This is consistent with the large number of OTUs detected in the SILVAngs pipeline, which used a threshold of 98 %. Along with the extra ~ 1.4 million reads, these differences might explain the

much higher number of OTUs detected using the SILVAngs approach compared to the analysis in QIIME. Other factors might also potentially have had an influence. A consequence of the large difference in OTU abundances is reflected in the alpha diversity estimates. The SILVAngs pipeline reported the bias corrected Chao1, applicable to very large sample sizes or when rare species are nearly homogeneous in terms of detection probabilities (Chao and Chiu 2016) while in the custom analysis the original Chao1 estimate (Chao 1984) was used. According to Siegwald *et al*. (2017), QIIME overestimates Chao1 at the family level but at the genus level, it is very accurate because the richness overestimation is compensated by the drop in resolution at the genus level. The high OTU abundance in the SILVAngs analysis resulted in much higher levels of the alpha diversity metric Chao1 for all sample types than the Chao1 estimates from the analysis in QIIME (Figure 2). In addition, comparisons between the sample types showed different patterns for QIIME and for SILVAngs, which can be caused by spurious formations of OTUs in the SILVAngs pipeline. For instance, 195,771 (or 40.4 %) of the 484,761 OTUs were assigned to the same genus, *Mycoplasma*, which was highly abundant in the intestinal samples. The alpha diversity metrics are used as illustrators of various characteristics of the microbial community and potential connections to environmental factors. Therefore, these results show that comparisons of samples within a study can infer different biological meanings depending on the methods used even though they are in well-established bioinformatics pipelines.

a) b)

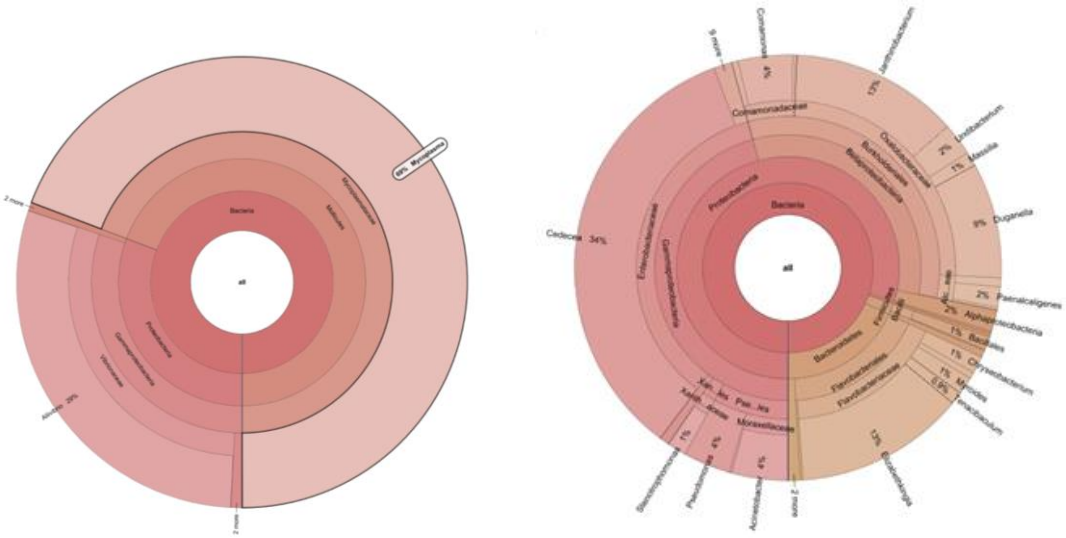

Figure 3. Representative krona plots. Krona plots from the SILVAngs analysis of (a) distal intestinal sample and (b) skin mucus sample.

The taxonomic classification resulted in a much diminished difference between QIIME and SILVAngs. The analysis in QIIME resulted in 362 taxonomic identities while SILVAngs determined 521. The alpha diversity from SILVAngs based on OTUs (Figure 2) did not seem compatible with the taxonomic paths displayed in the various tissue types. Representative krona plots from the SILVAngs analysis are illustrated in Figure 3. This suggests that the real alpha diversity is more in line with that determined in QIIME.

The relative distribution of the high abundance bacteria in the various sample types showed that the microbial community was overall very similar for both analyses when the resolution was at the family level (figure 4). This is consistent with a comparison of sequence clustering tools performed by Kopylova *et al.* (2016) that showed different clustering results converge to the same conclusions with longer, higher quality reads and deep sequencing.

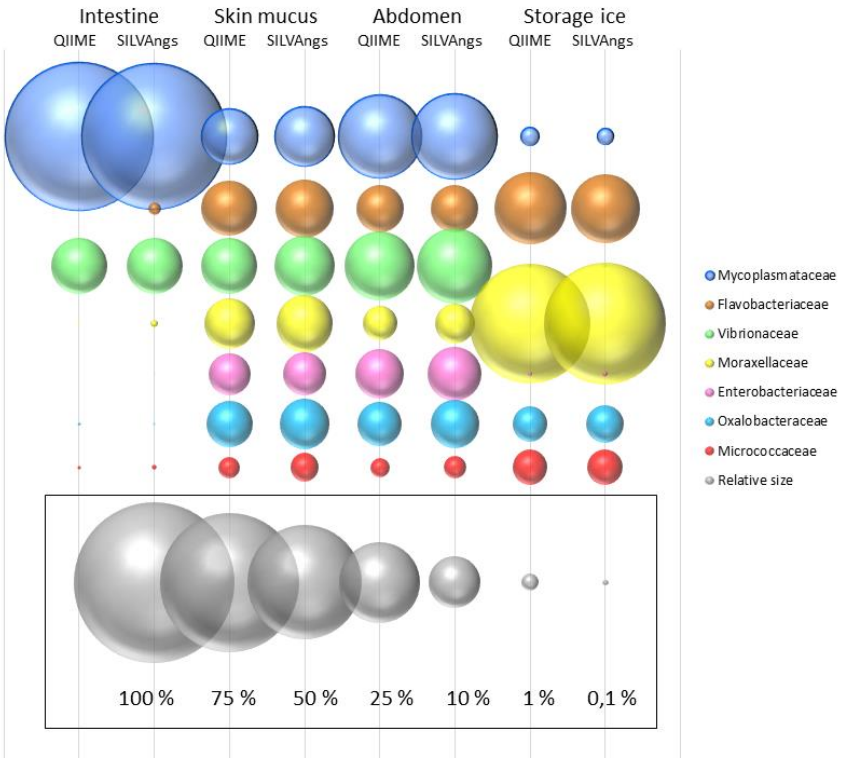

Figure 4. Relative abundances of selected families in the various sample types.

However, at a higher resolution, there were some instances of different taxonomic annotations (Figure 5). SILVAngs sometimes assigned most or all OTUs to genera within these families while

QIIME defined most at family level only. QIIME defined many reads to the families *Mycoplasmataceae* and *Enterobacteriaceae* without further resolution while SILVAngs defined these families' reads as *Mycoplasma* and *Cedecea*, respectively (Figure 5). In other families such as *Moraxellaceae*, *Oxalobacteraceae* and *Micrococcaceae*, the reads were in part assigned to genera by QIIME. Some genera in these families, such as *Acinetobacter* and *Janthinobacterium*, were at similar relative abundance as defined by SILVAngs while other genera were not. In addition, the QIIME analysis had many unassigned reads while SILVAngs had few. An analysis by Bokulich *et al.* (2018) showed that UCLUST and BLAST+ both performed well in novel taxon classification, meaning they did not over-, under-, or misclassify much. However, performances varied greatly depending on the parameter settings. In addition, these differences might be caused by the application of different databases in the two analyses, namely Greengenes and SILVA, consistent with the findings of Edgar (2018).

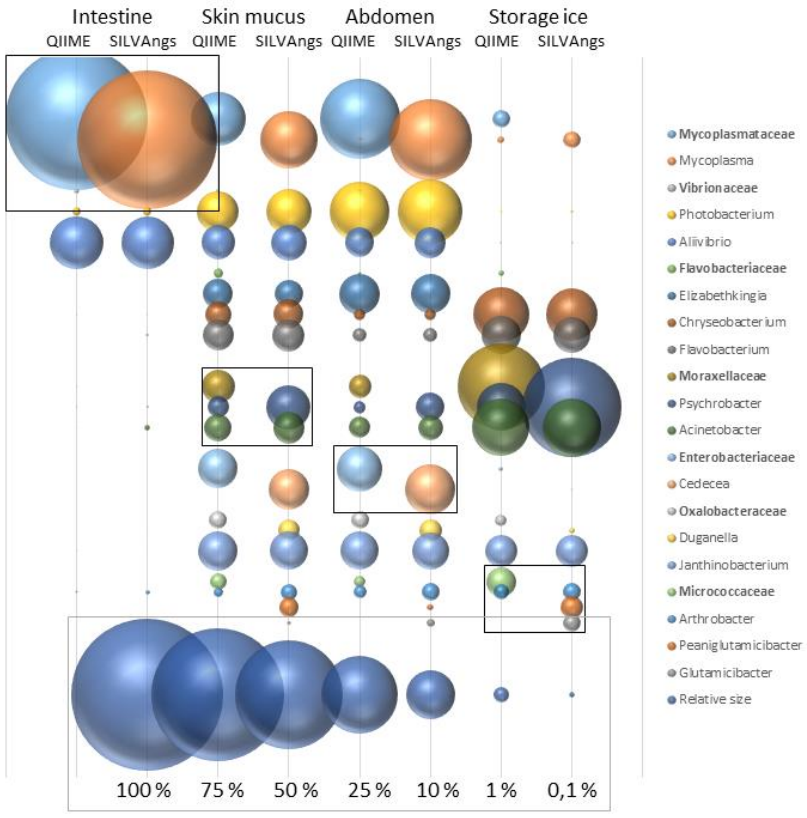

Figure 5. Illustration of similarities and differences in taxonomic resolution. The black boxes highlight some of the differences.

At the present Greengenes is the smallest of the commonly used databases. The latest version of Greengenes is from May 2013. Since then, the SILVA SSU database has almost doubled in size. In

this comparison, the SILVA SSU database was generally better able to define the microbial community at a higher resolution. However, some comparative analysis have demonstrated that Greengenes is more accurate in defining the metagenome from certain environments such as the ocean (Almeida *et al.* 2018) although others have revealed taxonomic annotation errors in Greengenes, which could affect analysis of marine samples (Lydon and Lipp 2018).

Overall, both bioinformatic analyses showed strengths and weaknesses. The clustering, OTU formation and alpha diversity estimation seemed more reliable in QIIME. However, the taxonomic resolution was better in the SILVAngs pipeline. The impact of the different interpretations from these bioinformatic pipelines could be large, if results are accepted without criticism. However, with some insight into the various aspects of these analyses it is possible to identify potential pitfalls and perform alternative analyses for comparison.

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References

Allali I, Arnold JW, Roach J, Cadenas MB, Butz N, Hassan HM, Koci M, Ballou A, Ali R, Azcarate-Peril MA (2017). A comparison of sequencing platforms and bioinformatics pipelines for compositional analysis of the gut microbiome. BMC Microbiology 17:194 doi:10.1186/s12866-017-1101-8.

Almeida A, Mitchell AL, Tarkowska A, Finn RD (2018). Benchmarking taxonomic assignments based on 16S rRNA gene profiling of the microbiota from commonly sampled environments. GigaScience 7, 1-10 doi: 10.1093/gigascience/giy054.

Angers-Loustau A, Petrillo M, Bengtsson-Palme J, Berendonk T, Blais B, Chan K-G, Coque TM, Hammer P, Heß, Kagkli DM, Krumbiegel C, Lanza VF, Madec J-Y, Naas T, O'Grady J, Paracchini V, Rossen JWA, Ruppé E, Vamathevan J, Venturi V, Van den Eede G (2018). The challenges of designing a benchmark strategy for bioinformatics pipelines in the identification of antimicrobial

resistance determinants using next generation sequencing technologies [version 2; referees:2 approved]. F1000Research 7:459 doi.org/10.12688/f1000research.14509.1.

Bokulich NA, Kaehler BD, Rideout JR, Dillon M, Bolyen E, Knight R, Huttley GA, Caporaso JG (2018). Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. *Microbiome* **6**, 90. Doi.org/10.1186/s40168-018-0470-z.

Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP (2016). DADA2: High resolution sample inference from Illumina amplicon data. *Nature Methods* **13(**7), 581-583. Doi: 10.1038/nmeth.3869.

Camacho C, Coulouris G, Avagyan V, Ma N, Papadopoulos J, Bealer K, Madden T (2009). BLAST+: architecture and applications. *BMC Bioinformatics 10(1):421.* Doi: 10.1186/1471-2105-10-421.

Caporaso JG, Bittinger K, Bushman FD, DeSantis TZ, Andersen GL, Knight R (2010a). PyNAST: a flexible tool for aligning sequences to a template alignment. *Bioinformatics* 26(2):266-267 doi: 10.1093/bioinformatics/btp636.

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Peña AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knight SD, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widman J, Yatsunenko T, Zaneweld J, Knight R (2010b)

Chao, A (1984). Non-parametric estimation of the number of classes in a population. *Scandinavian Journal of Statistics*, 11:265-270

Chao A, Chiu CH (2016). Species richness: estimation and comparison. Wiley StatsRef: Statistics Refernece Online, 1-26.

Chen C, Wan Y, Lei Y, Zobel J, Verspoor K (2016). Evaluation of CD-HIT for constructing non-redundant databases. *IEEE International Conference on Bioinformatics and Biomedicine.* Doi: 10.1109/BIBM.2016.7822604.

Chen W, Zhang CK, Cheng Y, Zhang S, Zhao H (2013). A comparison of methods for clustering 16S rRNA sequences into OTUs. *PloS ONE* 8(8): e70837. Doi: 10.1371/journal.pone.0070837.

D’argenio V (2018). Human microbiome acquisition and bioinformatic challenges in metagenomic studies. International Journal of Molecular Sciences 19:383. Doi:10.3390/ijms19020383.

Edgar RC (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26, 2460-2461. Doi: 10.1093/bioinformatics/btq461.

Edgar RC (2018). Taxonomy annotation and guide tree errors in 16S rRNA databases. PeerJ 6:e5030. doi.org/10.7717/peerj.5030.

Huse SM, Welch DM, Morrison HG, Sogin ML (2010). Ironing out the wrinkles in the rare biosphere through improved OUT clustering. *Environmental Microbiology* **12**, 1889-1898.

Jacobsen Á, Mikalsen S-O, Joensen H, Eysturskarð J (2019). Composition and dynamics of the bacterial communities present in the post-slaughter environment of farmed Atlantic salmon (*Salmo salar* L.) and correlations to gelatin degrading activity. *PeerJ* 7:e7040. Doi.org/10.7717/peerj.7040.

Kopylova E, Navas-Molina JA, Mercier C, Xu ZZ, Mahé F, He Y, Zhou H-W, Rognes T, Caporaso JG, Knight R (2016). Open-source sequence clustering methods improve the state of the art. *mSystems* **1**(1), e00003-15. Doi: 10.1128/mSystems.00003-15.

Kunin V, Engelbrektson A, Ochman H, Hugenholtz P (2010). Wrinkles in the rare biosphere: pyrosequencing errors can lead to artificial inflation of diversity estimates. *Environmental Microbiology* **12**, 118-123.

Lambert C, Braxton C, Charlebois RL, Deyati A, Duncan P, La Neve F, Malicki HD, Ribrioux S, Rozelle DK, Michaels B, Sun Wenping, Yang Z, Khan AS (2018).Considerations for optimization of the high-throughput sequencing bioinformatics pipelines for virus detection. Viruses 10:528. doi:10.3390/v10100528.

Li W, Fu L, Niu B, Wu S, Wooley J (2012). Ultrafast clustering algorithms for metagenomic sequence analysis. *Briefings in Bioinformatics* **13**(6), 656-668.Doi: 10.1093/bib/bbs035.

López-Garcia A, Pineda-Quiroga C, Atxaerandio R, Pérez A, Hernández I, Garcia-Rodríguez A, González-Recio O (2018): Comparison of Mothur and QIIME for the analysis of rumen microbiota composition based on 16S rRNA amplicon sequences. Frontiers in Microbiology 9:3010. doi: 10.3389/fmicb.2018.03010.

Lydon KA, Lipp EK (2018). Taxonomic annotation errors incorrectly assign the family Pseudoalteromonadaceae to the order Vibrionales in Greengenes: Implications for microbial community assessments. PeerJ 6:e5248 doi.org/10.7717/peerj.5248.

McDonald D, Price MN, Goodrich J, Nawrocki EP, DeSantis TZ, Probst A, Andersen GL, Knight R, Hugenholtz P. An improved greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. ISME J. 2012; 6(3):610–8.

Mukherjee et al. 2017: Bioinformatic approaches including predictive metagenomic profiling reveal characteristics of bacterial response to petroleum hydrocarbon contamination in diverse environments.

Quest C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO (2013). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Research* 41(D1):D590-D596 doi: 10.1093/nar/gks1219.

Siegwald L, Touzet H, Lemoine Y, Hot D, Audebert C, Caboche S (2017). Assessment of common and emerging bioinformatics pipelines for targeted metagenomics. PLoS ONE 12(1):e0169563 doi:10.1371/journal.pone.0169563.

Steinegger M, Söding J (2018). Clustering huge protein sequence sets in linear time. *Nature Communications* **9**, 2542. Doi: 10.1038/s41467-018-04964-5.

Yilmaz P, Parfrey LW, Yarza P, Gerken J, Pruesse E, Quast C, Schweer T, Peplies J, Ludwig W, Glöckner FO. The SILVA and "All-species Living Tree Project (LTP)" taxonomic frameworks. Nucleic Acids Res. 2014; 42(Database issue):643–8.

5. Discussion

In this project, we have shed light on the complex mechanism of gaping in salmon fillets. Although gaping in salmon fillets is not a new research area, we have used methods not previously applied to this issue and considered potential factors not previously studied in this context.

5.1 Cleaning of the abdominal cavity

Our first objective was to establish if cleaning of the abdominal cavity post-mortem and/or temperature from slaughter to processing had any influence on the occurrence or severity of gaping in the salmon fillets. To our knowledge, there have not been any previous reports of studies on salmon fillet gaping that have included potential effects of blood and intestinal fluids left in the abdominal cavity during cold storage. In addition, it has not been customary to mention the cleaning method or intensity of the salmon post-slaughter in experimental setups analyzing gaping in fillets. Possibly, the cleaning of the abdominal cavity might not have been considered of sufficient importance. Alternatively, a consistent and proper cleaning of the abdominal cavity as part of the standardization of the sampling procedure has been thought to be too obvious to mention. However, the cleaning of the abdominal cavity has been mentioned as a general recommendation in relation to bacterial load (Erkan 2007; Borderias and Sánchez-Alonso 2011). The results of our study clearly showed that the cleaning of the abdominal cavity post-mortem had a significant effect on the occurrence and severity of gaping in the fillets (Paper I). The correlation detected between bodily fluids left in in the abdominal cavity of salmon during cold storage and gaping in the fillets implies that it might be beneficial to investigate the post-mortem activity of enzymes present in the blood or intestinal fluids of the salmon. Establishing potential effects of various enzymes in the blood and intestinal fluids on the extracellular matrix components could promote the understanding of the mechanisms leading to gaping fillets. It would also be of importance to resolve if potential ECM degrading enzymes in the intestinal fluids from the salmon are inherent or derived from bacteria.

5.1.1 Standardization

It is important to notice that the correlation detected between cleaning of the abdominal cavity post-slaughter and gaping does not exclude the influence of other variables, most of which are mentioned in the Introduction section. It is certainly possible that several factors can induce gaping in salmon fillets, potentially interacting with each other. For example, the immune cells in the blood named polymorphonuclear leucocytes contain metalloproteinase 8 (MMP-8), which is a collagenase that preferentially degrades collagen I (Gangbar *et al*. 1990; Herman *et al*. 2001). Stress causes an

increase in the number and activity of polymorphonuclear leucocytes (Suzuki *et al.* 1999; Sasagawa *et al.* 1993), and coagulation of blood induces release and activation of the MMP-8 (Jung 2008; Manello 2008). Bacteria potentially derived from the intestinal fluids can possibly also activate host MMPs or inactivate proteinase inhibitors (Lähteenmäki *et al.* 2001) in addition to having collagenases of their own with broad substrate specificity (Duarte *et al.* 2016). In addition, physical handling can release many of the autolytic enzymes that are compartmentalized in discrete membrane bound packages (in particular in the lysosomes) which may disrupt when subjected to physical abuse (Huss 1995). Such a multifactorial induction of gaping is quite possible and might explain why results from various studies, potentially not including all relevant co-factors, often seem to give conflicting results. For this reason, we would recommend to include cleaning of the abdominal cavity in the methods description of experimental setups and sampling to ensure standardization so that it does not influence the results of other parameters tested. More importantly, the results demonstrate that the salmon farming industry might benefit from optimizing the post-slaughter cleaning of the abdominal cavity. There seems to be a large variation in the prevalence of gaping in the salmon farming industry (Andersen et al. 1994; Pittman 2013), and implementing a higher level of scrutiny to the cleaning of the abdominal cavity post-slaughter might reduce loss and wastage as well as increase profitability in the salmon farming industry.

5.2 Extracellular matrix

In gaping tissue, the location of tearing is in the extracellular matrix. However, there is no consensus on which molecules and mechanisms are involved. Collagens, PGs, and GAGs are intrinsic parts of the ECM and are likely somehow involved in the detachment of the myocommata from the myomere. Differences in molecular structure might reveal something about potentially inherent differences, forms of degradation and causes of degradation related to gaping. However, collagens, PGs, and GAGs are complex and versatile compounds. Hussel et al. (2018) stated, "The interstitial matrix and basement membrane are highly complex structures and understanding the effect of changes in any particular components abundance or distribution is a difficult task". With this in mind, we chose to investigate glycosaminoglycans because they are intricate parts of many ECM components and structures and molecular changes might have an impact of relevance (Paper II). We chose to analyze the GAGs by mass spectrometry because such analysis have the potential to reveal detailed information about structural diversity and abundance of GAGs in various tissue types (Shi and Zaia 2009). To our knowledge, no previous studies have analyzed

glycosaminoglycans by mass spectrometry in relation to gaping in salmon fillets. Nor have any other ECM components been analyzed by mass spectrometry in relation to gaping in fillets.

5.2.1 Estimation of chain length

Various methods have been applied for the estimation of GAG chain lengths. Deligny *et al*. (2016) used 35S labeled HS, gel chromatography and scintillation. Veraldi *et al.* (2018) used fractionation by ultrafiltration through MWCO 10-kDa filters. Pegeot *et al.* (2015) used signal intensity of xylose residue of the linkage tetrasaccharide between the GAG and the protein core, measured by NMR, and compared to the sum of intensities of GlcNAc and GlcNS. Brown *et al.* (2007) used DMMB + agarose gel filtration chromatography (superose 6).

In this study, the estimation of the HS chain length was calculated by dividing the total amount of disaccharides with the saturated amount of disaccharides (Figure 4.1). Enzymatic cleavage of the GAG chains by lyases produces oligosaccharides with $\Delta^{4,5}$-unsaturated uronic acid residues while the non-reducing chain terminus is not cleaved and these oligosaccharides remain saturated (Zaia 2013). Estimation of the HS chain length, including only disaccharides, has previously been used in other studies (Shi and Zaia 2009), and applied here as the only HS oligosaccharides measured were disaccharides. The HS chains in the connective tissue samples were estimated to be very short in comparison with the reported common range (Kreuger *et al.* 2006). In contrast to this, the estimation of the CS chain length, which was within the reported common range (Little *et al.* 2008), was calculated by including all CS oligosaccharides measured adjusted to disaccharide units and all saturated oligosaccharides measured (Figure 4.1). Most of the oligosaccharides measured were found at low abundances, between less than 0.1 % and 5.4 % of the dp2 abundance, but the unsaturated dp3 [1,1,1,1,1] was detected at higher concentrations than the total dp2 and therefore made an impact on the chain calculations.

HS dp2 chain length estimation:

Total dp2 / sat. dp2

CS dp2 chain length estimation:

(dp2 + 0.5xdp1 + 1.5xdp3 + 2xdp4 + 2.5xdp5 + 3xdp6 + 4xdp8) / (sat. dp1 + sat. dp3 + sat. dp5)

Figure 4.1. HS and CS chain length estimation. Sat. = saturated.

A calculation based on disaccharides only, assumes a complete digestion of the GAG chains. In reality, there might be other HS oligosaccharides of various sizes present after the digestion, as was the case with CS. Therefore, an attempt to base the chain length estimation on other small oligosaccharides of the digested HS as well as the disaccharides might be worth considering in future studies. However, CS oligosaccharides were included for comparative analysis, which also made it possible to include these in the chain estimation. HS oligosaccharides were not included in the analysis because HS is more heterogeneous than CS and measurement of all HS oligosaccharides is a more comprehensive task. The short HS chain length might also be due to the action of endolytic heparanases (Lindahl and Kjellén 2013; Peterson and Liu 2012) during storage or due to other normal post-mortem processes. No differences were detected in chain length between gaping and intact tissue samples and it is therefore unlikely that complete degradation of CS or HS chains are reasons for gaping fillets.

5.2.2 HS chain composition

The difference in the HS chain composition stringency between gaping and intact samples is quite interesting, as HS within one tissue tend to have a characteristic domain structure and sulfation degree (Multhaupt and Couchman 2012). Because heparan sulfate proteoglycans have important functions for cell-ECM adhesion and basement membrane adhesion (Sarrazin *et al.* 2011), the detected differences might be relevant for the gaping issue. It is difficult to estimate whether these differences could be caused by some form of selective degradation potentially connected to inherent or bacterial enzymes in the bodily fluids left in the abdominal cavity. Alternatively, they might be inherent differences present in the tissue at slaughter. Comparisons with fresh salmon tissue samples could help determine the normal variation in HS chain structure in order to estimate the relevance of this finding.

5.2.3 Chondroitin sulfate sulfation degree

The MS analysis revealed several differences in the CS and HS compositions between intact and gaping tissue. One difference detected was in the CS dp2 distribution where gaping samples had a higher proportion of non-sulfated CS disaccharides and a lower proportion of singly and doubly sulfated CS disaccharides compared to the intact tissue samples. In addition, there was a significantly higher amount of monosulfated CS in the intact tissue samples. These results suggested the action of endosulfatases because the gaping samples had a lower CS sulfation degree without the CS chains being more degraded. To our knowledge, no eukaryotic CS endosulfatases have been identified, though these might still be discovered in the future. However, bacterial CS

endosulfatases have been detected utilizing different degradation pathways (Ulmer *et al.* 2014). CS endosulfatase-producing bacteria have been found in both the marine environment (Wang *et al.* 2015) and in human intestines (Ulmer *et al.* 2014). Based on present knowledge it is therefore possible that the CS endosulfatase activity does not originate from inherent enzymes in the blood or intestinal fluids, but rather from bacteria. However, investigations into the potential of inherent enzymes in the salmon blood and intestinal fluids might also be relevant. Alternatively, the findings of different CS dp2 compositions in gaping and intact samples are not necessarily cause and effect, but might be due to concurrent and independent events. If it is accepted that bacterial endosulfatases potentially are responsible for the lower sulfation degree in gaping tissue and that it might have an effect on the presence and severity of gaping, then one has to investigate more carefully the enzymatic activity. Mapping any potential relevant bacterial activity in more detail could enhance the understanding of the molecular mechanisms of gaping in salmon fillets. Another aspect is the overall high proportion of non-sulfated CS disaccharides detected in these samples at nearly 40 %, which is considerably different than the CS dp2 distribution detected in other fish species (Zhang *et al.* 2009; Higashi *et al.* 2015; Maccari *et al.* 2015; Pan *et al.* 2018; Vázguez *et al.* 2018). Again, comparisons with fresh salmon tissue are needed to clarify if this difference is inherent or caused by post-mortem degradation.

5.2.4 Spatial distribution of ECM components in the tissue

In order to achieve some descriptive information on the spatial distribution of selected ECM components in the tissue, immunohistochemistry analysis was performed on muscle tissue samples with and without gaping, staining with CS-4S, CS-6S and Collagen I antibodies (method description in Supplement X). The IHC analysis was originally intended to be included in Paper II, but the different approaches added too much complexity to the manuscript and the IHC results were left out for future use. We report here some observations from the analysis we consider worth noticing. Tissue sections were stained for collagen I, as this is the main component in the myocommata and for CS-4S and CS-6S because the MS analysis could not distinguish between these two forms. The CS-4S and CS-6S phenotypes infer different characteristics on the CS chain and the tissue (e.g. Bayliss *et al*. 1999; Miyata *et al.* 2012). Both CS-4S and CS-6S were abundantly present in both gaping and intact tissue. Both the endomysium and the larger connective tissue were brightly stained (Figure 4.2). No obvious difference in abundance or tissue distribution of the two CS sulfation types was apparent. In addition, there were no obvious differences between intact and

gaping tissue samples. However, CS was clearly present in the myocyte's basement membrane on both myomeres facing the myocommata.

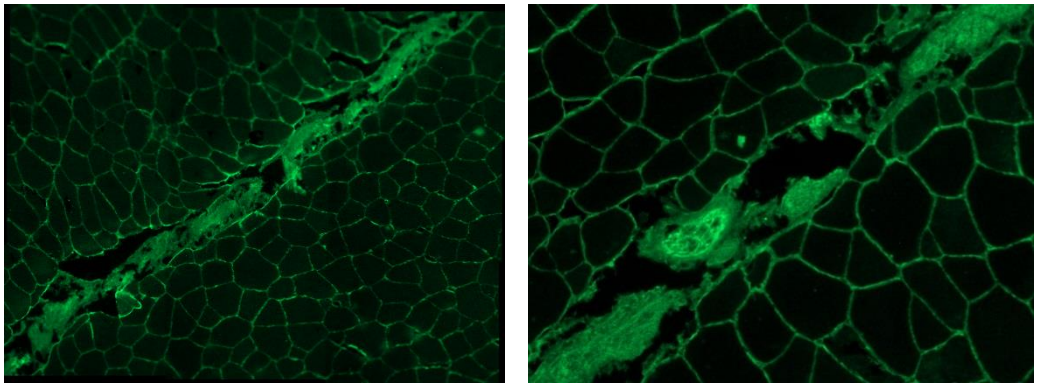

Figure 4.2. Immunofluorescence images. Intact salmon muscle tissue stained with CS-6S (a) and gaping tissue stained with CS-4S (b) antibodies. Images were processed in ImageJ. The IHC analyses were performed in collaboration with Amanda G Vang, Karen W. Sanden and Mona E. Pedersen.

In contrast, the IHC of collagen I often showed a presence in only one of the two myomere edges facing the myocommata (Figure 4.3). In places where the myocommata was detached from one of the myomeres, the myocommata itself was brightly stained with collagen I antibodies, but the myocytes not attached to the myocommata were relatively bare.

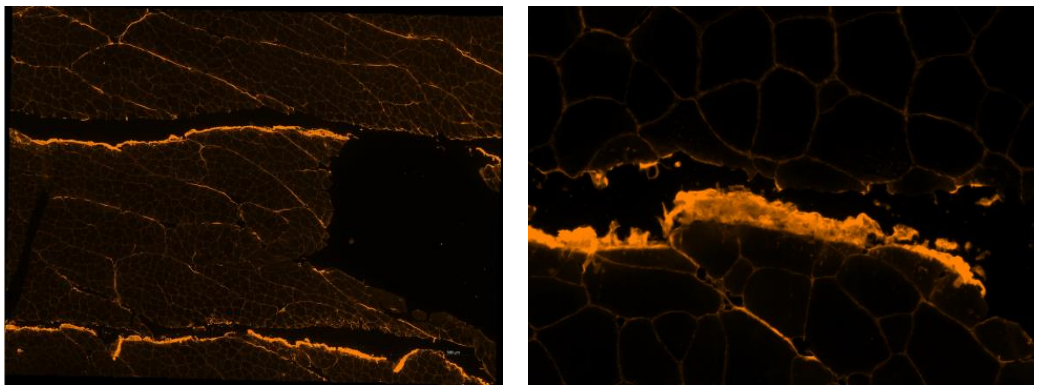

Figure 4.3. Immunofluorescence images. Salmon muscle tissue with gaping stained with collagen I antibody. Images with xx (a) and xx (b) magnification processed in ImageJ. The IHC analyses were performed in collaboration with Amanda G Vang, Karen W. Sanden and Mona E. Pedersen.

A study by Torgersen *et al.* (2014) on soft texture in salmon fillets using immunohistochemistry to analyze presence and distribution of collagen I, perlecan and aggrecan showed similar results, although their focus was on the endomysium. Soft fillets often showed lower fluorescent intensity and a detachment from one of the myocytes while still attached to the myocytes opposite. However, in our study this form of detachment was not prevalent for CS and not restricted to samples from gaping tissue. Therefore, no correlation was detected between CS-4S, CD-6S or collagen I spatial distribution in the connective tissue and gaping in the salmon fillets. Potentially, the observations mentioned could be normal post-mortem degradation unrelated to gaping or it might be a consequence of tissue handling.

5.3 Bacterial enzymes

The results from the first experimental setup with cleaning of the abdominal cavity post-slaughter and the results from the mass spectrometry analysis of CS and HS suggest that the involvement of bacterial enzymes in gaping of salmon fillets is a possibility. As mentioned earlier a few recommendations have been made to clean the abdominal cavity of salmon post-slaughter in relation to general spoilage of fillets (Erkan 2007; Borderias and Sánchez-Alonso 2011). However, to our knowledge there have not been any previous attempts to analyze the possibility of bacterial degradation in relation to gaping in salmon fillets.

The experimental setup analyzing the microbial communities in the intestines of salmon at slaughter and in the skin mucus, abdominal cavity and storage ice during cold storage confirmed this potential (Paper III). Bacterial communities detected in the intestinal samples and in the abdominal cavity during storage were generally different as was the gelatinase activity. However, when the bacterial composition in the abdominal cavity of salmon in one storage box were similar to that in intestinal samples while being different from the other abdominal cavity samples, then the gelatinase activity was also similar to that in the intestinal samples. Although these results suggest a potential involvement of intestinal fluids in gaping, there are several missing links in the hypothetical chain linking intestinal bacteria to gaping in the fillets. Further research is required to establish more precisely the changes in the ECM leading to gaping, which enzymes cause these changes, and the origin of these enzymes.

5.4 Dominating bacteria

Potential future studies investigating the possibility of specific bacteria degrading ECM components in the salmon fillets during storage, might find the bacterial family *Mycoplasmataceae* of interest. Our study found it to be highly dominant in the salmon intestines (Paper III and IV), which is consistent with the findings of others (Llewellyn *et al.* 2015; Fogarty *et al.* 2019). Bacteria within this family, in the genus *Mycoplasma*, are capable of gelatinolytic activity (Chekalowski *et al.*, 1973). However, some clarification is needed regarding the classification of OTUs as belonging to *Mycoplasmataceae* or *Mycoplasma.* In the experimental setup correlating intestinal bacterial communities to gelatinolytic activity, the dominating bacteria are classified as *Mycoplasmataceae*. The same raw sequencing data analyzed by another bioinformatic pipeline reported the dominating bacteria to be *Mycoplasma* (Paper V). This could be a discrepancy between for instance the different databases used in the different pipelines, Greengenes and SILVA. However, at the same time data from the initial pilot-study (Paper IV) showed that OTUs were assigned to both *Mycoplasmataceae* and *Mycoplasma* in Greengenes. *Mycoplasma* was mainly detected in the *M. molva* samples and *Mycoplasmataceae* in the *S. salar* samples. Other studies have also found *Mycoplasmataceae* to be dominating in salmon intestines and Llewellyn *et al.* (2015) also assigned OTUs to various genera in the *Mycoplasmataceae* family, including *Mycoplasma*. A further clarification of the influence of bioinformatic methods and databases on the assignment of OTUs to genera in the *Mycoplasmataceae* family would be a great value to further investigation into the significance of these bacteria. One option is whole genome sequencing (WGS) of microbiomes, which can enhance the understanding of composition and function of the microbiomes (Rinke *et al.* 2013; Almeida *et al.* 2019). It is more expensive and requires more computational effort than amplicon sequencing, but has enhanced species detection and has the ability to reveal more about the phylogenetic diversity and functional properties of the bacteria, including their enzymatic potential (Ranjan *et al.* 2016; Rausch *et al.* 2019).

5.4.1 Potential in the unknown

Other bacteria and enzymatic capabilities could also be of interest. A recent study by Medvecky *et al.* (2018) revealed the high potential of *Bacteroidetes,* which in our study was detected in all sample types, to produce collagenases, hyaluronidases, heparanases, and chondroitinases. This is a good representation of the potentially large variation of both bacterial species and enzymatic activity of relevance. The search for and characterization of bacteria with various enzymatic activities has been skewed towards potentially harmful or useful bacteria such as those causing

diseases and potentially dangerous spoilage or those producing beneficial enzymes for medicinal purposes. More benign environmental or intestinal bacteria, like *Bacteroidetes*, have traditionally attracted less research attention. However, with the massive increase of information from next-generation sequencing and related techniques, data from environmental and intestinal samples include the entire microbiome facilitating biomining at a larger and broader scale (Alma'abadi *et al.* 2015; Koutsandreas *et al.* 2016). In addition, WGS can determine the functional profile of various samples directly and allow the exploration of potential activity of even unknown bacteria (Rinke *et al.* 2013; Jovel *et al.* 2016). Overall, it seems that salmon intestines commonly contain bacteria capable of producing enzymes that degrade extracellular matrix components. These bacteria could potentially affect the fillet quality in case of inadvertent contamination. With the currently available techniques, future studies might reveal any such potential in more detail.

5.4.2 Reproducibility

Currently, microbiome studies can produce a massive amount of sequence data. At the same time, there is a lack of standardized data management and bioinformatic analysis. This has led to a concern about the reproducibility and replicability of such studies as well as recommendations for improvements (Poussin *et al.* 2018; Schloss 2018; Fricker *et al.* 2019). The terms reproducibility and replicability are sometimes confused, so for clarification the illustrative table from Schloss (2018) is reproduced here for guidance:

TABLE 1 Simple grid-based system for defining concepts that can be used to describe the validity of a result[a]

Methods	**Same experimental system**	**Different experimental system**
Same methods	Reproducibility	Replicability
Different methods	Robustness	Generalizability

[a]This is a generalization of the approach used by Whitaker (9), who used it to describe computational analyses

Generally speaking, we want to be able to compare results from any of our studies with other similar studies. Each study is usually based on different samples and although attempts are being made to apply standardized bioinformatic pipelines, there will most likely be differences due to updated software, reference databases etc. These comparisons are termed generalizations according to the concept system illustrated above. Testing replicability and robustness of your results can

show how reliable these are before comparing with other studies for generalization. The comparison in our study between two different bioinformatics methods performed on the same raw data was thus a test of robustness.

There are many potential obstacles in the analysis of NGS data, which can make replicability and robustness hard to achieve. However, OTU formation remains a major challenge, as there are no universal guidelines for correct OTU formation. An OTU is a cluster of similar sequence reads with a representative sequence formed with the purpose of reducing computational power needed to perform subsequent analysis. The assumption is that these clustered reads are derived from same or closely related organisms (He *et al.* 2015). However, some argue that this assumption of correlation between OTUs and species is unreliable (Stackebrandt and Ebers 2006). In addition, the instability of OTU formation due to variable sequencing depth was demonstrated early in the NGS era (Roesch *et al.* 2007) and is still an unresolved issue (He *et al.* 2015). Commonly used methods have been shown to produce many more OTUs than what is known to be present in mock communities (Kopylova *et al* 2016). In our test of robustness, the lack of OTU stability was clearly demonstrated. In the comparisons of the QIIME and SILVAngs bioinformatics (Paper V), one analysis produced 8.464 OTUs and the other 484.761 OTUs. It was not surprising that using different programs for OTU formation resulted in different number of OTUs, but the scale of difference was surprising and the effect it had on alpha diversity estimation. It is alarming that these commonly used methods applied to the same raw data could give so different results.

As an alternative, Callahan *et al.* (2017) recommended to replace the OTU as standard unit of amplicon sequencing analysis with amplicon sequence variants (ASVs) because they are reusable and reproducible in contrast to the more arbitrary nature of OTUs. Reproducibility and replicability is becoming ever more important and standard requirements in NGS studies. *De novo* OTUs are constructed by clustering reads that are sufficiently similar to one another without any external reference, the major drawback being that the result is data set dependent. Closed reference OTUs are not data set dependent but reference database dependent, as reads are clustered against a reference sequence collection. Contrary to this, ASVs have an intrinsic biological meaning. Some argue that current ASV methods provide better resolution and accuracy than OTU methods (Eren et al 2015, Callahan et al 2016). For analysis to be reproducible, the fundamental units must be reproducible, and OTUs are not. ASVs are reproducible and are not limited by different data sets or incomplete reference datasets.

Reference databases are another important factor in any chosen bioinformatics pipeline. The results from our study showed that the taxonomic assignments were relatively robust at family level. This reflects the conclusion of others (Kopylova *et al.* 2016; Allali *et al.* 2017). However, the robustness deteriorated at higher taxonomical resolution, as there were more pronounced differences at genus and species level. These differences could be due to underclassification, overclassification or the representation of relevant reference genomes. Although some databases are considered to be of better quality than others, none are ideal for all kinds of samples, and it is a general concern that reference databases need to be more comprehensive and accurate (Jovel *et al.* 2013; Breitwieser *et al.* 2017). Until these circumstances are markedly improved it is important to be aware of these challenges when analyzing your data and comparing with other studies.

5.5 Limitations and recommendations

5.5.1 Sampling

A potential limitation of the results presented in paper I is the relatively small sample size. Thirty individuals might be considered a bit low, and a future experiment might benefit from a higher number of samples. However, there are some practical issues to consider if a similar study is attempted with a larger sample size. In order to standardize as much as possible of the sampling procedure, the salmon have to be from the same pen and taken for slaughter within the same time frame. This ensures similar treatment and temperatures as well facilitating processing by the same staff under similar conditions. Therefore, the quality assessments have to be performed on the same day, because storage time needs to be consistent. In addition, the quality assessments should be performed by the same personnel in order to avoid bias. These conditions set a time limit for how many individual fish can be used in a single experimental setup. In order to improve flexibility in the experimental setup an option could be to perform a similar study with a reduced number of measurements in order to make room for more individuals being processed in a single day or replicate setups could be performed as a form of validation. The application of image analysis has also been studied (Balaban *et al.* 2011; Merkin *et al.* 2013; Pittman *et al.* 2013) and might be an option worth considering for future studies.

Although the MS analysis included a larger sample size significant differences were mainly detected when comparing intra-individual intact and gaping samples rather than when including all samples. The cause of this could to be the large individual variation in glycosaminoglycan content and structure. If this is a general issue, it might be difficult to identify potential differences in GAG

content and composition related to gaping between individuals. It could also make it difficult to determine any potential GAG thresholds related to gaping in salmon in general without a very large sample size. On the other hand, it could indicate that the effect of this degradation partly depends on each individual's connective tissue structure. Therefore, it is recommended that future studies compare intra-individual samples or have large sample sizes in order to enhance the probability of detecting potential inter-individual differences. In addition, comparisons with fresh salmon tissue could help differentiate between inherent composition and post-mortem changes.

5.5.2 Immunohistochemistry quantification

Immunohistochemistry is widely used for identification and quantification of various components in the ECM. However, standardized methods for antibody validation across research applications are not universally applied (Rickelt and Hynes 2018). As a result, the quality and consistency of data generated using antibodies can vary greatly and increasing concerns have been raised about reproducibility of scientific data using commercially available antibodies (Baker 2015; Bradbury and Pluckthun 2015). The need for improved standardization of IHC protocols has been addressed and recommendations and guidelines for IHC have been proposed (Rickelt and Hynes 2018; Weller 2018). Of most importance, every experiment should include positive and negative controls to assess performance of antibody and reagents used, ideally using a set of tissue samples with known variable expression levels of the protein of interest. In addition, there is very scarce information about the quantification process in the literature presenting IHC analysis of fish fillets. Although IHC analysis complement MS analysis well, this lack of possibilities to do validated quantitative measurements restricts the application of IHC to mainly qualitative analysis. This was consistent with the results from our study, which showed large variation within and between slides and samples. The establishment of positive controls with various fluorescent intensities for the salmon tissue type in question would require a substantial effort and this was not within the scope of this PhD project. However, making validated and quantitative IHC measurements possible would be of great value for research in salmon fillet quality issues such as gaping, because information about the spatial distribution is lost in most other analytical methods.

5.6 Concluding remarks

This study established that intestinal fluids left in the abdominal cavity of post-slaughtered salmon during cold storage are a possible cause of gaping in the salmon fillets. The results from two

experimental setups showed that firstly this increased the prevalence of gaping and secondly that this increased the potential of gelatin degrading activity in the abdominal cavity. The first experimental setup did not distinguish between the effect of blood and intestinal fluids, but the second experimental setup established a correlation between increased gelatinase activity in the abdominal cavity and the presence of intestinal fluids. However, this does not rule out the possibility of other enzymatic activity stemming from the blood having a negative effect on the connective tissue in the salmon fillets. In addition, it did not differentiate between the action of inherent enzymes in the intestinal fluids and enzymatic activity of bacteria. However, the results from the mass spectrometry analysis suggested that bacterial activity was a possible explanation for some differences in CS detected between intact and gaping tissue. Therefore, the combined results show that it is possible that bacteria transferred to the abdominal cavity from the intestines during slaughter of the salmon induce gaping in the salmon fillets. The results do not reject the possible action of inherent enzymes in either blood or intestinal fluids. A closer examination of enzymatic activity in blood and intestinal fluids left in the abdominal cavity of salmon post-slaughter might reveal in more detail which enzymes potentially harmful to the connective tissue are present and from where they originate. This might also enhance the understanding about possible mechanisms of degradation of the extracellular matrix in the salmon fillets. Detailed analysis of other extracellular matrix components similar to what was done in this study might also reveal some correlations between changes in amount or structure and gaping. Analyses of a broad range of ECM components might be important in order to reveal if some changes are causes of gaping while others might be concurrent events, independent or not. Ultimately, the connection(s) from specific enzymatic activity to specific changes in ECM components and to the effect on gaping prevalence might be achieved.

6. References

1) Aimes RT, Quigley JP (1995). Matrix metalloproteinase-2 is an interstitial collagenase. Inhibitor-free enzyme catalyzes the cleavage of collagen fibrils and soluble native type I collagen generating the specific ¾ - and ¼ -length fragments. *Journal of Biological Chemistry* **270**, 5872-5876. Doi: 10.1074/jbc.270.11.5872.
2) al Jamal R, Roughley PJ, Ludwig MS, (2001). Effect of glycosamionglycan degradation on lung tissue viscoelasticity. *American Journal of Physiology – Lung Cellular and Molecular Physiology* **280**, L306-L315.
3) Allali I, Arnold JW, Roach J, Cadenas MB, Butz N, Hassan HM, Koci M, Ballou A, Mendoza M, Ali R, Azcarate-Peril MA (2017). A comparison of sequencing platforms and bioinformatics pipelines for compositional analysis of the gut microbiome. *BMC Microbiology* **17**, 194. Doi: 10.1186/s12866-017-1101-8.
4) Alma'abadi AD, Gojobori T, Mineta K (2015). Marine metagenome as a ressource for novel enzymes. *Genomics Proteomics Bioinformatics.* **13**, 290-295. Dx.doi.org/10.1016/j.gpb.2015.10.001.
5) Almeida A, Mitchell AL, Boland M, Forster SC, Gloor GB, Tarkowska A, Lawley TD, Finn RF (2019). A new genomic blueprint of the human gut microbiota. *Nature* **568**, 499-504. Doi.org/10.1038/s41586-019-0965-1.
6) Andersen B, Steinsholt K, Stroemsnes A, Thomassen M (1994). Fillet gaping in farmed Atlantic salmon (*Salmo salar* L.) *Norwegian Journal of Agricultural Sciences* **8**, 165-179.
7) Andersen, C. M. & Wold, J. P. (2003). Fluorescence of muscle and connective tissue from cod and salmon. *Journal of Agricultural and Food Chemistry* **51**(2), 470-476.
8) Aubin J, Callier M, Rey-Valette H, Mathé S, Wilfart S, Legendre M, Slembrouck J, Caruso D, Chia E, Masson G, Blancheton JP, Ediwarman, Haryadi J, Prihadi TH, de Matos Casaca J, Tamassia STJ, Tocqueville A, Fontaine P (2019). Implementing ecological intensification in fish farming: definition and principles from contrasting experience. *Reviews in Aquaculture* **11**, 149-167. Doi: 10.1111/raq.12231.
9) Bahuaud D, Mørkøre T, Østbye T-K, Veiseth-Kent E, Thomassen MS, Ofstad R (2010). Muscle structure response and lysosomal cathepsin B and L in farmed Atlantic salmon (*Salmo salar* L.) *pre-* and *post-rigor* fillets exposed to short and long-term crowding stress. *Food Chemistry* **118**, 602-615.

10) Balaban MO, Şengör GFU, Soriano MG, Ruiz EG (2011). Quantification of gaping, bruising, and blood spots in salmon fillets using image analysis. *Journal of Food Science* **76**(3), E291-E297. Doi.org/10.1111/j.1750-3841.2011.02060.x.
11) Bame KJ (2001). Heparanases: endoglycosidases that degrade heparan sulfate proteoglycans. *Glycobiology* **11**(6), 91R-98R. doi.org/10.1093/glycob/11.6.91R.
12) Bastiaansen-Jenniskens YM, Koevoet W, Jansen W, Verhaar JAN, DeGroot J, VanOsch GJVM (2009). Inhibition of glycosaminoglycan incorporation influences collagen network formation during cartialge matrix production. *Biochemical and Biophysical Research Communications* **379**, 222-226.
13) Bayliss MT, Osborne D, Woodhouse S, Davidson C (1999). Sulfation of chondroitin sulfate in human articular cartilage. *The Journal of Biological Chemistry* **274**(22), 15892-15900.
14) Bhuimbar MV, Bhagwat PK, Dandge PB (2019). Extraction and characterization of acid soluble collagen from fish waste: Development of collagen –chitosan blend as food packaging film. *Journal of Environmental Chemical Engineering* **7**(2), 102983. Doi.org/10.1016/j.jece.2019.102983.
15) Birkedal-Hansen H, Moore WGI, Bodden MK, Windsor LJ, Birkedal-Hansen B, DeCarlo A, Engler JA (1993). Matrix metalloproteinases: a review. *Critical reviews in Oral Biology and Medicine* **4**, 197-250.
16) Bjørnevik M, Espe M, Beattie C, Nortvedt R, Kiessling A (2004). Temporal variation in muscle fibre area, gaping, texture, colour and collagen in triploid and diploid Atlantic salmon (*Salmo salar* L). *Journal of the Science of Food and Agriculture* **84**(6), 530-540. Doi.org/10.1002/jsfa.1656.
17) Borderías and Sánchez-Alonso (2011). First processing steps and the quality of wild and farmed fish. *Journal of Food Science* **76**(1):R1-R5. Doi: 10.1111/j.1750-3841.2010.01900.x.
18) Bremner HA, Hallet IC (1985). Muscle fiber-connective tissue junctions in the fish blue grenadier (*Macruronus novaezelandiae*). A scanning electron microscopy study. *Journal of Food Science* **50**, 975-980.
19) Brietwieser FP, Lu J, Salzberg SL (2017). A review of methods and databases for metagenomic classification and assembly. *Briefings in Bioinformatics*, 1-15. Doi: 10.1093/bib/bbx120.

20) Callahan BJ, McMurdie PJ, Holmes SP (2017). Exact sequence variants should replace operational taxonomic units in marker-gene data analysis. *The ISME Journal* **11**, 2639-2643.
21) Chakravarti S, Magnuson T, Lass JH, Jepsen KJ, LaMantia C, Caroll H (1998). Lumican regulates collagen fibril assembly: Skin fragility and corneal opacity in the absence of lumican. *Journal of Cell Biology* **141**, 1277-1286.
22) Chautard E, Ballut L, Thierry-Mieg N, Ricard-Blum S (2009). MatrixDB, a databasefocused on extracellular protein-protein and protein-carbohydrate interactions. *Bioinformatics* 25(5):690-691.
23) Clerc O, Mariethoz J, Rivet A, Lisacek F, Pérez S, Ricard-Blum S (2019). A pipeline to translate glycosaminoglycan sequences into 3D models. Application to the exploration of glycosaminoglycan conformational space. *Glycobiology* 29(1):36-44.
24) Costell M, Gustafsson E, Aszódi A, Mörgelin M, Bloch W, Hunziker E, Addicks K, Timpl R, Fässler R (1999). Perlecan maintaines the integrity of cartilage and some basement membranes. *The Journal of Cell Biology* **147**(5), 1109-1122. Doi: 10.1083/jcb.147.5.1109.
25) Couchman JR and Pataki CA (2012). An introduction to proteoglycans and their localization. *Journal of Histochemistry & Cytochemistry* **60**(12), 885-897. Doi: 10.1369/0022155412464638.
26) Danielson KG, Baribault H, Holmes DF, Graham H, Kadler KE, Iozzo RV (1997). Targeted disruption of decorin leads to abnormal collagen fibril morphology and skin fragility. *Journal of Cell Biology* **136**, 729-743.
27) Deligny A, Dierker T, Dagälv A, Lundequist A, Eriksson I, Nairn AV, Moremen KW, Merry CLR, Kjellén L (2016). NDST2 (*N*-Deacetylase/*N*-Sulfotransferase-2) enzyme regulates heparan sulfate chain length. *The Journal of Biological Chemistry* **291**(36), 18600-18607. Doi: 10.1074/jbc.M116.744433.
28) Duarte AS, Correia A, Esteves AC (2016). Bacterial collagenases – A review. *Critical Reviews in Microbiology* **42**(1), 106-126. Doi: 10.3109/1040841X.2014.904270.
29) Ellis T, Turnbull JF, Knowles TG, Lines JA, Auchterlomie NA (2016). Trends during development of Scottish salmon farming: An example of sustainable intensification? *Aquaculture* **458**, 82-99. Doi: 10.1016/j.aquaculture.2016.02.012.
30) Erkan N (2007). Sensory, chemical, and microbial attributes of sea bream (*Sparus aurata*): effects of washing and ice storage. *International Journal of Food Properties* **10**, 421-434.

31) Espe M, Ruohonen K, Bjørnevik M, Frøyland L, Nortvedt R, Kiessling A (2004). Interactions between ice storage time, collagen composition, gaping and textural properties in farmed salmon muscle harvested at different times of the year. *Aquaculture* **240**(1-4), 489-504.

32) Eysturskarð J, Haug IJ, Elharfaoui N, Djabourov M, Draget KI (2009). Structural and mechanical properties of fish gelatin as a function of extraction conditions. *Food Hydrocolloids* **23**, 1702-1711.

33) Eysturskarð J, í Kongsstovu S, Færø D, Jacobsen Á, Joensen H (2017). Fucus vesiculosus extract inhibits the proteolytic activity and gene expression of matrix metalloproteinases in Atlantic salmon (Salmo salar L.). *Aquaculture International* **25**(2), 1813-1819. Doi: 10.1007/s10499-017-0157-7.

34) FAO (2016). Sustainable intensification of aquaculture in the Aia-Pacific region. Documentation of successful practices. Miao W. and Lal K.K. eds., Bangkok, Thailand.

35) FAO (2018). The state of the world fisheries and aquaculture 2018 – meeting the sustainable development goals. Rome. Licence: CC BY-NC-SA 3.0 IGO.

36) Farach-Carson MC, Carson DD (2007). Perlecan – a multifunctional extracellular proteoglycan scaffold. *Glycobiology* **17**, 897-905.

37) Fletcher GH, Hallet IC, Jerret AR, Holland AJ (1997). Changes in the fine structure of the myocommata-muscle fibre junction related to gaping in rested and exercised muscle from king salmon (*Oncorhynchus tchawytacha*). *LWT – Food Science and Technology* **30**, 246-252.

38) Fogarty C, Burgess CM, Cotter PD, Cabrera-Rubio R, Whyte P, Smyth C, Bolton DJ (2019). Diversity and composition of the gut microbiota of Atlantic salmon (*salmo salar*) farmed in Irish waters. *Journal of Applied Microbiology*. Doi.org/10.1111/jam.14291.

39) Fricker AM, Podlesny D, Fricke WF (2019). What is new and relevant for sequencing-based microbiome research? A mini-review. *Journal of Advanced Research* **19**, 105-112.Doi.org/10.1016/j.jare.2019.03.006.

40) Fry JP, Mailloux NA, Love DC, Milli MC, Cao L (2018). Feed conversion efficiency in aquaculture: do we measure it correctly? *Environmental Research Letters* **13**, 024017. Doi: 10.1088/1748-9326/aaa273.

41) Fuller M, MeiklePJ, Hopwood JJ (2004). Glycosaminoglycan degradation fragments in mucopolysaccharidosis I. *Glycobiology* **14**(5), 443-450. Doi: 10.1093/glycob/cwh049.

42) Gangbar S, Overall CM, McCulloch CA, Sodek J (1990). Identification of polymorphonuclear leukocyte collagenase and gelatinase activities inmouthrinse samples: correlation with peridontal disease activity in adult and juvenile peridontitis. *Journal of Peridontal Research* ***25****, 257-267.*

43) Garber AF, Amini F, Grezan SA, Swift BD, Hodkinson SE, Nickerson J, Bridger CJ (2019). Genetic and phenotypic evaluation of harvest traits from a comprehensive commercial Atlantic salmon, *Salmo salar* L., broodstock program. *Aquaculture* **503**, 242-253. Doi.org/10.1016/j.aquaculture.2019.01.001.

44) Gelse K, Pöschl E, Aigner T (2003). Collagens – structure, function, and biosynthesis. *Advanced Drug Delivery Reviews* **55**, 1531-1546. Doi: 10.1016/j.addr.2003.08.002.

45) Geng Y, McQuillan D, Roughley PJ (2006). SLRP interaction can protect collagen fibrils from cleavage by collagenases. *Matrix Biology* **25**(8), 484-491. Doi: 10.1016/j.matbio.2006.08.259.

46) Gianelli G, Falk-Marzillier J, Schiraldi O, Stetler-StevensonWG, Quaranta V. Induction of cell migration by matrix metalloproteinase-2 cleavage of laminin-5. *Science* **277**, 225-228.

47) Gill SE, Parks WC (2011). Matrix metalloproteinases and their inhibitors in turnover and degradation of extracellular matrix. *In: Extracellular Matrix Degradation*, Parks WC and Mecham RP eds., Springer, Berlin Heidelberg. Doi: 10.1007/978-3-642-16861-1.

48) Goody MF, Sher RB, Henry CA (2015). Hanging on for the ride: Adhesion to the extracellular matrix mediates cellular responses in skeletal muscle morphogenesis and disease. *Developmental Biology* **401**, 75-91. Dx.doi.org/10.1016/j.ydbio.2015.01.002.

49) Gu Y, Lu M, Wang Z, Wu X, Chen Y (2017). Expanding the catalytic promiscuity of heparinase III from Pedobacter heparinus. *Chemistry* **23**(11), 2548–2551.

50) Hagen Ø, Johnsen CA (2016). Flesh quality and biochemistry of light-manipulated Atlantic cod (*Gadus morhua*) and the significance of collagen cross-links on fillet firmness and gaping. *Food Chemistry* **190**, 786-792. Doi.org/10.1016/j.foodchem.2015.06.007.

51) Hallet IC, Bremner HA (1988). Fine structure of the myocommate-muscle fibre junction in hoki (*Macruronus novaezelandiae*). *Journal of the Science of Food and Agriculture* **44**, 245-261.

52) Han W, Wang W, Zhao M, Sugahara K, Li F (2014). A novel eliminase from a bacterium that degrades hyaluronan and chondroitin sulfate. *Journal of Biological Chemistry* **289**, 27886-27898.

53) Hannesson KO, Tingbø MG, Olsen RL, Enersen G, Bævre AB, Ofstad R (2007). An immunological study of glycosaminoglycans in the connective tissue of bovine and cod skeletal muscle. *Comparative Biochemistry and Physiology – Part B: Biochemistry & Molecular Biology* **146**, 512-521.

54) Hansen AÅ, Rødbotten M, Eie T, Lea P, Rudi K, Mørkøre T (2012). The effect of crowding stress on bacterial growth and sensory properties of chilled Atlantic salmon fillets. *Journal of Food Science* **77**(1), S84-S90. Doi.org/10.1111/j.1750-3841.2011.02513.x.

55) Hariharan IK, Wake DB, Wake MH (2016). Indeterminate growth: Could it represent the ancestral condition? *Cold Spring Harbor Perspectives in Biology* **8**, a019174. Doi: 10.1101/cshperspect.a019174.

56) Harrington DJ (1996). Bacterial collagenases and collagen-degrading enzymes and their potential role in human disease. *Infection and Immunity* **64**(6), 1885-1891.

57) Hatlen, B., Oaland, Ø., Tvenning, L., Breck, O., Jakobsen, J. V. & Skaret, J. (2013). Growth performance, feed utilization and product quality in slaughter size Atlantic salmon (*Salmo salar* L.) fed a diet with porcine blood meal, poultry oil and salmon oil. *Aquaculture Nutrition* **19**(4), 573-584.

58) He Y, Caporase JG, Jiang X-T, Sheng Hua-Feng, Huse SM, Rideout JR, Edgar RC, Kopylova E, Walters WA, Knight R, Zhou H-W (2015). Stability of operational taxonomic units: an important but neglected property for analyzing microbial diversity. *Microbiome* **3**, 20. Doi: 10.1186/s40168-015-0081-x.

59) Henriksson PJG, Belton B, Jahan KM, Rico A (2018). Measuring the potential for sustainable intensification of aquaculture in Bangladesh using life cycle assessment. *PNAS* **115**(12), 2958-2963. Doi: 10.1073/pnas.1716530115.

60) Herman MP, Sukhova GK, Libby P, Gerdes N, Tang N, Horton DB, Kilbride M, Breithart RE, Chun M, Scönbeck U (2001). Expression of neutrophil collagenase (matrix Metalloproteinase-8) in human atheroma. A novel collagenolytic pathway suggested by transcriptional profiling. *Circulation* **104**, 1899-1904.

61) Higashi K, Takeuchi Y, Mukuno A, Tomitori H, Miya M, Linhardt RJ, Toida T (2015). Composition of glycosaminoglycans in Elasmobranchs including several deep-sea sharks: Identification of chondroitin/dermatan sulfate from the dried fins of *Isurus oxyrinchus* and *Prionace glauca*. *PLoS One* **10**, No. e0120860.

62) Hognes ES, Ziegler F, Sund V (2011). Carbon footprint and area use of farmed Norwegian salmon. *SINTEF Fisheries and Aquaculture Report* **A22471**. Doi: 10.13140/RG.2.1.4168.2808.

63) Honda T, Kaneiwa T, Mizumoto S, Sugahara K, Yamada S (2012). Hyaluronidases have strong hydrolytic activity toward chondroitin 4-sulfate comparable to that for hyaluronan. *Biomolecules* **2**, 549-563. Doi: 10.3390/biom2040549.

64) Huss HH (1995). Quality and quality changes in fresh fish. FAO Fisheries Technical Paper 348. Rome. ISBN 92-5-103507-5.

65) Hussel T, Lui S, Jagger C, Morgan D, Brand O (2018). The consequence of matrix dysfunction on lung immnity and the microbiome in COPD. *Eur Respir Rev* **27**, 180032. doi.org/10.1183/16000617.0032-2018.

66) Iozzo RV, Schaefer L (2015). Proteoglycan form and function: A comprehensive nomenclature of proteoglycans. *Matrix Biology* **42**, 11-15.

67) Johnston AI, Li X, Vieira VLA, Nickell D, Dingwall A, Alderson R, Campbell P, Bickerdike R (2006). Muscle and flesh quality traits in wild and farmed Atlantic salmon.*Aquaculture* **256**, 323-336. Doi: 10.1016/j.aquaculture.2006.02.048.

68) Jones AC, Mead A, Kaiser MJ, Austen MCV, Adrian AW, Auchterlonie NA, Black KD, Blow LR, Bury C, Brown JH, Burnell GM, Connolly E, Dingwall A, Derrick S, Eno NC, Gautier DJH, Green KA, Gubbins M, Hart PR, Holmyard JM, Immink AJ, Jarrad DL, Katoh E, Langley JCR, Lee DO'C, Le Vay L, Leftwich CP, Mitchell M, Moore A, Murray AG, McLaren EMR, Norbury H, Parker D, Parry SO, Purchase D, Rahman A, Sanver F, Siggs M, Simpson SD, Slaski RJ, Smith K, Syvret MLQ, Tibbott C, Thomas PC, Turnbull J, Whiteley R, Whittles M, Wilcockson MJ, Wilson J, Dicks LV, Sutherland WJ (2015). Prioritization of knowledge needs for sustainable aquaculture: a national and global perspective. *Fish and Fisheries* **16**, 668-683. Doi: 10.1111/faf.12086.

69) Jovel J, Patterson J, Wang W, Hotte N, O'Keefe S, Mitchel T, Perry T, Kao D, Mason AL, Madsen KL, Wong GK-S (2016). Characterization of the gut microbiome using 16S or shotgun metagenomics. *Frontiers in Microbiology* **7**, 459. Doi: 10.3389/fmicb.2016.00459.

70) Jung K (2008). Measurements of matrix Metalloproteinases and their tissue inhibitors in serum produces doubtful results. *The Journal of Infectious Diseases* **198**, 1722-1723.

71) Kalamajski S, Oldberg Å (2010). The role of small leucine-rich proteoglycans in collagen fibrillogenesis. *Matrix Biology* **29**, 248-253.

72) Kashiwagi M, Enghild JJ, Gendron C, Hughes C, Caterson B, Itoh Y, Nagase H (2004). Alterd proteolytic activities of ADAMTS-4 expressed by C-terminal processing. *Journal of Biological Chemistry* **279**, 10109-10119.

73) Kause A, Quinton C, Airaksinen S, Ruohonen K, Koskela J (2011). Quality and production trait genetics of farmed European whitefish. *Coregonus lavaretus. Journal of Animal Science* **89**, 959-971.

74) Kawai K, Kamochi R, Oiki S, Murata K, Hashimoto W (2018). Probiotics in human gut microbiota can degrade host glycosaminoglycans. *Scientific Reports* **8**, 10674.

75) Kiessling A, Espe M, Ruohonen K, Mørkære T (2004). Texture, gaping and colour of fresh and frozen Atlantic salmon flesh as affected by pre-slaughter iso-eugenol or CO_2 anaesthesia. *Aquaculture* **236**, 645-657.

76) Kiessling A, Ruohonen K, Bjørnevik M (2006). Muscle fibre growth and quality in fish. *Arch Tierz., Dummerstorf* **49**, 137-146.

77) Kousoulaki, K., Mørkøre, T., Nengas, I., Berge, R. K. & Sweetman, J. (2016). Microalgae and organic minerals enhance lipid retention efficiency and fillet quality in Atlantic salmon (*Salmo salar* L.). *Aquaculture* **451**, 47-57.

78) Koutsandreas T, Ladoukakis E, Pilalis E, Zarafeta D, Kolisis FN, Skretas G, Chatziioannou AA (2019). ANASTASIA: An automated metagenomic analysis pipeline for novel enzyme discovery exploiting next generation sequening data. *Frontiers in Genetics* **10**, 469. Doi: 10.3389/fgene.2019.00469.

79) Krekoski CA, Neubauer D, Graham JB, Muir D (2002). Etalloproteinase-dependent predegeneration *in vitro* enhances axonal regeneration within acellular peripheral nerve grafts. *Journal of Neuroscience* **22**(23), 10408- 10415. Doi.org./10.1523/JNEUROSCI.22-23-10408.2002.

80) Kreuger J, Spillman D, Li J-P, Lindahl U (2006). Interactions between heparan sulfate and proteins: the concept of specificity. *Journal of Cell Biology* **174**, 323-327.

81) Launay G, Salza R, Multedo D, Thierry-Mieg N, Ricard-Blum S (2015). MatrixDB, the extracellular matrix interaction database: updated content, a new navigator and expanded functionalities. *Nucleic Acids Research* 43:D321-D327 doi: 10.1093/nar/gku1091.

82) Lavéty J, Afolabi OA, Love RM (1988). The connective tissue in fish. IX. Gaping in farmed species. *International Journal of Food Science and Technology* **23**, 23-30.

83) Lindahl U, Kjellén L (2013). Pathophysiology of heparan sulphate: many diseases, few drugs. *Journal of Internal Medicine* **273**, 555-571.

84) Little DC, Newton RW, Beveridge MCM (2016). Aquaculture: a rapidly growing and significant source of sustainable food? Status, transition and potential. *Proceedings of the Nutrition Society* **75**(3), 274-286. Doi:10.1017/S0029665116000665.

85) Little PJ, Ballinger ML, Burch ML, Osman N (2008). Biosynthesis of natural and hyperelongated chondroitin sulfate glycosaminoglycans: New insights into an elusive process. *The Open Biochemistry Journal* **2**, 135-142.

86) Llewellyn MS, McGinnity P, Dionne M, Letourneau J, Thonier F, Carvalho GR, Greer S, Derome N (2016). The biogeography of the Atlantic salmon (*Salmo salar*) gut microbiome. *The ISME Journal*. **10**(15), 1280-1284. Doi: 10.1038/ismej.2015.189.

87) Lodish HF, Berk A, Kaiser CA, Krieger M, Scott MP, Bretscher A, Ploegh H, Matsudaira PT (2008). Molecular Cell Biology, 6th ed. W.H. Freeman and Company, New York.

88) Love DC, Fry JP, Milli MC, Neff RA (2015). Wasted seafood in the United States: Quantifying loss from production to consumption and moving toward solutions. *Global Environmental Change* **35**, 116-124. Doi: 10.1016/j.gloenvcha.2015.08.013.

89) Ly M, Laremore TN, Linhardt RJ (2010). Proteoglycomics: recent progress and future challenges. *OMICS* **14**(4), 389- 399. Doi: 10.1089/omi.2009.0123.

90) Lähteenmäki , Kuusela P, Korhonen T (2001). Bacterial plasminogen activators and receptors. *FEMS Microbiology Reviews* **25**, 531-552.

91) Maccari F, Galeotti F, Volpi N (2015). Isolation and structural characterization of chondroitin sulfate from bony fishes. *Carbohydrate Polymers* **129**, 143-147.

92) Merkin GV, Stien LH, Pittman K, Nortvedt R (2013). Digital image analysis as a tool to quantify gaping and morphology in smoked salmon slices. *Aquaculture Engineering* **54**, 64-71. Doi: 10.1016/j.aquaeng.2012.11.003.

93) Multhaupt HAB, Couchman JR (2012). Heparan sulfate biosynthesis. Methods for investigation of the heparanosome.*Journal of Histochemistry and Cytochemistry* **60**(12), 908-915.

94) Manello F (2008). Serum or plasma samples? The "Cindarella" role of blood collection procedures. Preanalytical methodological issues influence the release and activity of circulating matrix metalloproteinases and their tissue inhibitors, hampering diagnostic

trueness and leading to misinterpretation. *Arteriosclerosis, Thrombosis, and Vascular Biology* **28**, 611-614.

95) Margeirsson S, Jonsson GR, Arason S, Thorkelsson G (2007). Influencing factors on yield, gaping, bruises and nematodes in cod (*Gadus morhua*) fillets. *Journal of Food Engineering*. **80**, 503-508. Doi: 10.1369/0022155412460056.

96) Marine Harvest (2018). Salmon farming industry handbook 2018. https://mowi.com/wp-content/uploads/2019/04/2018-salmon-industry-handbook-1.pdf

97) Masola V, Bellin G, Gambaro G, Onisto M (2018). Heparanase: A multitasking protein involved in extracellular matrix (ECM) remodelling and intracellular events. *Cells* **7***(12)*, 236. Doi: 10.3390/cells7120236.

98) Matmaroh K, Benjakul S, Prodpran T, Encarnacion AB, Kishimura H (2011). Characteristics of acid soluble collagen and pepsin soluble collagen from scale of spotted golden goatfish (*Parupeneusheptacanthus*). *Food Chemistry* **129**(3), 1179-1186. Doi.org/10.1016/j.foodchem.2011.05.099.

99) Miyata S, Komatsu Y, Yoshimura Y, Taya C, Kitagawa H (2012). Consistant cortical plasticity by upregulation of chondroitin 6-sulfation. *Nature Neuroscience* **15**(3), 414-422.

100) Monaco S, Sparano V, Gioia M, Sbardella D, Di Pierro D, Marini S, Coletta M (2006). Enzymatic processing of collagen IV by MMP-2 (gelatinase A) affects neutrophil migration and it is modulated by extracatalytic domains. *Protein Science* **15**, 2805-2815. Doi: 10.1110/ps.062430706.

101) Moreno HM, Montero MP, Gómez-Guillén MC, Fernández-Martín F, Mørkøre T, Borderías J (2012). Collagen characteristics of farmed Atlantic salmon with firm and soft fillet texture. *Food Chemistry* **134**(2), 678-685. Doi: 10.1016/j.foodchem.2012.02.160.

102) Mott JD, Werb Z (2004). Regulation of matrix biology by matrix metalloproteinases. *Current Opinion in Cell Biology* **16**(5), 558-564.

103) Mouw JK, Ou G, Weaver VM (2014). Extracellular matrix assembly: a multiscale deconstruction. *Nature Reviews Molecular Cell Biology* **15**(12), 771-785. Doi: 10.1038/nrm3902.

104) Murphy G, Nguyen Q, Cockett MI, Atkinson SJ, Allan JA, Knight CG, Willenbrock F, Docherty AJP (1994). Assessment of the role of the fibronectine-like domain of gelatinase A by analysis of a deletion mutant. *The Journal of Biological Chemistry* **269**(9), 6632-6636.

105) Møretrø T, Moen B, Heir E, Hansen AÅ, Langsrud S (2016). Contamination of salmon fillets and processing plants with spoilage bacteria. *International Journal of Food Microbiology* **237**, 98-108. Doi.org/10.1016/j.ijfoodmicro.2016.08.016.

106) Mørkøre, T. & Austreng, E. (2004). Temporal changes in texture, gaping, composition and copper status of Atlantic salmon (*Salmo salar* L.) fed moist feed or extruded dry feed. *Aquaculture* 230, 425-437.

107) Mørkøre T, Mazo PI, Tahirovic V, Einen O (2008). Impact of starvation and handling stress on rigor development and quality of Atlantic salmon (*Salmon salar* L.). *Aquaculture* **277**(3-4), 231-238. Doi.org/10.1016/j.aquaculture.2008.02.036.

108) Mørkøre T, Rørvik K-A (2001). Seasonal variation in growth , feed utilisation and product quality of farmed Atlantic salmon (*Salmo salar*) transferred to seawater as 0 + smolts or 1 + smolts. *Aquaculture* **199**, 145-157.

109) Ndeh D, Munoz JM, Cartmell A, Bulmer D, Wills C, Henrissat B, Gray J (2018). The human gut microbe *Bacteroides thetaiotaomicron* encodes the founding member of a novel glycosaminoglycan degrading polysaccharide lyase family PL29. *Journal of Biological Chemistry* **293**, 17906-17916. Doi: 10.1074/jbc.RA118.004510.

110) Nordberg M (2018). Seasonal variation in fillet quality of Atlantic salmon (*Salmo salar*). Master Thesis NMBU.

111) Odaka C, Tanioka M, Itoh T (2005). Matrix metalloproteinase-9 in macrophages induces thymic neovascularization following thymocyte apoptosis. *Journal of Immunology* **174**(2), 846-853.

112) Ofstad R, Egelandsdal B, Kidman S, Mykleburst R, Olsen RI, Hermansson AM (1996). Liquid loss as effected by post mortem ultrastructural changes in fish muscle: cod (*Gadus morhua* L.) and salmon (*Salmo salar*). *Journal of the Science of Food and Agriculture* **71**, 301-312.

113) Pan Y, Wang P, Zhang F, Yu Y, Zhang X, Lin L, Linhardt RJ (2018). Glycosaminoglycans from fish swim bladder: isolation, structural characterization and bioactive potential. *Glycoconjugate Journal* **35**, 87-94.

114) Pegeot M, Sadir R, Eriksson I, Kjellen L, Simorre J-P, Gans P, Lortat-Jacob H (2015). Profiling sulfation/epimerization pattern of full-length heparan sulfate by NMR following cell culture 13C-glucose metabolic labeling. *Glycobiology* **25**(2), 151-156. Doi.org/10.1093/glycob/cwu114.

115) Peterson S, Liu J (2012). Deciphering mode of action of heparanase using structurally defined oligosaccharides. *Journal of Biological Chemistry* **287**, 34836-34843.

116) Philis G, Ziegler F, Gansel LC, Jansen MD, Gracey EO, Stene A (2019). Comparing Life Cycle Assessment (LCA) of salmonid aquaculture production systems: Status and perspectives. *Sustainability* **11**, 2517. Doi: 10.3390/su11092517.

117) Pittman, K., Merkin, G. V. & Brandebourg, T. (2013). Bridging the gap to sustainable salmon farming: overcoming the gaping problem. *Journal of Fisheries & Livestock Production* **1**(2), 104. doi: 10.4172/jflp.1000104

118) Poon J, Plaas AH, Keene DR, McQuillan DJ, Last K, Fosang AJ (2005). N-linked keratan sulfate in the aggrecan interglobular domain potentiates aggrecenase activity.*Journal of Biological Chemistry* **280**, 23615-23621.

119) Porter S, Clark IM, Kevorkian L, Edwards DR (2005). The ADAMTS metalloproteinases. *Biochemical Journal* **386**, 15-27. Doi: 10.1042/BJ20040424.

120) Poussin C, Sierro N, Boué S, Battey J, Scotti E, Belcastro V, Peitsch MC, Ivanov NV, Hoeng J (2018). Interrogating the microbiome: experimental and computational considerations in support of study reproducibility. *Drug Discovery Today* **23**(9). Doi.org/10.1016/j.drudis.2018.06.005.

121) Pöschl E, Schlötzer-Schrehardt U, Brachvogel B, Saito K, Ninomiya Y, Mayer U (2004). Collagen IV is essential for basement membrane stability but dispensable for initiation of its assembly during early development. *Development* **131**, 1619-1628. Doi: 10.1242/dev.01037.

122) Prabhakar V, Capila I, Bosques CJ, Pojasek K, Sasisekharan R (2005). Chondroitinase ABC I from *Proteus vulgaris*: cloning, recombinant expression and active site identification. *Biochemical Journal* **386**, 103-112.

123) Pratta MA, Tortorella MD, Arner EC (2000). Age-related changes in aggrecan glycosylation affect cleavage by aggrecenase. *Journal of Biological Chemistry* **275**, 39096-39102.

124) Pruteanu M, Hyland NP, Clarke DJ, Kiely B, Shanahan F (2011). Degradation of the extracellular matric components by bacterial-derived metalloproteases: Implications for inflammatory bowel diseases. *Inflammatory Bowel Diseases* **17**(5), 1189-1200.Doi: 10.1002/ibd.21475.

125) Quiñones RA, Fuentes M, Montes RM, Soto D, León-Muñoz (2019). Environmental issues in Chilean salmon farming: a review. *Reviews in Aquaculture* **11**(2), 375-402. Doi.org/10.1111/raq.12337.

126) Ranjan R, Rani A, Metwally A, McGee HS, Perkins DL (2016). Analysis of the microbiome: Advantages of whole genome shotgun versus 16S amplican sequencing. *Biochemical and Biophysical Research Communications* **469**(4), 967-977. Doi: 10.1016/j.bbrc.2015.12.083.

127) Rausch P, Rühlemann M, Hermes BM, Doms S, Dagan T, Dierking K, Domin H, Fraune S, von Frieling J, Hentschel U, Heinsen F-A, Höppner M, Jahn MT, Jaspers C, Kissoyan KAB, Langfeldt D, Rehman A, Reusch TBH, Roeder T, Schmitz RA, Schulenburg H, Soluch R, Sommer F, Stukenbrock E, Weiland-Bräuer N, Rosenstiel P, Franke A, Bosch T, Baines JF (2019). Comparative analysis of amplicon and metagenomic sequencing methods reveals key features in the evolution of animal metaorganisms. *Microbiome* **7**, 133. Doi.org/10.1186/s40168-019-0743-1.

128) Rickelt S, Hynes RO (2018). Antibodies and methods for immunohistochemistry of extracellular matrix proteins. *Matrix Biology* 71-72:10-27 doi.org/10.1016/j.matbio.2018.04.011.

129) Rinke C, Schwientek P, Sczyrba A, Ivanova NN, Anderson IJ, Cheng J-F, Darling A, Malfatti S, Swan BK, Gies EA, Dodsworth JA, Hedlund BP, Tsiamis G, Sievert SM, Liu W-T, Eisen JA, Hallam SJ, Kyrpides NC, Stepanauskas R, Rubin EM, Hugenholtz P, Woyke T (2013). Insights into the phylogeny and coding potential of microbial dark matter. *Nature* **499**, 431-437. Doi: 10.1038/nature12352.

130) Roth B, Slinde E, Arildsen J (2006). Pre or post mortem muscle activity in Atlantic salmon (*Salmo salar*). The effect on rigor mortis and the physical properties of flesh. *Aquaculture* **257**, 504-510.

131) Roesch LFW, Fulthorpe RR, Riva A, Casella G, Hadwin AKM, Kent AD, Daroub SH, Camargo FAO, Farmerie WG, Triplett EW (2007). Pyrosequening enumerates and contrasts soil microbial diversity. *The ISME Journal* **1**, 283-290. Doi.org/10.1038/ismej.2007.53.

132) Rowe RG, Weiss SJ (2008). Breaching the basement membrane: who, when and how? *Trends in Cellular Biology* **18**, 560-574.

133) Rørå AMB, Ruyter B, Skorve J, Berge RK, Slinning K-E (2005). Influence of high content of dietary soybean oil on quality of large fresh, smoked and frozen Atlantic salmon

(*Salmo salar*). *Aquaculture International* **13**(3), 217-231. Doi.org/10.1007/s10499-004-1074-0.

134) Sarrazin S, Lamanna WC, Esko JD (2011). Heparan sulfate proteoglycans. *Cold Spring Harbor Perspectives in Biology* **3**, a004952. Doi: 10.1101/cshperspect.a004952.

135) Sasagawa S, Matsubara J, Satow Y (1993). Stress-related induction of hepatic metallothionein synthesis and increase in peripheral polymorphonuclear leukocytes in mice. *Immunopharmacology and Immunotoxicology* **15**, 217-226.

136) Schloss P (2018). Identifying and overcoming threats to reproducibility, replicability, robustness, and generalizability in microbiome research. *mBio* **9**(3), e00525-18. Doi.org/10.1128/mBio.00525-18.

137) Schmidtchen A, Frick I-A, Björck L (2001). Dermatan sulphate is released by proteinases of common pathogenic bacteria and inactivates antibacterial α-defensin. *Molecular Microbiology* **39**(3), 708-713. Doi.org/10.1046/j.1365-2958.2001.02251.x.

138) Shi X, Zaia J (2009). Organ-specific heparan sulfate structural phenotypes. *Journal of Biological Chemistry* **284**(18), 11806-11814. Doi: 10.1074/jbc.M809637200.

139) Shipley JM, Doyle GAR, Fliszar CJ, Ye Q-Z, Johnson LL, Shapiro SD, Welgus HG, Senior RM (1996). The structural basis for the elastoloytic activity of the 92-kDa and 72-kDa gelatinases. *The Journal of Biological Chemistry* **271**(8), 4335-4341.

140) Sissener NH, Waagbø R, Rosenlund G, Tvenning L, Susort S, Lea TB, Oaland Ø, Chen L, Breck O (2016). Reduced n-3 long chain fatty acid levels in feed for Atlantic salmon (*Salmo salar* L.) do not reduce growth, robustness or product quality through an entire full scale commercial production cycle in seawater. *Aquaculture* **464**, 236-245.

141) Skjervold, P. O., Fjæra, S. O., Østby, P. B., Einen, O. (2001). Live-chilling and crowding stress before slaughter of Atlantic salmon (*Salmo salar*). *Aquaculture* **192**(2-4), 265-280.

142) Stanton H, Melrose J, Little CB, Fosang AJ (2011). Proteoglycan degradation by the ADAMTS family of proteinases. *Biochemica et Biophysica acta* **1812**, 1616-1629. Doi: 10.1016/j.bbadis.2011.08.009.

143) Suzuki K, Totsuka M, Nakaji S, Yamada M, Kudoh S, Liu Q, Sugawarw K, Yamaya K, Sato K (1999). Endurance exercise causes interaction among stress hormones, cytokines, neutrophil dynamics, and muscle damage. *Journal of Applied Physiology* **87**, 1360-1367.

144) Sveinsdottir K, Hyldig G, Martinsdottir E, Jørgensen B, Kristbergsson K (2003). Quality Index Method (QIM) scheme developed for farmed Atlantic salmon (*Salmo salar*). *Food Quality and Preference* **14**, 237-245. Doi.org/10.1016/S0950-3293(02)00081-2.

145) Tatara Y, Suto S, Itoh K (2017). Novel roles of glycosaminoglycans in the degradation of type I collagen by cathepsin K. *Glycobiology* **27**(12), 1089-1098. Doi.org/10.1093/glycob/cwx083.

146) Taylor RG, Fjaera SO, Skjervold PO (2002). Salmon fillet texture is determined by myofiber-myofiber and myofiber-myocommata attachment. *Food Chemistry and Toxicology* **67**(6), 2067-2071. Doi:

147) Teletchea F, Fontaine P (2014). Levels of domestication in fish: implications for the sustainable future of aquaculture. *Fish and Fisheries* **15**, 181-195. Doi: 10.1111/faf.12006.

148) Teletchea F (2018). Fish domestication: An overview. In Animal domestication, Teletchea F ed., IntechOpen. Doi: 10.5772/intechopen.79628.

149) The World Bank (2013). Fish to 2030: Prospects for fisheries and aquaculture. *World Bank Report* **83177-GLB**. http://www.fao.org/3/i3640e/i3640e.pdf

150) Tingbø MG, Kolset SO, Ofstad R, Enersen G, Hannesson KO. Identification and distribution of heparan sulfate proteoglycans in the white muscle of Atlantic cod(*Gadus morhua*) and spotted wolffish (*Anarhichas minor*). *Comparative Biochemistry and Physiology – Part B: Biochemistry & Molecular Biology* **143**, 441-452.

151) Torgersen JS, Koppang EO, Stien LH, Kohler A, Pedersen ME, Mørkøre T (2014). Soft texture of Atlantic salmon fillets is associated with glycogen accumulation. *PloS ONE* **9**(1):e85551. doi.org/10.1371/journal.pone.0085551.

152) Tripathi CK, Banga J, Mishra V (2012). Microbial heparin/heparan sulphate lyases: potential and applications. *Applied Microbial Biotechnology* **94**(2), 307–321.

153) Troell M, Naylor RL, Metian M, Beveridge M, Tyedmers PH, Folke C, Arrow KJ, Barrett S, Crépin A-S, Ehrlich PR, Gren Å, Kautsky N, Levin SA, Nyborg K, Österblom H, Polasky S, Scheffer M, Walker BH, Xepapadeas T, de Zeeuw A (2014). Does aquaculture add resilience to the global fodd system? *PNAS* **111**(37), 13257-13263. Doi.org/10.1073/pnas.1404067111.

154) Ulmer JE, Vilén EM, Namburi RB, Benjdia A, Beneteau J, Malleron A, Bonaffé D, Driguez P-A, Descroix K, Lasalle G, Le Narvor C, Sandström C, Spillman D, Berteau O (2014). Characterization of glycosaminoglycan (GAG) sulfatases from human gut symbiont

Bacteroides thetaiotamicron reveals the first GAG-specific bacterial endosulfatase. *Journal of Biological Chemistry* **289**, 24289-24303.

155) Vargová V, Pytliak M, Mechírová V (2012). Matrix metalloproteinases, *In: Matrix metalloproteinase inhibitors – Specificity of binding and structure-activity relationships*, Gupta SP ed., Springer, Basel. Doi: 10.1007/978-3-0348-0364-9.

156) Vázguez JA, Fraguas J, Novosa-Carvallal R, Reis RL, Antelo LT, Pérez-Martín RI, Valcarcel J (2018). Isolation and chemical characterizationof chondroitin sulfate from cartilage by-products of blackmouth catshark (*Galeus melastomus*). *Marine Drugs* **16**, 344.

157) Veraldi N, Parra A, Urso E, Cosentino C, Locatelli M, Corsini S, Pedrini E, Naggi A, Bisio A, Sangiorgi L (2018). Structural features of heparan sulfate from multiple osteochondromas and chondrosarcomas. *Molecules* **23**, 3277. Doi: 10.3390/moleculrs23123277.

158) Waite R, Beveridge M, Brummett R, Castine S, Chaiyawannakarn N, Kaushik S, Mungkung R, Nawapakpilai S, Phillips M (2014). Improving productivity and environmental performance of aquaculture. Working paper, Installment 5 of *Creating a sustainable food future.* Washington, DC: World Resource Institute (available at www.worldresourcesreport.org)

159) Wang W, Han W, Cai X, Zheng X, Sugahara K, Li F (2015). Cloning and characterization of a novel chondroitin sulfate/dermatan sulfate 4-*O*-endosulfatase from a marine bacterium. *Journal of Biological Chemistry* **290**, 7823-7832.

160) Wang S, Guan J, Zhang Q, Chen X, Li F (2019). Identification and signature sequences of bacterial $\Delta^{4,5}$Hexuronate-2-*O*-Sulfatases. *Frontiers in Microbiology* **10**, 704. Doi: 10.3389/fmicb.2019.00704.

161) Yang X, Xiao X, Liu D, Wu R, Wu C, Zhang J, Huang J, Liao B, He H (2017). Optimization of collagenase production by *Pseudoalteromonas* sp. SJN2 and application of collagenases in the preparation of antioxidative hydrolysates. *Marine Drugs* **15**(12), 377. Doi: 10.3390/md15120377.

162) Ytrestøyl T, Aas TS, Åsgård T (2015). Utilisation of feed resources in production of Atlantic salmon (*Salmo salar*) in Norway. *Aquaculture* **448**, 365-374. Doi: 10.1016/j.aquaculture.2015.06.023.

163) Zaia J (2013). Glycosaminoglycan glycomics using mass spectrometry. *Molecular and Cellular Proteomics* **12**(4), 885-892. Doi.org/10.1074/mcp.R112.026294.

164) Zeng Z-S, Cohen AM, Guillem JG (1999). Loss of basement membrane type IV collagen is associated with increased expression of metalloproteinases 2 and 9 (MMP-2 and MMP-9) during human colorectal tumorigenesis. *Carcinogenesis* **20**(5), 749-755.

165) Zhang F, Zhang Z, Thistle R, McKeen L, Hosoyama S, Toida T, Linhardt RJ, Page-McCaw P (2009). Structural characterization from zebrafish in different ages. *Glycoconjugate Journal* **26**, 211-218.

166) Zhang Y, Liu W, Li G, Shi B, Miao Y, Wu X (2007). Isolation and partial characterization of pepsin-soluble collagenfrom the skin of grass carp (*Ctenopharyngodon idella*). *Food Chemistry* **103**(3), 906-912. Doi.org/10.1016/j.foodchem.2006.09.053.

167) Zhang Y-Z, Ran L-Y, Li C-Y, Chen X-L (2015). Diversity, structures, and collagen-degrading mechanisms of bacterial collagenolytic proteases. *Applied and Environmental Microbiology* **81**(18), 6098-6107. Doi: 10.1128/AEM.00883-15.

7. Apendix

7.1 Supplementary material for thesis

7.1.1 Supplementary file S1. Method description of immunofluorescent analysis.

Immunohistochemistry samples. Ten salmon with an average weight of 4.1 kg were kindly provided by Bakkafrost farming company (Faroe Islands). Following standard procedure of slaughter, except a less rigorous cleaning procedure of the abdominal cavity in order to induce gaping, the ten individuals were stored in transport boxes with chipped ice in a cooling facility for seven days at 1.5 ºC. The salmon were filleted and samples of intact and gaping tissue were excised from the fillets. The tissue samples, approximately 1 cm x 1 cm x ½ cm, were immersed in OCT and carefully frozen in liquid nitrogen. The samples were since stored at -80 ºC until further analysis.

Immunoflourescence. Immunostaining were performed with primary antibodies from mouse for C-4-S (mab2030, Millipore), C-6-S (mab2035, Millipore) and from rabbit for collagen (ab20033, abcam). Secondary antibodies used was from mouse (A21133, Invitrogen) and rabbit (q22074, Termo scientific). Cross-section of 10 µm were cut in a cryostat (Leica CM 3050, Leica Microsystem Wetzlar, GmBH, Heidelberg, Germany) and mounted on a poly-L-lysine coated glass slide which were kept in -20 until use. The section was then fixed with ice-cold aceton and permeabilized using 0.5 % triton in PBS for 15 min, To generate the antigenic epitopes, the sections were digested with chondroitinase ABC lyase (cABC) from *Proteus vulgaris* (0.5 units/ml) in 0.1 M Tris-HCl buffer, pH 8. After cABC treatment for 2 h at 37°C, non-specific binding were blocked by using 5% non-fat dry milk powder dissolved in 1xPBS. Primary antibodies (1:100 diluted) in 2% non-fat dry milk in PBS was added and incubated over night at 4°C before washing with 1xPBS for

30 min. Subsequent incubation with secondary antibodies was performed for 2 hours, washing with 1x PBS for 30 min before using Dako fluorescent mounting medium (Glostrup, Denmark). Negative controls were incubated with secondary antibodies only. The section were cover-slipped using a Prolong Gold antifade (Life Technologies). The slides were examined by fluorescence microscopy analysis (apotome mode) (ZEISS Axio Observer Z1 microscope, Jena, Germany).

7.2 Supplementary material for paper II

Characterization of glycosaminoglycans in gaping and intact connective tissue of farmed Atlantic salmon (*Salmo salar*) fillets by mass spectrometry

Ása Jacobsen[1*], Xiaofeng Shi[2], Chun Shao[2], Jonhard Eysturskarð[1], Svein-Ole Mikalsen[3], Joseph Zaia[2]

[1]Aquaculture Research Station of the Faroe Islands, vid Áir, FO-430 Hvalvík, The Faroe Islands

[2]Department of Biochemistry, Center for Biomedical Mass Spectrometry, Boston University School of Medicine, 670 Albany Street, Boston, MA 02118, USA

[3]Department of Science and Technology, University of the Faroe Islands, Vestara Bryggja 15, Tórshavn, The Faroe Islands

[*]Corresponding author

Email addresses:

ÁJ: asa@fiskaaling.fo

XS: stephen.shi@gmail.com

CS: shaochunletdreamfly@gmail.com

JE: jonhard@fiskaaling.fo

SOM: sveinom@setur.fo

JZ: jzaia@bu.edu

Table S1. Details of HS disaccharides measured.

HS disaccharide	Ion species	m/z	Retention time (min)
D0A0	1-	378	108
	Dimer, 1-	757	108
	Adduct, 1-	468	108
D2A0/D0A2	1-	458	98
D2A6	1-	538	92
	2-	268.5	92
D0S0	1-	416	103
D2S0/D0S6	1-	496	96
	2-	247.5	96
	SO_3 loss, 1-	416	96
D2S6	1-	576	89
	2-	287.5	89
	SO_3 loss, 1-	496	89
	SO_3 loss, 2-	247.5	89
U0A0	1-	396	108
U2A0/U0A6	1-	476	98
U2A6	1-	556	92
	2-	277.5	92
U0S0	1-	434	103
U2S0/U0S6	1-	514	96
	2-	256.5	96
	SO_3 loss, 1-	434	96
U2S6	1-	594	89
	2-	296.5	89
	SO_3 loss, 1-	514	89
	SO_3 loss, 2-	256.5	89

Figure S1

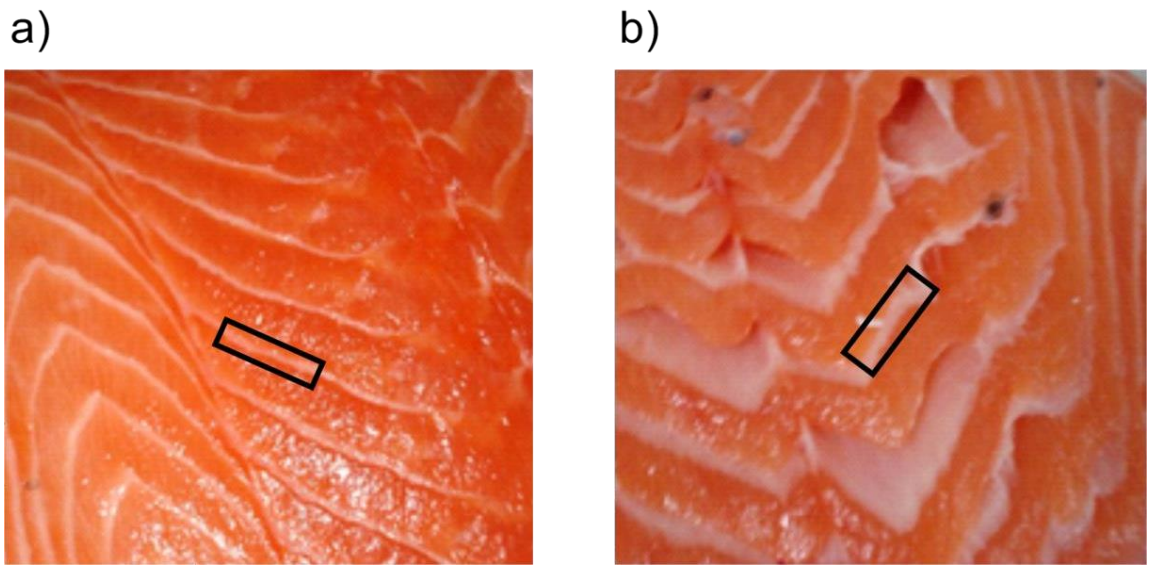

Figure S1. Fotographs of (a) intact and (b) gaping salmon fillets. Samples used in this study were obtained by cutting out the connective tissue and adjoining muscle as illustrated by the areas outlined in black. Photograph courtesy of Durita Nielsen. Copyright 2019.

Figure S2

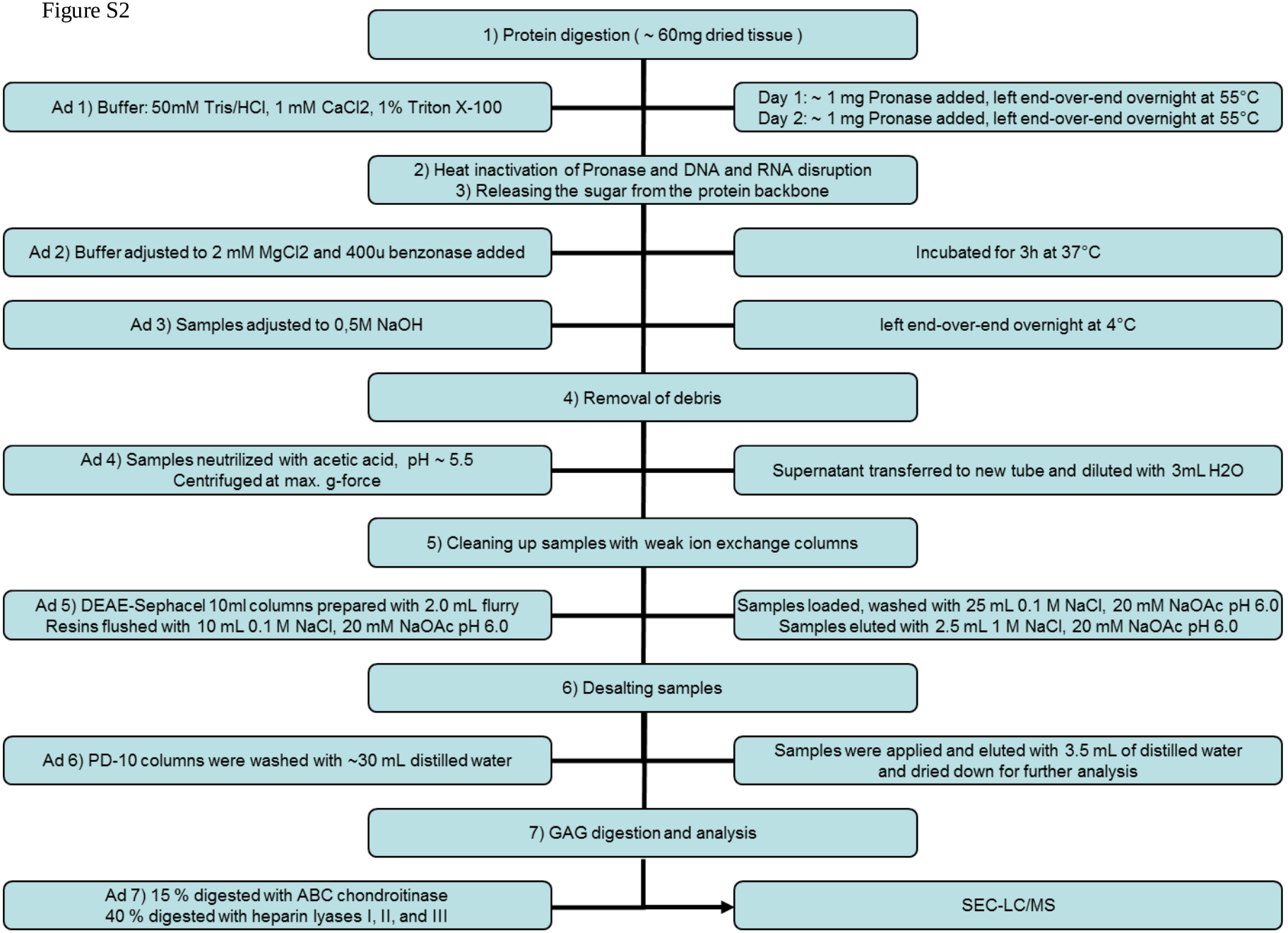
1) Protein digestion (~ 60mg dried tissue)
Ad 1) Buffer: 50mM Tris/HCl, 1 mM CaCl2, 1% Triton X-100
Day 1: ~ 1 mg Pronase added, left end-over-end overnight at 55°C
Day 2: ~ 1 mg Pronase added, left end-over-end overnight at 55°C
2) Heat inactivation of Pronase and DNA and RNA disruption
3) Releasing the sugar from the protein backbone
Ad 2) Buffer adjusted to 2 mM MgCl2 and 400u benzonase added
Incubated for 3h at 37°C
Ad 3) Samples adjusted to 0,5M NaOH
left end-over-end overnight at 4°C
4) Removal of debris
Ad 4) Samples neutrilized with acetic acid, pH ~ 5.5
Centrifuged at max. g-force
Supernatant transferred to new tube and diluted with 3mL H2O
5) Cleaning up samples with weak ion exchange columns
Ad 5) DEAE-Sephacel 10ml columns prepared with 2.0 mL flurry
Resins flushed with 10 mL 0.1 M NaCl, 20 mM NaOAc pH 6.0
Samples loaded, washed with 25 mL 0.1 M NaCl, 20 mM NaOAc pH 6.0
Samples eluted with 2.5 mL 1 M NaCl, 20 mM NaOAc pH 6.0
6) Desalting samples
Ad 6) PD-10 columns were washed with ~30 mL distilled water
Samples were applied and eluted with 3.5 mL of distilled water
and dried down for further analysis
7) GAG digestion and analysis
Ad 7) 15 % digested with ABC chondroitinase
40 % digested with heparin lyases I, II, and III
SEC-LC/MS

Figure S2. Outline of the GAG extraction and digestion procedures performed in the study

7.3 Supplementary material for Paper III

Characterization of glycosaminoglycans in gaping and intact connective tissue of farmed Atlantic salmon (*Salmo salar*) fillets by mass spectrometry

Ása Jacobsen[1*], Xiaofeng Shi[2], Chun Shao[2], Jonhard Eysturskarð[1], Svein-Ole Mikalsen[3], Joseph Zaia[2]

[1]Aquaculture Research Station of the Faroe Islands, vid Áir, FO-430 Hvalvík, The Faroe Islands

[2]Department of Biochemistry, Center for Biomedical Mass Spectrometry, Boston University School of Medicine, 670 Albany Street, Boston, MA 02118, USA

[3]Department of Science and Technology, University of the Faroe Islands, Vestara Bryggja 15, Tórshavn, The Faroe Islands

[*]Corresponding author

Email addresses:

ÁJ: asa@fiskaaling.fo

XS: stephen.shi@gmail.com

CS: shaochunletdreamfly@gmail.com

JE: jonhard@fiskaaling.fo

SOM: sveinom@setur.fo

JZ: jzaia@bu.edu

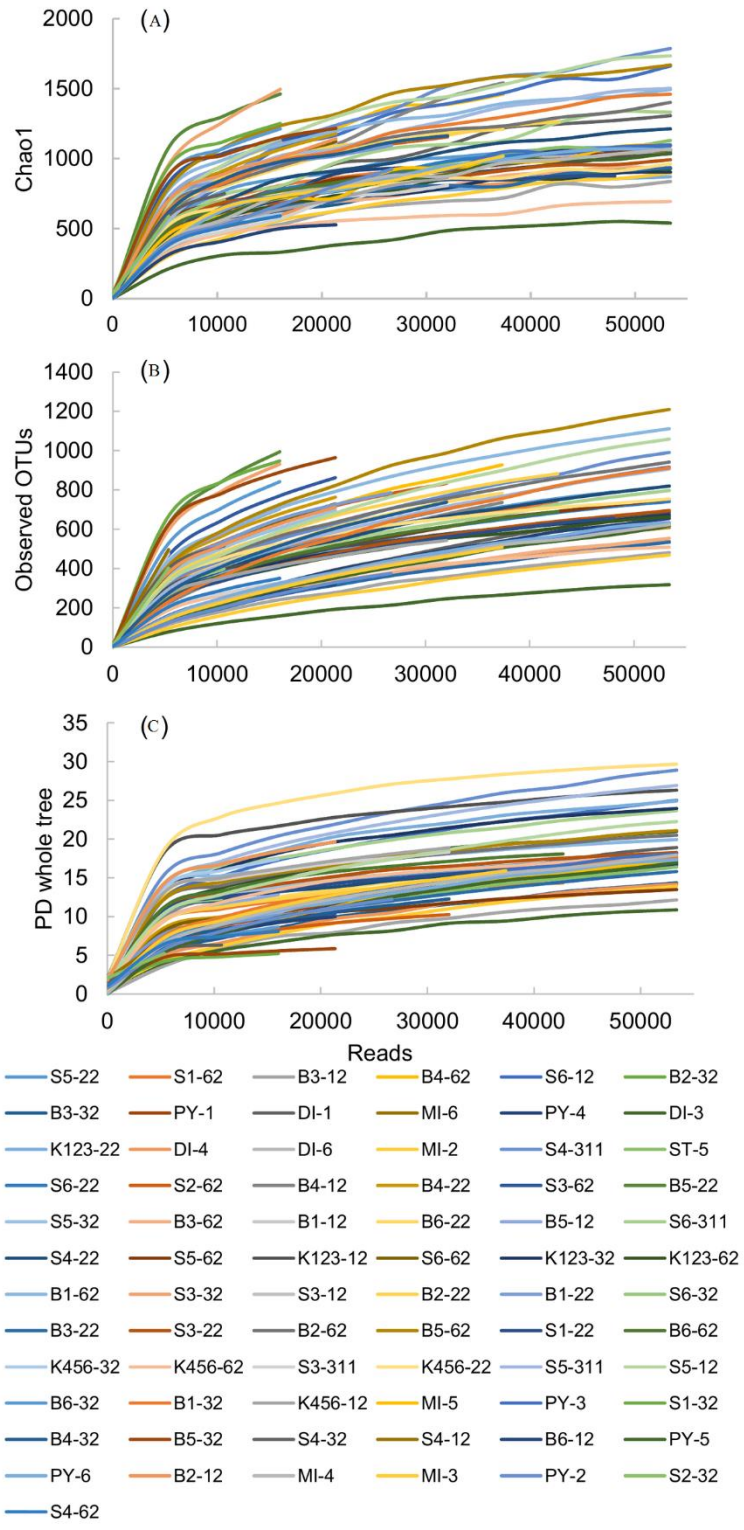

Figure S1. Rarefaction curves. Rarefaction curves for all samples (A) Chao1, (B) observed OTUs, and (C) PD whole tree alpha diversity estimates. Doi: 10.7717/peerj.7040/supp-1.

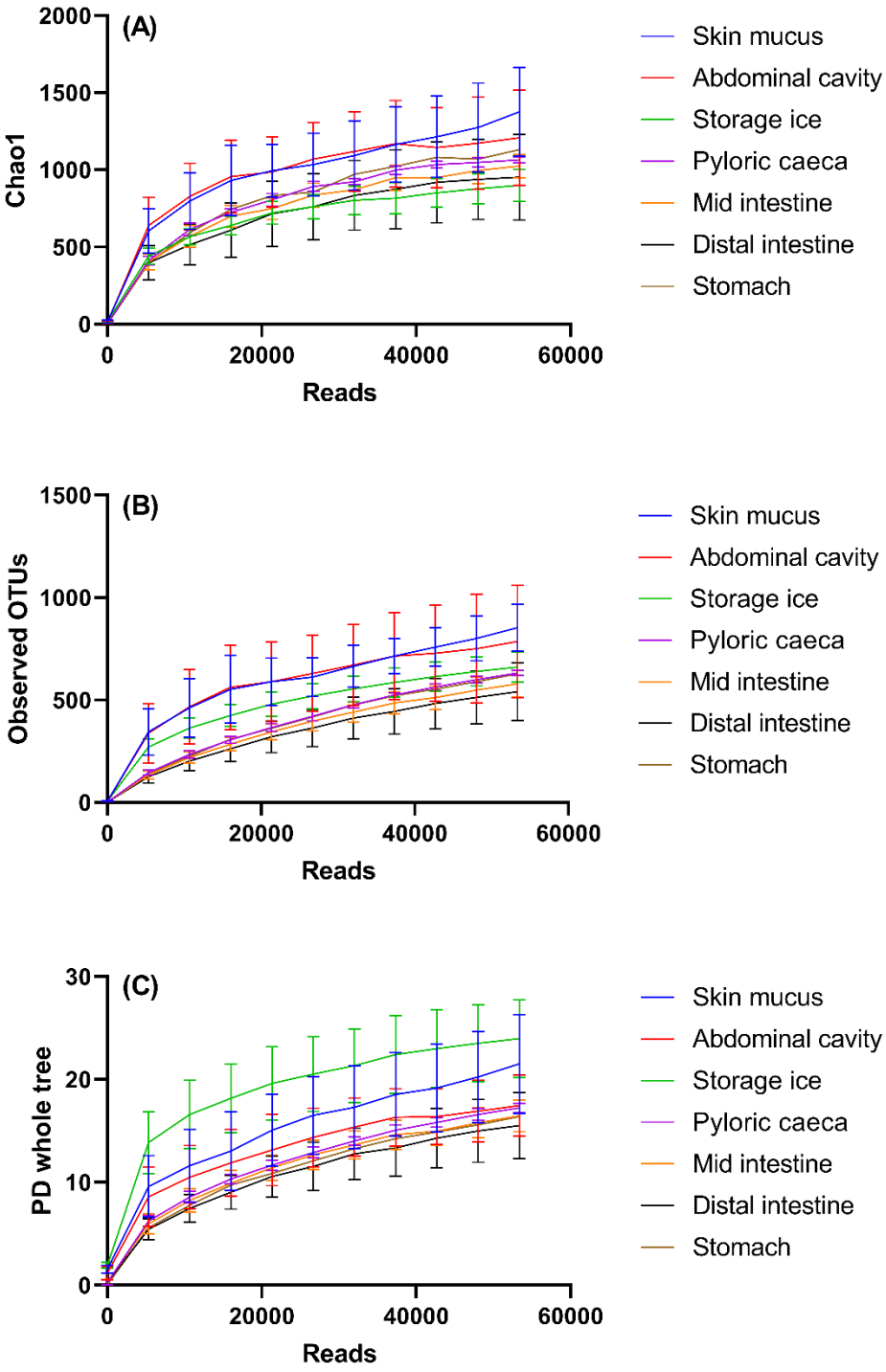

Figure S2. Group-wise rarefaction curves. Mean rarefaction curves with standard deviation of Chao1 (A), Observed OTUs (B) and PD whole tree (C) alpha diversity estimates for all sample types. Doi: 10.7717/peerj.7040/supp-2.

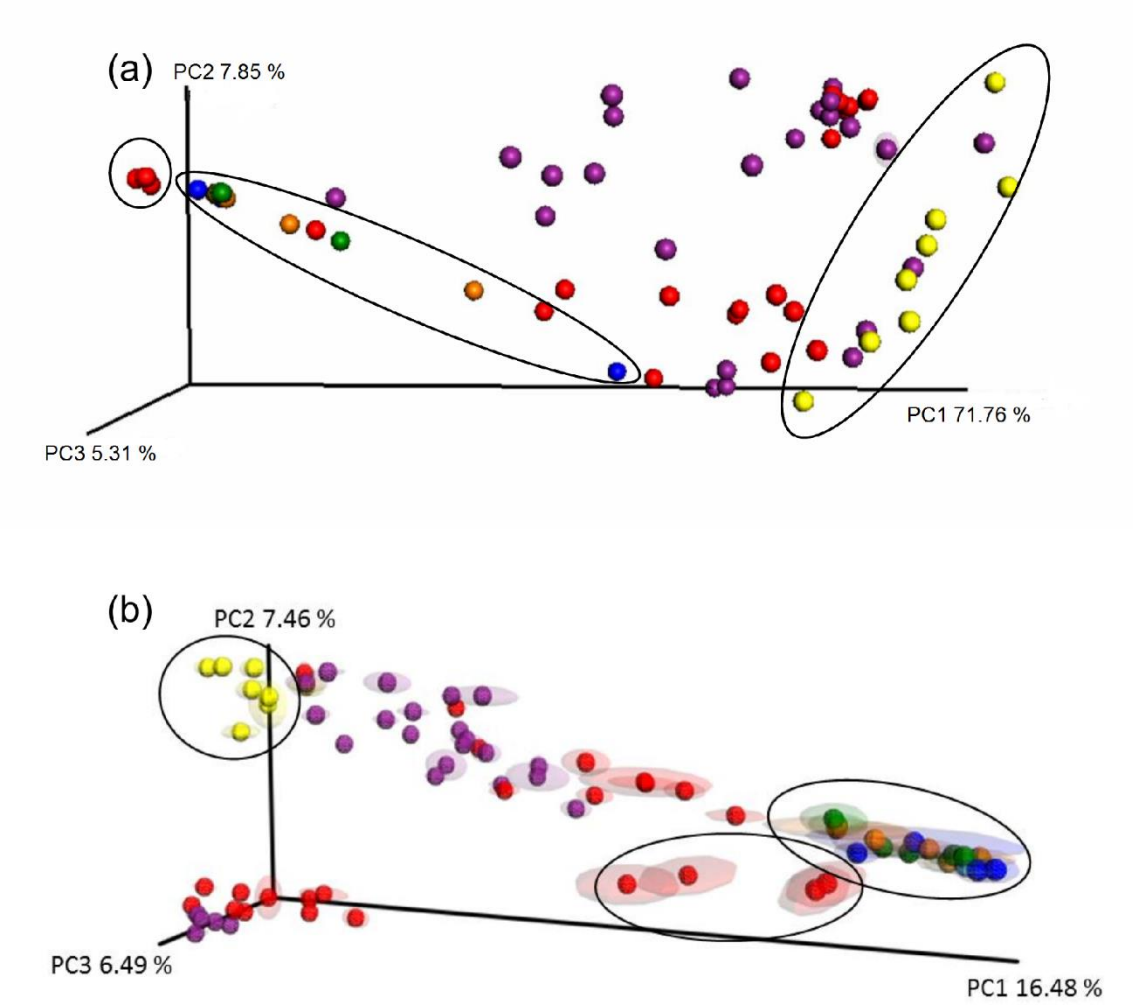

Figure S3. PC plots of beta diversities. PC plots of weighted (A) and unweighted (B) UniFrac calculations. Each coloured circle represents a sample. Red: abdominal samples, purple: skin samples, yellow: ice samples, green/blue/orange: digestive tract samples. The grouped ice samples and digestive tract samples are encircled in both plots. The encircled red abdominal samples are those from storage box no. 3. Doi: 10.7717/peerj.7040/supp-3.

Table S1. OTU table. Doi: 10.7717/peerj.7040/supp-4.

Due to a format not suitable for A4 print, readers are referred to the assigned DOI.

Table S2. Gelatinase activity measurements. Doi: 10.7717/peerj.7040/supp-5.

Initial screening:

Time(min)	Py-a	Py-b	MI-a	MI-b	DI-a	DI-b	S-a	S-b	B-a	B-b
0	1247,88	1364,05	1252,13	1307,3	193,2308	128,4498	17,7599	15,7962	-0,6313	-3,4124
10	1371,843	1559,503	1386,673	1467,583	391,2533	275,9793	27,8401	24,1293	1,0752	-2,3451
20	1459,237	1705,647	1480,807	1566,767	493,0454	361,2764	35,1569	30,7224	2,9892	-1,3005
30	1532,402	1812,792	1547,702	1635,862	554,2295	418,3445	41,3567	35,3398	4,5981	-0,6323
40	1594,492		1593,802	1694,762	597,1055	460,2395	45,941	39,2853	6,3869	-0,059
50	1654,909		1648,059	1747,389	632,4397	493,9657	49,5194	42,0399	8,1084	0,4519
60	1705,417		1683,057	1786,867	662,3481	522,5191	52,9257	45,0043	10,0893	1,0583
70	1749,768		1715,798		685,8834	546,2234	55,3555	47,1963	12,1553	1,3876
80	1785,569		1745,439		702,6238	566,3288	57,8044	48,8214	14,506	1,9561
90	1818,849		1770,829		717,9149	584,1439	60,3971	50,6609	16,6483	2,2723
100			1790,88		735,6437	601,3037	62,7348	52,1142	19,1572	2,7903
110			1816,348		751,5638	614,8278	64,4977	54,004	21,988	3,2488
120					763,4481	626,8111	66,667	55,3097	24,2354	3,7052
130					774,3269	636,6029	68,3626	56,28	27,0849	4,2559
140					784,6556	646,3006	69,2905	57,185	29,6224	4,5653
150					792,6535	655,0465	71,1355	58,6686	32,1362	4,9967
160					800,9055	663,3345	72,6265	59,5428	35,0942	5,406
170					809,2947	668,8217	73,5257	60,6316	37,9267	5,6535
180					816,8838	674,1518	75,2838	61,8477	40,5116	5,9995
190					821,3216	678,7926	76,0636	62,6074	43,294	6,3445
200					826,9562	682,7442	77,5902	63,7253	46,4956	6,7049
210					832,1176	685,9146	78,4726	64,4236	48,6117	7,144
220					837,3699	690,0089	78,8089	65,0793	50,7589	7,4735
230					841,9979	692,5059	80,4359	65,982	52,8517	7,7982
240					846,0013	693,5423	81,8093	66,8397	55,1273	8,1061
250					848,5438	695,7708	82,8098	66,9817	56,9396	8,3949

Time(min)	Py	MI	DI	S	B
0	1305,965	1279,715	160,8403	16,77805	-2,02185
10	1465,673	1427,128	333,6163	25,9847	-0,63495
20	1582,442	1523,787	427,1609	32,93965	0,84435
30	1672,597	1591,782	486,287	38,34825	1,9829
40	1594,492	1644,282	528,6725	42,61315	3,16395
50	1654,909	1697,724	563,2027	45,77965	4,28015
60	1705,417	1734,962	592,4336	48,965	5,5738

70	1749,768	1715,798	616,0534	51,2759	6,77145
80	1785,569	1745,439	634,4763	53,3129	8,23105
90	1818,849	1770,829	651,0294	55,529	9,4603
100		1790,88	668,4737	57,4245	10,97375
110		1816,348	683,1958	59,25085	12,6184
120			695,1296	60,98835	13,9703
130			705,4649	62,3213	15,6704
140			715,4781	63,23775	17,09385
150			723,85	64,90205	18,56645
160			732,12	66,08465	20,2501
170			739,0582	67,07865	21,7901
180			745,5178	68,56575	23,25555
190			750,0571	69,3355	24,81925
200			754,8502	70,65775	26,60025
210			759,0161	71,4481	27,87785
220			763,6894	71,9441	29,1162
230			767,2519	73,20895	30,32495
240			769,7718	74,3245	31,6167
250			772,1573	74,89575	32,66725

Abdominal samples:

	Abdomen day 2					
Time (min)	B1a	B2a	B3a	B4a	B5a	B6a
0	6078,217	555,9667	49791,07	-2820,78	-2875,43	-5447,18
10	6744,933	1249,883	72629,23	-1845,92	-2048,77	-4587,22
20	7178,767	1567,017	94989,37	-1294,18	-1460,18	-4038,38
30	7533,683	2000,933	115702,4	-869,067	-1088,02	-3689,07
40	8101,55	2295,75	135707,2	-255,85	-628,7	-3144,8
50	8588,8	2570,6	154665,9	174,45	-208,9	-2784,85
60	8963,417	2836,317	172539,6	601,8167	119,2667	-2456,73
70	9272,517	3058,717	189568,9	1086,017	526,2667	-1978,98
80	9740,917	3257,667	205126,6	1432,917	771,5167	-1737,13
90	10047,12	3459,017	220286,4	1933,167	1065,817	-1366,98
100	10292,92	3709,367	233957,3	2292,567	1357,417	-1047,48
110	10534,93	3963,483	247100,5	2729,383	1541,133	-742,617
120	10724,57	4078,767	259106,2	3170,167	1968,067	-450,783
130	11171,87	4361,467	271074,7	3428,467	2220,667	-190,583
140	11219,07	4445,967	281124,5	3952,067	2587,967	34,56667
150	11152,05	4726,9	291780,5	4272,7	2720,55	361,25
160	11881,45	4824,85	301698,4	4547,5	2907,2	564,55
170	11343,7	4958,55	310057,9	4727,4	3340,4	892,45
180	11821,98	5109,533	318409,2	5074,583	3340,233	1185,583

190	11618,38	5356,033	326000,7	5367,783	3721,383	1396,933
200	11927,75	5478,85	333714,1	5564,3	4101,95	1613,55
210	12086,77	5563,167	340511,3	5766,367	4377,017	1862,417
220	12284,6	5629,15	346929,4	6059,65	4486,2	2116,1
230	12430,33	6146,333	352976,9	6304,333	4824,433	2322,533
240	12536,88	5887,633	358477,8	6567,383	4904,933	2511,183
250	12738,48	6032,033	364855,6	6868,933	5220,433	2743,583
260	12957,6	6411,5	370109,6	6798,95	5361,1	2924,1
270	13032,53	6519,583	374462,6	7123,383	5585,533	3127,383
280	13242,57	6680,267	379364,7	7340,967	5848,067	3253,767
290	13378,52	6811,867	384060,3	7599,617	6037,517	3515,967
300	13483,62	6830,417	388769,8	7799,317	6316,667	3616,367
310	13599,18	7274,483	392144,7	7856,933	6432,883	3852,433
320	13733,43	7673,383	396914,1	8263,433	6674,183	3999,933
330	13849,23	7614,483	400692,8	8427,683	6842,133	4170,883
340	13840,35	7823	403712,2	8582,7	6888,25	4341,1
350	14089,23	7947,333	406402	8707,283	7118,033	4404,283
360	14182,65	8130,25	410045,3	8785	7294,05	4626,85
370	14261,17	8115,867	411883,2	9068,467	7334,667	4761,517
380	14392,67	8083,467	417085,1	9197,617	7531,467	4905,117
390	14507,98	8019,033	420015,8	9324,533	7703,333	5056,033
400	14582,08	8062,233	423369,7	9543,083	7925,233	5261,733
410	14664,25	8032,35	423979,9	9663,8	7941	5362,6
420	14790,3	8359,2	428547	9859,85	8210,45	5520,45
430	14811,75	8621	432172,1	10046,05	8336,2	5643,2
440	14899,02	8691,617	432773,7	10121,97	8442,867	5804,367
450	14987,9	8793,4	436469,5	10313,1	8610,5	5907,4
460	14993,45	8892,05	439956,1	10495,5	8705,95	6024,9
470	15078,03	8849,983	442391,7	10549,68	8823,733	6196,433
480	15143,02	9161,267	443093,1	10676,62	8945,067	6302,967
490	15226,35	9230,4	444828,2	10841,25	9099,3	6390,3
500	15243,63	9228,633	445000,1	10975,78	9162,683	6472,783
510	15243,95	9095,55	449225,8	11105,3	9309,65	6579,4
520	15318,42	9364,417	451608,8	11253,92	9462,317	6743,617
530	15331,6	9530,7	453428,8	11332,3	9613,9	6808,45
540	15433,13	9644,883	454852,1	11325,23	9713,783	6882,733
550	15435,85	9686,55	457879,6	11631,25	9791,7	7002,35
560	15490,42	9781,917	459600	11779,87	9925,867	7105,817
570	15529,97	9773,267	460452,4	12063,97	10023,02	7187,567
580	15586,17	9727,467	462056,8	12295,72	10172,07	7283,567
590	15679,98	9739,283	463466,7	12218,33	10186,38	7364,333
600	15794,5	9742	464829,6	12152,65	10292	7443,35
610	15832,53	9887,983	466271,3	12561,53	10404,78	7589,383
620	15816,85	9964,8	467561,2	12589,7	10483,8	7649,45
630	15871,7	9983,45	468292,7	12553,55	10609,9	7746,3

640	15696,23	9833,583	469967,8	12858,73	10721,43	7838,383
650	15728,3	9926,6	471126,6	13184,95	10780,85	7916,1
660	15819	10289,35	471600,9	13161	10924,25	7990,55
670	15821,37	10179,32	472124,9	13419,52	10997,92	8087,067
680	15768,42	9858,667	473160,7	13327,47	11098,47	8118,017
690	15813,85	10359,15	473606,7	13584,85	11185,15	8206,85
700	15847,48	10388,93	473794,2	13485,58	11333,33	8237,333
710	15801,4	10038,95	475624,3	13652,85	11460,3	8319,5
720	15816,58	10568,88	476817	14090,28	11546,33	8350,983
730	15779,27	10165,27	477228	14143,07	11643,52	8443,717
740	15784,63	10457,83	478044,3	14157,18	11695,73	8496,033
750	15779,4	10630,6	478621,6	14354,7	11812,25	8557,3
760	15832,38	10265,63	478834,2	14466,83	11969,73	8668,983
770	15826,65	10671,75	479271,3	14601,45	12039,2	8708,35
780	15837,05	10588,6	479934,6	14591,2	12135,75	8746,5
790	15846,43	10600,13	479828,9	14740,28	12237,98	8824,633
800	15892,32	10838,92	481266,5	14848,07	12357,67	8902,667
810	15874,72	10376,02	480617,2	14975,67	12458,97	8990,467
820	15873,68	10852,83	480838,8	15069,68	12533,78	9042,883
830	15901,43	10827,28	480674,9	15149,68	12692,33	9099,633
840	15871	10629,85	480030,8	15223,75	12793,15	9163,3
850	15963,83	11021,78	480283,3	15341,43	12905,93	9242,733
860	15881,47	10578,57	480000,2	15509,27	12931,37	9250,067
870	15917,17	11038,27	479649,6	15642,82	13069,32	9347,767
880	15945,17	10653,82	479833,7	15663,62	13185,17	9376,317
890	15945,82	10967,02	479470,6	15876,17	13263,17	9471,917

	Abdomen day 3					
Time (min)	B1b	B2b	B3b	B4b	B5b	B6b
0	21594,62	14831,92	10616,02	-1427,78	-3798,88	9830,867
10	22473,83	15676,08	17256,83	-437,267	-3092,87	10563,33
20	23106,92	16029,47	23639,27	48,06667	-2727,73	10957,67
30	23579,03	16038,78	29992,63	377,7833	-2472,12	11311,93
40	23860,2	16223,1	36096,3	893,55	-2149,55	11658,75
50	23970,95	16362,3	42199,1	1268,7	-1893,3	11939,2
60	24300,77	16527,92	48301,77	1581,167	-1690,68	12218,22
70	24504,27	16654,87	54508,37	1900,417	-1471,18	12509,52
80	24848,82	16705,52	60381,72	2329,967	-1303,38	12703,02
90	25276,17	16768,52	66188,82	2630,067	-1096,68	12942,22
100	25228,27	16823,62	72007,27	2874,467	-966,033	13207,62
110	25309,13	16853,73	77564,53	3199,133	-710,817	13415,03

120	25486,42	16867,72	83122,67	3444,767	-594,083	13589,92
130	25463,22	16880,87	88579,67	3636,217	-438,383	13782,37
140	25522,12	16927,97	93837,97	3925,917	-290,783	14015,17
150	25716,15	17150,05	98761,5	4236,95	-129,7	14230,95
160	25865,65	17131,05	103833,4	4462,95	0,6	14359,25
170	25944,75	17248,9	108763,4	4677,35	163	14444,85
180	25842,53	17284,93	113384,2	4877,583	291,3833	14626,63
190	25935,23	17314,18	118030,2	5134,583	396,8833	14767,78
200	25993,9	17357	122473,1	5316,5	559,7	14890,4
210	26078,92	17444,92	126977,8	5475,817	699,4167	14878,07
220	26164,9	17567,25	131342,4	5656,7	771,35	14959,8
230	26450,98	17542,23	135261,9	5881,233	912,7833	15115,33
240	26329,18	17611,28	139159,3	5979,233	1032,433	15197,23
250	26283,18	17579,48	143193,6	6218,183	1143,133	15308,43
260	26748,05	17691,25	146938,6	6355,5	1228,55	15418,9
270	26459,88	17869,88	150604,1	6502,833	1341,783	15547,73
280	26524,57	17892,27	154412,7	6671,667	1429,017	15632,62
290	26717,67	17933,27	158122,8	6840,517	1555,717	15799,22
300	26651,92	18014,87	161666,3	6967,967	1671,667	15909,87
310	26727,08	18017,38	165144,2	7060,133	1761,433	15945,78
320	26876,93	18118,53	168622,6	7235,533	1880,083	16050,83
330	26932,13	18120,68	171443,8	7295,283	1919,583	16171,63
340	27070,5	18245,2	174827,2	7423,1	2047,05	16252,65
350	27205,63	18307,83	178111,5	7590,683	2108,333	16316,28
360	27290,9	18346,85	181199,3	7649,3	2223,15	16404,7
370	27390,27	18332,02	184006,2	7731,067	2315,567	16448,47
380	27543,97	18364,37	186879,1	7741,317	2412,117	16591,57
390	27508,93	18493,98	189794,8	7947,083	2513,633	16628,33
400	27643,93	18560,28	192819,2	8151,383	2594,283	16742,73
410	27648,3	18580,95	195212,4	8211,65	2645,4	16807,45
420	27758,95	18618	198090	8371,65	2728,6	16880,9
430	27692,6	18650,2	200546,6	8427,9	2803,1	16946,85
440	27695,72	18648,87	202885,7	8537,317	2853,267	17004,27
450	27803,45	18778	205534	8641,35	2957,85	17039,35
460	27847,4	18754,45	207788,1	8704,7	3000,85	17071,2
470	27885,83	18852,93	210002,7	8796,033	3084,233	17108,78
480	27961,07	18847,42	212285,6	8945,917	3160,217	17261,67
490	27938,35	18910,75	214616,7	9013,55	3209,2	17305,25
500	28007,98	18935,53	216674,1	9104,033	3244,933	17337,63
510	28091,6	19006,95	218776,8	9213,65	3311,2	17406,25
520	28224,12	18965,27	220832,3	9298,867	3386,817	17424,12
530	28204,7	19036,2	222967,8	9365,75	3453,1	17484,4
540	28228,08	19059,88	224764,1	9444,683	3464,133	17501,43
550	28315,85	19010,85	226520,6	9503,35	3542,05	17578,45
560	28354,57	19093,02	228497,5	9585,267	3598,367	17659,02

570	28420,57	19210,77	230067,4	9651,067	3659,967	17710,77
580	28443,42	19056,87	232018,3	9753,217	3680,267	17741,87
590	28393,73	19035,83	233603,2	9794,883	3755,483	17769,93
600	28388,7	19221,25	235070,1	9877,2	3801,9	17817,7
610	28467,58	19131,23	236777,3	9912,633	3859,283	17845,53
620	28486,5	19371,75	238313,7	10019,85	3910,15	17936,45
630	28535,65	19157,15	239933,7	10086,8	3945,3	17958,1
640	28656,38	19300,48	241021,3	10134,73	3992,383	18071,18
650	28719,15	19284,6	242722,1	10209,15	4051,7	18093,55
660	28729,05	19267,65	244168,4	10273,15	4117,7	18144,95
670	28829,87	19088,67	245494,4	10314,47	4133,317	18166,27
680	28815,37	19247,27	246565,2	10335,52	4141,067	18228,62
690	28973,9	19384,3	248147,2	10407,6	4204,8	18262,55
700	28796,48	19447,48	249352,7	10451,53	4231,633	18277,58
710	28875,6	19179,65	250583,3	10516,2	4250,7	18306,35
720	28990,33	19298,43	251727,5	10565,28	4305,483	18323,03
730	28973,57	19387,87	252433	10598,17	4322,617	18180,02
740	29015,53	19433,33	253712,3	10669,38	4356,533	18216,13
750	28967,25	19382,3	255086,6	10728,3	4388,85	18184,75
760	29074,93	19546,68	255635,2	10777,13	4449,483	18216,83
770	29099,85	19389,4	256820,3	10831,25	4462,85	18289,8
780	29120,8	19263	257982,1	10841,55	4494,1	18257,4
790	29184,78	19423,43	258487,4	10901,83	4558,333	18272,18
800	29187,32	19590,37	259551	10976,92	4597,817	18342,62
810	29211,02	19651,92	260501,2	11014,97	4617,017	18400,97
820	29346,78	19704,53	261233,3	11058,18	4639,333	18420,03
830	29428,78	19696,98	262029,4	11112,58	4672,283	18456,98
840	29438,95	19757,15	263070,8	11144,25	4704,6	18514,85
850	29557,18	19774,58	263675,8	11214,38	4746,033	18495,18
860	29482,22	19748,47	264479,2	11241,37	4768,467	18552,22
870	29495,37	19623,62	265292,1	11288,67	4782,417	18581,12
880	29444,42	19724,87	266054,7	11327,27	4871,367	18652,92
890	29492,57	19775,02	266682,1	11386,62	4877,617	18645,87

	Abdomen day 4					
Time (min)	B1c	B2c	B3c	B4c	B5c	B6c
0	37981,97	872,8067	33259,77	14555,32	8039,367	1478,517
10	38159,08	1279,828	39383,48	15370,93	9578,283	2453,783
20	37752,62	1319,847	44558,42	15808,27	10572,92	3022,417
30	37465,98	1195,443	49267,58	16196,38	11340,78	3471,283
40	37295	1313,11	54051,55	16572,65	12252	3984,85
50	36858	1500,85	58416,7	16960,05	13004,1	4309,1
60	36341,67	1323,602	62784,82	17214,67	13516,67	4694,067
70	35989,47	1603,492	67346,87	17450,57	14125,72	5099,517

80	36374,37	1303,287	71768,07	17794,52	14621,07	5375,117
90	35975,22	1489,512	76313,87	18133,87	15187,92	5691,567
100	35717,22	1417,792	80658,77	18426,27	15671,47	5961,267
110	35594,83	1407,018	84884,03	18738,33	16098,43	6300,133
120	35129,92	1525,837	88964,17	18921,07	16564,42	6557,067
130	34913,57	1443,382	93323,67	19126,92	17023,47	6753,567
140	36987,72	1521,192	97418,47	19348,67	17390,42	7030,717
150	34549,35	1556,34	101526,5	19572,8	17776,6	7263,6
160	34515,9	1654,73	105507,9	19647,6	18086,55	7477,6
170	34459,05	1677,825	109495,4	20166,6	18514,5	7668,15
180	34238,28	1731,393	113571,7	20235,18	18922,48	7832,183
190	34143,28	1769,433	117308,2	20409,73	19112,08	8081,183
200	34076,65	1803,565	121199,1	20607,35	19433,95	8253,7
210	34115,97	1899,072	124852,3	20730,32	19682,22	8443,217
220	33970,2	1975,72	128589,9	20880,5	19905,25	8637,05
230	33934,48	2124,593	131851,9	20986,03	20087,58	8781,133
240	33904,88	2164,278	135424,8	21175,73	20240,38	8959,733
250	33781,83	2240,823	138863,1	21363,28	20212,43	9115,883
260	33828,05	2197,52	142346,6	21570,25	20220,75	9305
270	33798,08	2245,558	145697,1	21753,78	20271,28	9431,333
280	33772,02	2191,042	149062,2	21963,47	20505,57	9519,967
290	33658,92	2278,412	152440,8	22158,22	20576,62	9647,817
300	33622,57	2333,427	155423,3	22170,57	20729,62	9820,717
310	33497,63	2403,398	158739,7	22354,68	20799,93	9797,533
320	33442,33	2434,143	161807,6	22442,73	21111,53	10053,83
330	33371,98	2324,973	164938,8	22507,63	21175,73	10126,48
340	33327,8	2422,135	167960,7	22803,5	21323,4	10218,65
350	33429,18	2531,448	170981	22857,38	21462,23	10355,83
360	33439,7	2561,025	174005,8	22845,95	21564,1	10545,85
370	33254,67	2649,087	176888,7	23017,22	21730,32	10670,72
380	33222,97	2625,827	179675,6	23102,17	21837,17	10771,07
390	33166,78	2767,308	182575,3	23189,43	21994,23	10842,48
400	33179,78	2772,378	185476,7	23303,88	22201,78	11071,28
410	33046,9	2691,74	188140,9	23481,6	22346,3	11151,2
420	32924,8	2737,465	190721,5	23553,05	22527,8	11348,75
430	32886,95	2726,9	193349,6	23575,55	22757,85	11495,2
440	32781,12	2757,562	196118,2	23648,37	22808,37	11587,52
450	32637,6	2784,25	199059,5	23697,25	22998,8	11673,45
460	32720,6	2745,585	201503,1	23773,85	23128	11759,5
470	32708,28	2763,973	203777,2	23802,83	23233,33	11889,93
480	32643,47	2756,912	206569,6	23887,37	23439,92	11974,42
490	32570,8	2758,885	208695,7	23960,05	23589,35	12068,6
500	32491,08	2717,938	211487,1	23922,23	23748,88	12097,43
510	32243	2768,295	213680,3	23982,25	23905,15	12137,1
520	32164,47	2776,332	216299,8	24107,42	24121,47	12229,57

530	32027,45	2823,53	218349,8	24138,55	24278,4	12396,45
540	31882,88	2719,123	220919,6	24189,48	24395,73	12509,88
550	31887,05	2730,135	223119,6	24287,35	24538,65	12630,2
560	31866,57	2733,487	225437	24328,62	24625,02	12738,57
570	31665,47	2683,452	227962,4	24448,07	24886,62	12825,87
580	31735,47	2691,162	229952,8	24441,97	24985,77	12877,82
590	31527,43	2719,808	232517,7	24497,78	25072,43	12957,63
600	31499,35	2711,165	234414,6	24575,95	25158,75	13069,05
610	31431,43	2735,998	236316,3	24638,48	25321,88	13129,83
620	31383,35	2802,13	238675,7	24646,15	25424,8	13205,35
630	31292,65	2797,875	240909,7	24663,4	25556,65	13306,35
640	31329,13	2869,283	242826,3	24798,88	25701,08	13339,63
650	31191	2793,465	244668,6	24773,95	25837,9	13374,1
660	31372,75	2892,26	246743,9	24803,7	25995,85	13494,1
670	31155,27	2780,577	248586,4	24824,92	26123,97	13528,92
680	31160,07	2781,677	250276,2	24885,32	26136,67	13537,07
690	31127,1	2779,655	251887,2	24862,4	26155,35	13631,85
700	31040,53	2729,038	253947,7	24919,48	26273,63	13657,08
710	30955,55	2727,29	255667,3	24947,6	26376,05	13723,45
720	30964,68	2754,838	257215,5	24964,08	26520,23	13781,18
730	30969,87	2728,172	259080	24965,02	26669,22	13824,87
740	30915,03	2730,658	260726,3	24965,93	26790,53	13878,58
750	30862,25	2720,44	262188,1	25045,3	26874,65	13915,45
760	30933,13	2699,318	264025,2	25050,23	27022,73	13991,98
770	30892,05	2631,47	265585,8	25169,3	27164,1	14003,2
780	30833,1	2602,305	267099,1	25130,55	27252,9	14054,75
790	30793,68	2634,138	268603,9	25150,93	27395,43	14086,33
800	30814,02	2659,522	270344,5	25189,52	27465,42	14109,07
810	30786,22	2661,132	271970,7	25223,47	27650,87	14203,92
820	30789,93	2669,353	273403,3	25249,98	27803,03	14213,63
830	30725,78	2635,928	274863,4	25313,08	27936,08	14253,18
840	30626,8	2670,07	276347,8	25291,25	28090,1	14297,85
850	30657,23	2596,348	277841,8	25382,78	28215,98	14343,13
860	30684,37	2572,762	279287,7	25369,72	28356,92	14403,87
870	30697,37	2605,197	280637,1	25373,32	28523,57	14383,52
880	30607,57	2626,302	281849,2	25442,27	28605,27	14404,97
890	30478,77	2575,187	283082,1	25471,27	28786,57	14437,17

	Abdomen day 7					
Time (min)	B1d	B2d	B3d	B4d	B5d	B6d
0	57631,52	56520,32	55076,02	45059,47	54728,32	33405,37
10	57446,63	58629,43	56125,33	45222,23	55256,58	33671,78
20	56966,92	60005,87	56027,02	45503,37	55146,72	33535,77
30	56349,88	61081,53	55647,03	45450,18	54642,33	33085,78

40	55516,2	62126,25	55469,65	45433,3	54312,5	32911,5
50	54704,85	62558,2	55226,25	45298,35	53834,8	32427,65
60	53959,07	62927,02	55035,22	45515,12	53388,92	31932,82
70	53009,32	62942,27	54929,27	45244,77	52807,07	31359,47
80	52171,42	63143,12	54492,07	44944,92	52912,42	30903,12
90	51342,97	63236,87	54670,52	44967,82	52622,67	30604,12
100	50951,72	63108,27	54447,02	45021,72	52583,67	30001,32
110	50151,18	62945,03	54467,93	44534,73	52176,18	29769,43
120	49530,07	63179,62	54327,27	44460,82	51574,92	29284,52
130	49128,97	63007,12	54090,92	44468,07	51589,77	29091,42
140	48840,92	62770,97	54337,12	44253,52	52063,17	28909,67
150	48456,6	62500,45	54355,05	44288,7	51757,15	28996,65
160	48021	62675,2	54307,5	44406,9	50554,6	29033,45
170	47465,9	62191,55	54560,5	44128,4	49893,95	28389,05
180	47030,93	61727,68	54752,28	44243,83	50369,58	27771,03
190	46500,13	61973,58	54717,73	44344,83	49438,13	27645,73
200	46256,8	61821,85	55071,6	44048,9	50056,35	27508,35
210	46033,17	61745,82	55207,57	44253,47	49222,37	27063,42
220	45476,1	61154,8	55325,9	43949,15	48860,1	26606,15
230	44988,63	61064,88	55457,23	44024,53	48834,83	26982,13
240	44681,08	61008,48	55624,63	43592,53	48676,68	26837,68
250	44117,43	60642,03	55863,83	43614,83	48563,68	25992,83
260	43875,25	60424,45	56202,15	43425,05	48215,1	26083,9
270	43556,13	60084,38	56496,48	43559,63	48090,13	25994,43
280	43130,67	60063,27	56291,47	43404,42	47690,02	26232,82
290	42786,17	59763,22	56705,02	43949,47	47975,82	25672,92
300	42490,37	59613,42	56913,12	43427,47	47642,07	25705,82
310	42063,13	58940,33	57069,78	43736,58	47706,48	25217,83
320	41907,68	58707,48	57351,38	43202,58	47907,73	25067,68
330	41749,08	58302,18	57457,98	43880,58	47643,83	24955,33
340	41537,8	57937,4	57800,9	44308,8	47476,15	24461,2
350	41275,43	57842,23	57991,73	43789,88	47263,13	24287,63
360	40965,9	57441,5	57879,9	43476,85	47557,2	24157,6
370	40909,22	57166,07	58124,52	44297,27	47270,62	23968,22
380	40795,07	56798,52	58485,52	43444,42	47195,87	23723,32
390	40992,68	56469,68	58634,73	43572,78	47266,03	23975,28
400	42901,28	56335,98	58977,13	43267,13	47159,03	24233,08
410	43038,4	56084,6	58952,7	42808,85	47096,65	23752,75
420	42891,85	55994,65	59215,75	42768,4	46990,35	23520,05
430	43068,45	55481,45	59170,8	43044,95	46765,6	23624,15
440	43251,12	55222,42	59498,77	43021,62	46725,77	23182,92
450	43816,1	54953,5	59741,8	42770	46556,3	23167,2
460	42764,15	54637,6	60009,6	42421,25	46365,15	23329,5
470	42228,03	54536,18	60171,23	42480,88	46190,43	23224,43
480	42297,57	54231,17	60447,47	42471,77	46453,72	23282,27

490	43409,45	53827,7	60687,65	42767,8	46426,2	23022,7
500	39814,73	53924,78	60653,63	42174,43	46564,88	23061,28
510	39108,1	53048,1	61268,7	42144	46499,7	22962,35
520	38940,47	52488,82	61354,02	42251,82	46279,57	23206,72
530	38865,2	52271,1	61858	42035,1	46177	23028,35
540	38885,58	52001,93	61860,63	41971,58	46120,13	22953,53
550	38783,15	51843,6	61987,15	41865,45	46158,9	22744,45
560	38531,17	51641,27	62401,62	41896,82	46180,12	22624,57
570	38745,42	51415,92	62358,12	41880,82	46220,27	22756,62
580	38583,57	51219,72	62551,57	41925,97	46196,22	22758,22
590	38647,33	50968,48	63050,23	41866,13	45961,38	22953,58
600	38708,8	50621	62842,9	41963,05	46013	22710,25
610	38646,48	50516,08	63487,78	41865,88	45950,58	22619,68
620	38552,45	50358,65	63493,3	42012,8	46135,15	22690
630	38339,55	50092,4	63660,7	42032,1	45805,45	22696,7
640	38252,63	49564,48	63814,58	41847,33	45803,98	22331,73
650	38228,2	48956,55	63914,75	41848,6	45656,6	22439,85
660	38484,3	48912,75	64385,95	41965,05	45512,35	22233,4
670	38143,52	48633,87	64493,02	41947,02	45367,17	22207,37
680	37912,82	48346,27	64752,77	41775,32	45306,32	22108,87
690	37734,85	48168,15	64800,9	41868,45	45508,25	22117,2
700	38198,98	47807,73	65002,33	41867,68	45406,58	22073,03
710	38658,35	47473,2	65407,4	41838,55	45371,5	22012,55
720	38988,23	47181,23	65196,78	41590,33	45296,53	21987,03
730	40499,77	46819,72	65862,37	41721,27	45201,67	21899,97
740	39810,38	46642,08	66063,73	41507,33	45081,78	22230,38
750	38826,5	46458,25	66095,95	41414,8	44954,8	22099,4
760	38901,18	46336,13	66264,43	41244,63	44922,83	21840,48
770	39352,6	46147,1	66393,85	41331,9	44603,85	21717,95
780	38250,95	46098,05	66536,4	41341	44589,65	21752,7
790	37687,38	45956,28	66870,08	41301,28	44777,13	21614,33
800	37353,02	45739,02	67097,47	41265,82	44748,22	21602,02
810	35965,17	45673,82	67243,17	41260,87	44666,72	21737,52
820	36095,18	45255,93	67525,58	41535,18	44747,28	21591,88
830	36201,93	45034,68	67752,83	41311,03	44710,43	21385,03
840	35906,3	44783,6	67871,05	41195,35	44508,2	21391,8
850	33757,28	44580,48	68076,68	41249,48	44849,58	21372,58
860	30714,72	44352,02	68130,42	41391,07	44816,42	21206,72
870	30109,17	44171,67	68342,72	42610,12	44950,77	21300,42
880	29956,77	44146,62	68746,97	42805,92	45024,47	21164,92
890	29071,37	43937,82	68744,27	42607,62	44980,67	21137,22

DNA deposition

The sequencing data supporting the conclusion of this article is available in the NCBI Sequence Read Archive database, study accession no.: **SRP149649**

7.4 Supplementary material for Paper IV

Initial metagenomic screening of microbial communities in stomach and intestines of four fish species inhabiting coastal waters

Á. Jacobsen[1§], J. Eysturskarð[1], S.-O. Mikalsen[2]

[1]Fiskaaling, Aquaculture Research Station of the Faroes, við Áir 11, Hvalvík, Faroe Islands

[2]Department of Science and Technology, University of the Faroe Islands, Vestara Bryggja 15, Tórshavn, Faroe Islands

[§]Corresponding author. Tel.:+298 774748; e-mail: asa@fiskaaling.fo

Figure S1. Rarefaction curves for all samples based on observed OTUs.