Effects of dietary fermented sugar kelp (*Saccharina latissima*) on Atlantic salmon (*Salmo salar*) intestinal morphology and redox status

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Abstract

The aquaculture industry is the world's fastest growing food production sector and there is a demand for new feed ingredients from lower marine trophic levels to replace the marine and vegetable resources now used in aquafeeds. Seaweeds, such as sugar kelp (*Saccharina latissima*), have been suggested as one such candidate due to their low use of resources and potential for net zero environmental impact. The low protein and high crude fiber content have until now limited their use in feed for carnivore fish species like Atlantic salmon (*Salmo salar).* A practical solution to this may be fermentation. However, little is known about how fermented sugar kelp (FSK) affects the intestinal health and redox status of fish.

The aim of this study was to evaluate if an inclusion of FSK in feed for Atlantic salmon post-smolt would affect any gut morphological parameters and redox status in the first segment of the mid intestine. A dose- response study with FSK inclusions of 1, 2, 3, and 4% was performed with Atlantic salmon post- smolt in triplicate groups over 10 weeks. A commercial relevant fish meal- based diet was formulated as reference. Growth and feed utilization were studied, and a morphometric analysis as well as a semi- quantitative evaluation of the gut morphology in the mid intestine were performed. In the same intestinal segment, a quantifiable analysis of the mucous cells was performed. Additionally, the redox status in the mid intestine was evaluated with the GSH/GSSG ratio as a biomarker.

The results demonstrated that the Atlantic salmon fed FSK inclusions ≤2% performed similar to the control group on growth performance and feed utilization, but a dose dependent decrease in final body weight and specific growth rate (SGR) was seen in fish fed FSK inclusions ≥3%. No morphometric changes due to dietary modulations were detected in the mid- intestine, but an increased amount of submucosal connective tissue was seen in the fish fed FSK 2% through semi- quantitative evaluation. Dietary FSK neither affected the number nor the size of mucous cells. An enhanced total glutathione (GSH) was seen in the fish fed FSK 1%, but the GSH/GSSG ratio was not affected by diet. Based on the results from this study, the suitable inclusion of fermented sugar kelp in diet for Atlantic salmon is sat to 2%. However, to determine the real potential of fermented sugar kelp as a feed ingredient for Atlantic salmon, further research is needed.

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Abbreviations

ANFs- Anti-nutritional factors

ANOVA- One-way analysis of variance

CF- Condition factor

DI- Distal intestine

FM- Fish meal

FO- Fish oil

FSK- Fermented sugar kelp

GI- Gastrointestinal

GR- Glutathione reductase

GSH- Reduced glutathione

GSSG- Oxidized glutathione

HES- Hematoxylin-eosin- saffron

HSD- Tukey's Honest Significant Difference test

KWt- Kruskal Wallis rank sum test

LAB- Lactic acid bacteria

LR- Linear regression

MI- Mid intestine

NLRM- Nonlinear regression model

NSP- Non- starch polysaccharide

PAS- Periodic acid- Schiff

PC- Pylorus caeca

PPC- Pea protein concentrate

ROS- Reactive oxygen species

SBM- Soybean meal

SBMIE- Soybean meal-induced enteritis

SNK- Student Newman Keuls post- hoc test

SPC- Soy protein concentrate

1. Introduction

1.1 Background: Trends and future prospective in aquaculture

The aquaculture industry is now the fastest-growing food-producing sector globally with an annual growth rate of 5.3 percent from 2001 to 2018 (Costello *et al.*, 2020). Fisheries have historically been important contributors to food safety, nutrition, and livelihoods, but wild catches have for some time been near or at the threshold for what the aquatic ecosystems can sustain (Beveridge *et al.*, 2013; Blanchard *et al.*, 2017; FAO, 2020). Aquaculture is now the dominating contributor of fish for human consumption worldwide. In 2017, 52 percent of the fish for human consumption and 17 percent of the world population's animal protein intake came from aquaculture (FAO, 2020). With a production of 54.3 million tonnes, finfish species are the dominating fishes for aquaculture (FAO, 2020). China is the largest producer of farmed finfish species, mainly freshwater species such as several carps and Nile tilapia (*Oreochromis niloticus*) (FAO, 2020). In Europe, the finfish aquaculture is dominated by coastal aquaculture with Atlantic salmon (*Salmo salar*) as the most valuable species counting for about 19% of the total value of internationally traded fish products in 2018 (FAO, 2020). Other important species in European finfish aquaculture are species of trout (*Oncorhynchus mykiss, Salmo trutta),* gilthead sea bream (*Sparus aurat*a), and European seabass (*Dichentrachus labrax*).

1.2 Salmon farming in Norway

The development of salmonid aquaculture as known today started in Norway the mid 1960s when fish farmers turned their interest from rainbow trout (*Oncorhynchus mykiss*) to Atlantic salmon (*Salmo salar)* (Tilseth, Hansen and Møller, 1991), and became the world's leader in both production and export of farmed Atlantic salmon in 2018 (FAO, 2020).

The Norwegian Atlantic salmon production is now one of the most profitable and technologically advanced fish production industries in the world (FAO, 2020). The production reached 1.3 million tonnes and an export value of over 70 billions Norwegian kroner (NOK) in 2019 (ssb.no, 2020). Moreover, according to the prognosis by Olafsen *et al.*, 2012, the Norwegian aquaculture industry is estimated a 5- fold production potentially by 2050.

1.3 Feed in salmon aquaculture

Traditionally, farmed Atlantic salmon were fed formulated diets containing mainly fish meal (FM) and fish oil (FO) as respective protein- and fat sources (Torstensen *et al.*, 2008). These two marine ingredients are favorable for use in aquafeeds for carnivorous species due to their excellent amino acid profile, high nutrient digestibility, and as a source of long chain highly unsaturated fatty acids (HUFA) (Gatlin *et al.*, 2007; Turchini, Torstensen and Ng, 2009). The availability of these marine ingredients are, however, finite and has led to worries about overfishing (Clarkson *et al.*, 2017). The logical choice has been vegetable sources of protein and lipid due to their high availability and relatively low production costs (Moldal *et al.*, 2014). The composition of salmon feed has therefore changed drastically since 1990, and a significant proportion of the marine ingredients are now replaced by plant ingredients (Figure 1.1) (Ytrestøyl, Aas and Åsgård, 2015; Aas, Ytrestøyl and Åsgård, 2019). The dominating resources for Norwegian salmon feed production are now of vegetable origin, where soy protein concentrate (SPC) together with rapeseed oil and camelina oil are the ingredients used in largest amounts (Aas, Ytrestøyl and Åsgård, 2019).

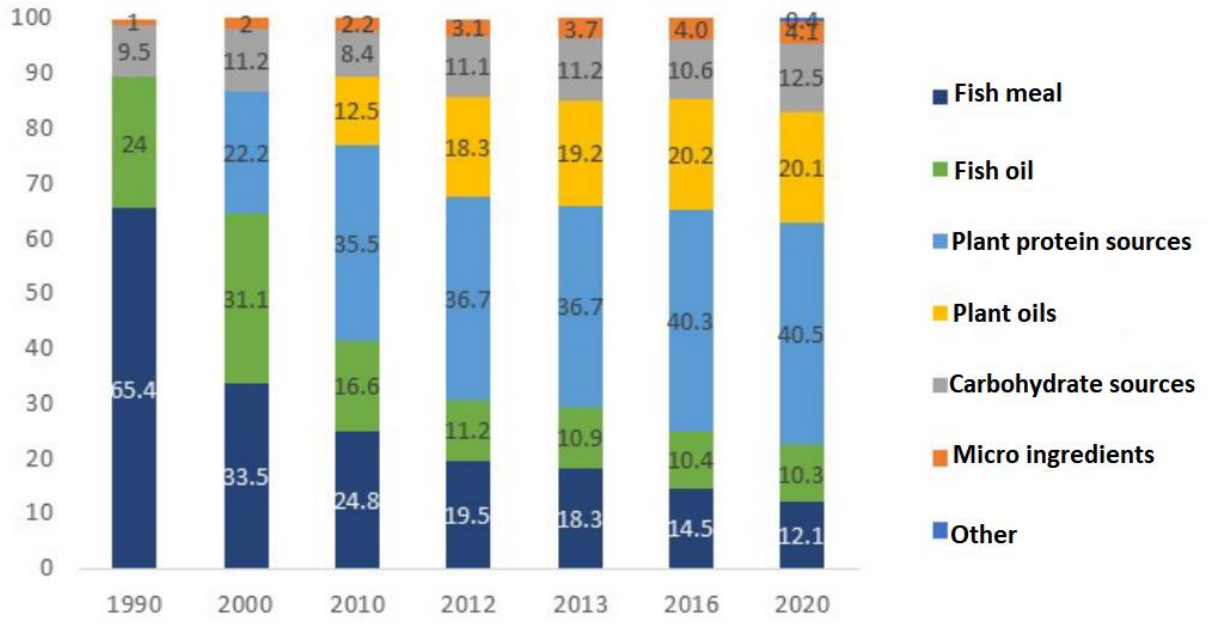

Figure 1.1 Ingredient sources (% of feed) in Norwegian salmon feed from 1990 to 2020 (Aas, Ytrestøyl and T. Åsgård, 2019)**.** Figure retrieved from Aas, Ytrestøyl and Åsgård, 2022 and translated by I.L.Øverbø . "Other" includes insect meal, one cell protein, fermented products, and micro algae.

Even though the shift from marine ingredients to vegetable feed ingredients has been beneficial from an economic point of view and has let the industry grow, it has not been a problem free transition (Aas, Ytrestøyl and Åsgård, 2019). One challenge is the existence of antinutritional factors (ANFs) in plant feed ingredients that may reduce nutrient digestibility and utilization,

and alter the function of the gastrointestinal tract (GI) (Krogdahl *et al.*, 2010; Moldal *et al.*, 2014; Bjørgen *et al.*, 2020). Another aspect with plant feed ingredients is the competition with land- based human food production, as an increasing proportion of cereal and soy production suitable for direct human consumption rather is channeled to fish feed production (Blanchard *et al.*, 2017). Due to these challenges associated with plant feed resources in salmon feed, much effort has been put into finding new sustainable and suitable ingredients from lower trophic marine sources for future fish feed production. Large volumes are needed for a new ingredient to make up large proportions of salmon feed, and only 0.4% of the ingredients used in Norwegian salmon feed in 2020 originated from "new" feed resources (Figure 1.1) (Aas, Ytrestøyl and Åsgård, 2022). Further, Norwegian raw materials contributed only 8% of the feed ingredients for Norwegian salmon feed in 2020, and this was the FM and FO (Aas, Ytrestøyl and Åsgård, 2022).

As more than 70% of the green house gas emissions originate from fish feed raw materials due to production and transport (Winther *et al.*, 2020), there are also incentives from the Norwegian Government to find suitable and local feed resources that facilitate for a more sustainable Aquaculture industry (Norwegian Ministry of Trade, Industry and Fisheries, 2021). One such alternative is seaweeds.

1.4 Sugar kelp: a potential feed ingredient

1.4.1 Production and chemical composition of sugar kelp

Saccharina latissima (Linnaeus), commonly known as sugar kelp ("sukkertare" in Norwegian), is a brown alga within the order of Laminariales and the family Laminariaceae (Lane *et al.*, 2006). Sugar kelp is found distributed along the northern hemisphere, all along the shore from Svalbard to Portugal in Europe (Bekkby and Moy, 2011). The cultivating of macroalgae has mainly been associated with Asian countries, but the first trials of kelp cultivating in Norway started around 2005 (Stévant, Rebours and Chapman, 2017). Farmed seaweeds are now dominating the global production of farmed aquatic algae and the production has more than tripled from 2000 to 2018: now representing 97.1 percent of the total 32.4 million tonnes wild-collected and cultivated algae (FAO, 2020). Cultivating of seaweeds is still dominated by countries in East and Southeast Asia with Japanese kelp (*Laminaria japonica*), Eauceuma seaweeds (Eucheuma spp.), and Gracilaria seaweeds (Gracilaria spp.) produced in the highest volumes (FAO, 2020). In Norway, the focus has been on large-scale cultivating of kelp species, mainly sugar kelp, that holds a potential for high biomass yields and can be cultivated without the use of land- area, fresh water, fertilizers, or pesticides (Skjermo *et al.*, 2014; Stévant,

Rebours and Chapman, 2017). Open aquaculture systems with fed species inevitably brings a loss of nutrients through feces and feed wastage, and seaweeds hold a potential for generating net positive environmental impacts as they absorb nutrients from the surrounding water (Venolia *et al.*, 2020). For that reason, *S.latissima* is grown on longlines as a part of integrated multitrophic aquaculture (IMTA) to capture nutrients from salmon cages (Granby *et al.*, 2020).

Brown seaweeds, like sugar kelp, consist mainly of carbohydrates, and the storage carbohydrates are primarily mannitol, laminarin and fucoidans, while alginate and cellulose serve as structural carbohydrates (Øverland, Mydland and Skrede, 2019). As to amino acid composition, *S. latissima* is rich in glutamic and aspartic acids, and the levels of leucine, phenylamine, threonine, tyrosine and valine are reported higher, while the levels of histidine and methionine are reported lower, compared to soybean meal (SBM) and FM (Biancarosa *et al.*, 2017). The crude protein content in *S. latissima* has been reported to count for about 8% (Biancarosa *et al.*, 2017) and 15% (Bruhn *et al.*, 2019) of the dry weight. Despite high levels of complex carbohydrates, sugar kelp can be used as a functional ingredient due to the richness in vitamins (Norambuena *et al.*, 2015) and essential minerals, particularly iodine, which can biofortify farmed fish when included in aquafeeds (Schiener *et al.*, 2015; Biancarosa *et al.*, 2018; Granby *et al.*, 2020). Macroalgae have been used in animal nutrition since the beginning of recorded writing, and seaweeds have been used for human consumptions for centuries in Asian countries (Evans and Critchley, 2014; FAO, 2020). In recent years, the interest in inclusion in feed for monogastric animals, including fish, has increased (Øverland, Mydland and Skrede, 2019; Thépot *et al.*, 2021).

1.4.2 Effects of brown seaweeds and its derived bioactive extracts on fish growth and intestinal health

Due to the high crude fiber and low protein content in brown seaweeds, their use in aquafeeds have by far been limited (Øverland, Mydland and Skrede, 2019). Thus, there are few available publications on inclusion of the whole form of sugar kelp or other brown seaweeds in feed for fish. Previous work have so far indicated that fish growth responses rely on the algae species and the inclusion level of seaweed (Ferreira *et al.*, 2020). Dried *S.latissima* have been fed rainbow trout at inclusions up to 2% without compromising growth performance or intestinal health during a 12 weeks trial in fish with initial weight of 200g (Granby *et al.*, 2020). However, the adverse health effects associated with higher inclusions of *S. latissima* as described by Granby *et al.* (2020) were seen at inclusions of 4% with reduced growth and specific growth

rate (SGR), and altering of the gut morphology. On the other hand, dietary inclusions with a seaweed meal derived from brown seaweeds (Laminaria sp.) in 3 and 10% inclusions in Atlantic salmon post- smolts have previously been reported to increase final body weight (FBW), weight gain (WG), and SGR in a feed trial of 30 days, while a 6% inclusion had no clear effect on growth (Kamunde, Sappal and Melegy, 2019). Supplementations with brown seaweeds have also been carried out in Atlantic cod (*Gadus morhua*) juveniles through inclusions of 10% egg wrack (*Ascophyllum nodosum*) in a 12- week trial, reporting reduced growth combined with altered gut morphology (Keating *et al.*, 2021). Thus, demonstrating a suitable inclusion level of fermented sugar kelp in the Atlantic salmon diet based on the growth and health parameters, particularly gut health, would be necessary and evaluated in this study.

There are however several previous publications focusing on the bioactive compounds found in brown seaweeds and their extracts as functional feed ingredients for fish and other monogastric animals (Øverland, Mydland and Skrede, 2019; Thépot *et al.*, 2021). The term "functional feed" is used to describe a feed that may benefit the animal beyond normal nutritional requirements (Tacchi *et al.*, 2011), and functional feed can be briefly broken into probiotics, prebiotics, and immunostimulants (Thépot *et al.*, 2021). Seaweeds are considered immunostimulants and can be used for non- specific (irrespective of antigenic specificity) immunostimulation through the diet (Vallejos-Vidal *et al.*, 2016; Thépot *et al.*, 2021). In Atlantic salmon blood, elevated levels of lysozyme have been seen when fed alginate derived from *Ascophyllum nodosum* (Gabrielsen and Austreng, 1998; Kiron, 2012), while the potential immunomodulatory effect of laminarin have been seen in rainbow trout as a significant increase in phagocytic activity in head kidney macrophages as well as an increase in production of TNFα and IL- 8 in gill tissue (Morales-Lange *et al.*, 2015). This immunomodulatory effect has also been seen in grouper (*Epinephelus coioides*) as increased expression of the immune response genes IL-1β, IL-8, and TLR2 in the mid intestine (Yin *et al.*, 2014). In fucoidan administered Nile tilapia (*Oreochromis niloticus*) increased growth and improved intestinal mucosal fold area have been reported (Mahgoub *et al.*, 2020), while a slight reduction in feed conversion efficiency have been reported in fucoidan supplemented rainbow trout (Papadopoulou *et al.*, 2022). Dietary supplementation with laminarin and fucoidan have also both been suggested to have a positive effect on gut health in pigs (Walsh *et al.*, 2013b, 2013a; Øverland, Mydland and Skrede, 2019).

To enhance the use of the whole form of brown seaweeds such as sugar kelp in aquafeeds, not only the bioactive compounds and extracts, while overcoming the high content of complex carbohydrates, a practical solution may be fermentation (Øverland, Mydland and Skrede, 2019).

1.5 Fermentation of seaweeds

Seaweeds, as land plants, contain polysaccharides that are limiting their digestibility (Silva *et al.*, 2015). The complex carbohydrates present in seaweeds, non- starch polysaccharides (NSPs), may act as ANFs (Silva *et al.*, 2015), and the digestibility of brown algae is particularly limited due to the alginate and fucoidan embedded in a matrix in the cell wall (Gupta and Abu-Ghannam, 2011). Fermentation has the potential to reduce these complex, indigestible carbohydrates and thus improve the seaweeds' nutritional value for fish (Wan *et al.*, 2019). Further, since the chemical composition of sugar kelp is seasonally dependent, it is generally harvested once a year to ensure optimal yield. Fermentation constitutes a method that enables for a stable year- round availability (Larsen *et al.*, 2021), which will be a prerequisite for use in commercial aquafeeds.

During fermentation, microbes metabolically convert the sugars present in stock material to acids, gases, and alcohol. Fermentation of seaweeds can be done spontaneously through ensiling (Herrmann *et al.*, 2015), but for commercial fermentation processes inclusion of bacteria in the order Lactobacillales (lactic acid bacteria (LAB) are often used (Wan *et al.*, 2019). Very few studies are conducted on fermentation of sugar kelp, and the main objectives have been on preservation for human consumption or for biorefining (Bruhn *et al.*, 2019; Akomea-Frempong *et al.*, 2021; Larsen *et al.*, 2021). Whether fermentation of sugar kelp affects the availability of certain nutrients and subsequently influences fish health remains to be studied.

The primary fermentation substrates in sugar kelp have been reported to be dominated by fucoidan and mannitol (Sørensen *et al.*, 2021a). Alginates are likely to be more resistant to microbial degradation (Uchida and Miyoshi, 2013; Campbell *et al.*, 2020), but the sugar alcohol mannitol is reported fermentable by Bruhn *et al.* (2019). Larsen *et al.* (2021) reported a reduction in the biomass glucose concentration during LAB fermentation of sugar kelp, and it is suggested that seaweeds contain endogenous enzymes which hydrolyze the complex carbohydrates like laminarin as fermentation substrate (Kiron, 2012; Campbell *et al.*, 2020).

Although there are no studies available on health effects of fermented seaweeds in salmonids, studies with lactic acid fermentation of SBM have, however, showed promising results. Available studies suggest a prebiotic- effect on the Atlantic salmon's intestinal microbiota compared to a FM- based control diet and SBM non- fermented diet (Catalán *et al.*, 2018). Fermented *Ulva prolifera* (green alga) have been fed red tilapia (*Oreochromis mossambicus* × *Oreochromis niloticus*), reporting increased SGR and enhanced serum non-specific immunity as well as increased digestive enzyme activity when compared to a SBM-based control diet (Yang *et al.*, 2016).

There are also additional benefits of fermentation that may improve its applicability in feed for Atlantic salmon. Very high iodine levels, which subsequently may cause adverse health effects in humans, have previously been reported in sugar kelp (1100mg kg^{-1}) (Biancarosa *et al.*, 2018). Some reduction in iodine, as well as a reduction in the chemical risk factor of trace metals such as sodium, cadmium, and mercury have been seen in LAB fermented sugar kelp (Bruhn *et al.*, 2019). Even though the publications on both fermentation of seaweeds and its use in aquafeeds are limited, the available literature is indicating that the breakdown of the complex carbohydrates might reduce the ANFs and thus enhance the digestibility. Further, it is possible that fermentation may reduce the associated chemical risk factors. Whether this is applicable when fed to Atlantic salmon remains to be studied.

1.6 Morphology and function of the salmonid gastrointestinal tract

The alimentary tract for Atlantic salmon is divided into the following characteristics: the mouth is followed by the oral cavity, the pharynx, the esophagus, the stomach, the intestine and the anus (Bjørgen *et al.*, 2020). The intestine (Figure 1.2) is divided into pyloric caeca (PC), mid intestine (MI) and the distal intestine (DI) (Moldal *et al.*, 2014). The MI may further be divided into a first and second segment, where several studies have shown that the second segment of the MI is more immunologically active than other segments in the GI tract (Bjørgen *et al.*, 2020). The enterocytes in the PC and the first segment of the MI are absorptive cells, while there is a strong uptake of macromolecules in the enterocytes characterized by supranuclear vacuoles in the second segment of the MI (Rombout *et al.*, 2011). The distal intestine is suggested to have an osmoregulatory function rather than a nutritional function (Rombout *et al.*, 2011). There is however no uniform nomenclature for gut segments in teleost fish used in the literature, and the gut is often loosely divided into the fore-, mid-, and hind- gut (Egerton *et*

al., 2018), the proximal and distal intestine (van den Ingh *et al.*, 1991; Silva *et al.*, 2015), or the anterior, middle, and posterior intestine (Dawood *et al.*, 2020).

Figure 1.2. The gastrointestinal tract of the Atlantic salmon. Schematic drawing derived from Løkka *et al.* 2013 of the salmon GI- tract with pyloric caeca. Ca is the cardiac stomach, 1) and 2) is pyloric caeca, 3) first segment of the MI posterior to the pyloric caeca, 4) second segment of the MI (indicated by black arrows), and 5) the posterior segment (DI).

The GI- tract is, together with the skin and gills, the main mucosal tissues in the fish (Sveen *et al.*, 2017). These mucosal tissues are the main routes of infections in fish, and are characterized by being covered in mucus which forms a physical barrier against potential harmful components (Jin *et al.*, 2015; Sveen *et al.*, 2017). The mucus consists of high- molecular- weight, gel-forming macromolecules where glycoproteins (mucins) are dominating. The mucus is produced by mucous cells (Figure 1.3) which remains the similar structure as goblet cells in mammalians (Shephard, 1994). An adaptive immune system that relies on the role of B and T cells is associated with the mucosal body surfaces in teleost fish, but the publications on this are scarce compared with mammalian literature (Salinas, 2015). It has thus been suggested that the number of mucous cells present in mucosal tissues in fish may reflect its health status (Pittman *et al.*, 2011; Sveen *et al.*, 2017).

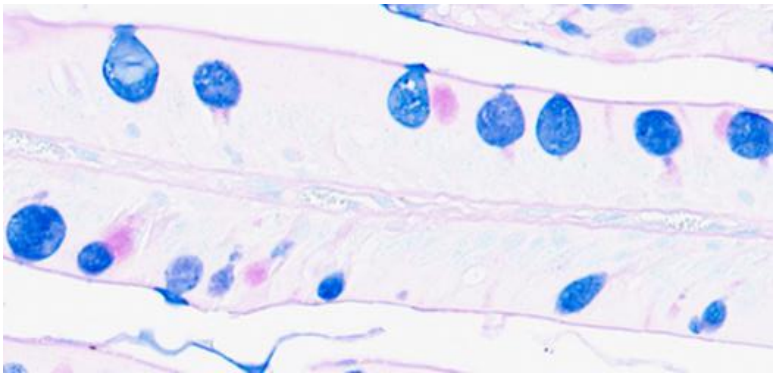

Figure 1.3: Mucus cells in the mid intestine of Atlantic salmon. The mucus cells are stained blue with Alcian Blue PAS. Illustration picture: I.L. Øverbø.

The gastrointestinal wall is built up by four layers (Figure 1.4). The first layer of the intestinal tract is the mucosa with its folds built up by stratified squamous epithelium (Kryvi and Poppe, 2016). The mucosal folds in the first segment of the MI are organized in simple folds while the second segment of the MI and the DI in addition have complex mucosal folds with branching (Løkka *et al.*, 2013). In contrast to the second segment of the MI and the DI, the enterocytes of the mucosal folds in PC and in the first segment of the MI do not have supranuclear vacuoles (Krogdahl, Bakke-McKellep and Baeverfjord, 2003; Bjørgen *et al.*, 2020; Verdile *et al.*, 2020). In the PC, increased vacuolization in the PC is reflecting an abnormal lipid droplet accumulation within the enterocytes (Li *et al.*, 2019). The role of the supranuclear vacuoles in the distal most segments of the intestine in well- fed Atlantic salmon intestine have not yet been described, but they disappear when the tissue is inflamed and during starving (Bjørgen *et al.*, 2020). In addition, the mucosal folds have a thin, delicate core of connective tissue called lamina propria (Kryvi and Poppe, 2016), which is located in the center of the mucosal folds (Knudsen *et al.*, 2007).

The second layer is the submucosa, which is located beneath the mucosa (Kryvi and Poppe, 2016). Submucosa consists of both loose and compact connective tissue, where the compact connective tissue segment is called stratum compactum (Knudsen *et al.*, 2007; Kryvi and Poppe, 2016). Both submucosa and lamina propria are susceptible for cellular (leucocyte) infiltration when inflamed (Krogdahl *et al.*, 2015). The third layer is the muscular layer termed tunica muscularis. Muscularis consists of an inner layer with circular muscle fibers, and an outer layer with longitudinal arranged muscle fibers (Kryvi and Poppe, 2016). The outermost and fourth layer toward the abdominal cavity is the serosa which is a thin layer of connective tissue lined by mesothelial cells (Løkka *et al.*, 2013; Kryvi and Poppe, 2016).

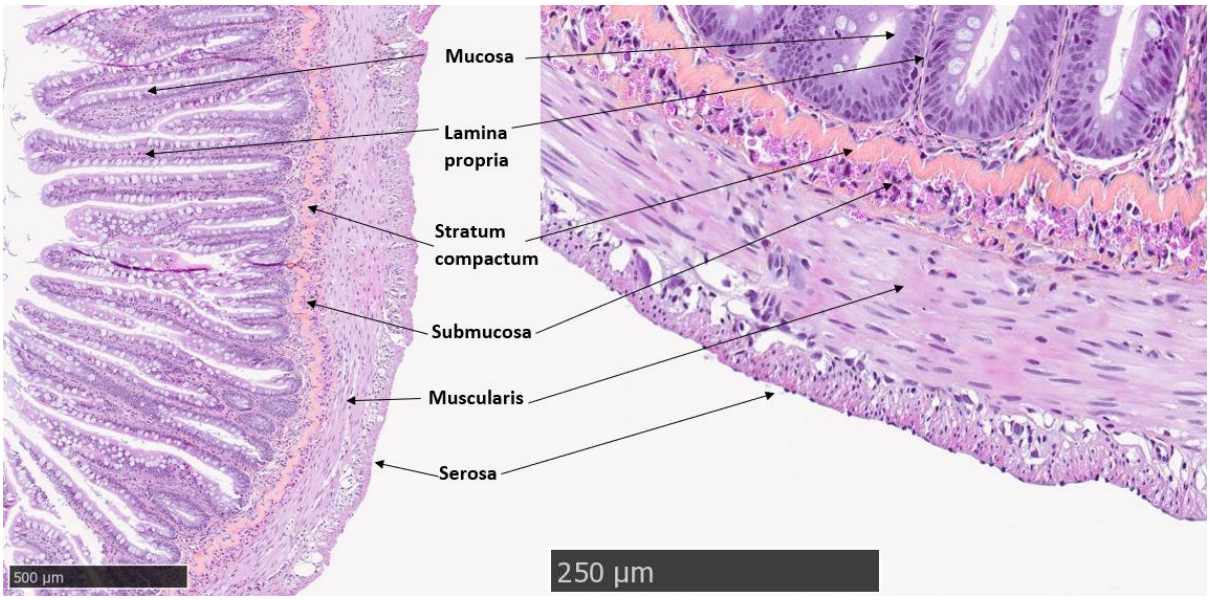

Figure 1.4: Morphology of the mid- intestine of Atlantic salmon. The picture shows the four layers of the intestine. The first layer is mucosa with its folds and the underlying lamina propria in the core of the mucosal folds. The second layer is the submucosa with stratum compactum. The third layer is muscularis, while the outermost and fourth layer is denoted serosa. Illustration picture: I.L. Øverbø.

1.7 Adverse reactions to dietary changes on intestinal morphology

Salmon species are originally strict carnivores and the formulated diet they are fed often differs significantly from their natural diet. The inclusion of plant- derived materials can unfortunately cause adverse effects and challenges with regard to fish health and nutritive value (Naylor *et al.*, 2009; Moldal *et al.*, 2014), and intestinal health is often used as a parameter to measure dietary impact.

It has been well documented during the last three decades that SBM induces a pathological condition in the second segment of the MI of Atlantic salmon, known as soybean meal induced enteritis (SBMIE) (van den Ingh *et al.*, 1991; Baeverfjord and Krogdahl, 1996; Knudsen *et al.*, 2007). The mechanisms underlying SBMIE are not fully understood (Djordjevic *et al.*, 2021), but the ANF soyasaponins, present in high levels in SBM, have been pointed out as the main compound causing the inflammatory response (Krogdahl *et al.*, 2015). Other plant ingredients have however also been seen to induce a similar inflammation response in the intestine of Atlantic salmon, although milder than the symptoms typical for SBMIE (Penn *et al.*, 2011; De Santis *et al.*, 2015). It has thus been suggested that the ANFs present in the plant ingredients cause the inflammatory response, but that fish cope better with lower levels of a mixture of ANFs than high levels of individual ANF such as the presence of soyasaponins in SBM (De Santis *et al.*, 2015).

The inflammatory reaction associated with SBMIE includes loss of supranuclear vacuoles in the absorptive enterocytes, widening of lamina propria with infiltration of inflammatory cells, increased amount of connective tissue due to infiltration of inflammatory cells between the base of the mucosal folds and stratum compactum, and shortening of mucosal folds (van den Ingh *et al.*, 1991; Knudsen *et al.*, 2007). An increased number of mucus cells in the distal intestine have also been observed in Atlantic salmon fed full- fat soybean meal (van den Ingh *et al.*, 1991). When evaluating the intestinal health from histological sections in Atlantic salmon feeding trials, the changes previously associated with SBMIE are often assessed. In Atlantic salmon, shorter intestinal folds have been seen when fed vegetable oils (Moldal *et al.*, 2014), and hyper vacuolization of the enterocytes (steatosis) in the pyloric caeca have been observed when fed lupin meal and wheat gluten meal (Gu *et al.*, 2014). Such hyper vacuolization in the pyloric caeca has also been seen with total replacement of FM with black soldier fly (*Hermetia illucens*) (BSF) meal, but also when fed the FM and SPC based control diet (Li *et al.*, 2020). Vacuolization of the enterocytes in pyloric caeca and the first segment of the MI have also been observed in Artic charr fed linseed oil (Olsen *et al.*, 1999, 2000). Altered morphology can potentially reveal changes in nutrient absorption as the gut morphology plays a key role in the digestive function (Keating *et al.*, 2021), and are thus important parameters to evaluate when considering new ingredients.

Some studies with brown seaweeds in aquafeeds have evaluated intestinal health parameters on histological sections. The changes observed have been lower muscularis thickness and some vacuolization of the enterocytes in the first segment of the MI in rainbow trout fed 4% *S. latissima* (Granby *et al.*, 2020). Reduced length of mucosal folds in combination with increased thickness of mucosal folds have been seen in Atlantic cod fed high inclusions of the brown seaweed *Ascophyllum nodusum* (Keating *et al.*, 2021). Intestinal morphology have also been evaluated in rainbow trout fed inclusions of red seaweeds where reduced length of mucosal folds have been seen in the anterior intestine both when fed high inclusions of *Gracilaria vermiculophylla* (Araújo *et al.*, 2016) and *Gracilaria pygmaea* (Sotoudeh and Mardani, 2018). All the mentioned reported changes have been when fed inclusion levels of seaweed ≥4% of diet.

The presence of ANFs, known to interfere with nutrient utilization and affect the health of the animal (Francis, Makkar and Becker, 2001), have been pointed out as a limiting factor for the use of macroalgae in aquafeeds (Sotoudeh and Mardani, 2018; Wan *et al.*, 2019). To overcome some of the negative intestinal health effects of ANFs from plant meals such as soybean, lupin

and various pulses, various processing methods have been imposed (Wan *et al.*, 2019). Among them, soybean protein concentrate (SPC) and pea protein concentrate (PPC) are often used (Johny *et al.*, 2020). SPC is prepared from the cake press remaining after oil is removed and washed with water /alcohol mixtures to remove soluble carbohydrates while protein, fiber, and some non-soluble carbohydrates remains (Hardy and Brezas, 2019). How processing of seaweeds through fermentation influences the intestinal morphology remains to be studied.

1.8 Evaluation of intestinal morphology

By scoring different health parameters in the gut on a pre- defined numeric scale, you can get a numerical answer to a qualitative evaluation of histological sections, thus make it possible to run statistical analysis on an originally qualitative measure of intestinal health. A semi-quantitative scoring system adapted from Urán *et al.*, (2004) has been used by Knudsen *et al*., (2007) to enable statistical analysis on a qualitative evaluation of histological parameters associated with SBMIE, including a qualitative assessment of supranuclear vacuoles, lamina propria of simple folds, connective tissue between the base of folds and stratum compactum, and appearance of mucosal folds. Such semi- quantitative scoring system has been used in several studies on functional ingredients in Atlantic salmon (Bakke-McKellep *et al.*, 2007; Wang *et al.*, 2020; Agboola *et al.*, 2021), but this method has not yet been used to investigate the effects of seaweeds in aquafeeds on intestinal morphology.

Evaluating of health parameters in the intestine such as length of mucosal folds, thickness of mucosal folds and thickness of the intestinal wall through morphometry of histological sections have been widely used to assess morphological changes associated with dietary modulations in Atlantic salmon (Baeverfjord and Krogdahl, 1996; Sanden *et al.*, 2005; Moldal *et al.*, 2014; Egerton *et al.*, 2020). Morphometry of histological intestinal sections have also been used in studies with seaweeds in aquafeeds for rainbow trout (Granby *et al.*, 2020), in Atlantic cod (Keating *et al.*, 2021), and in Nile tilapia (Silva *et al.*, 2015).

Quantification of mucous cells is also an efficient method for revealing abnormal responses in mucosal tissues, as the number of mucous cells present in the tissue may be an indicator of its health status (Pittman *et al.*, 2011; Sveen *et al.*, 2017; Dang *et al.*, 2020). This has been conducted in several dietary studies in finfish as a parameter for intestinal health for over three decades, both when evaluating SBMIE (van den Ingh *et al.*, 1991; Urán *et al.*, 2008), and more recently also when investigating the effect of seaweeds in aquafeeds (Moutinho *et al.*, 2018; Sotoudeh and Mardani, 2018; Granby *et al.*, 2020).

1.9 Oxidative stress and redox status

Oxidative stress occurs when the generation of reactive oxygen species (ROS) in a system exceeds the ability to neutralize and eliminate them (Olsvik *et al.*, 2011). Examples of ROS are e.g., hydrogen peroxide (H_2O_2), superoxide (O_2^-) and hydroxyl radical ($\cdot OH$) (Evans and Halliwell, 2001). ROS are naturally generated during normal cellular metabolism, and mitochondrial respiration is the most important endogenous source of ROS (Fridovich, 2004; Olsvik *et al.*, 2011). Glutathione (GSH) is an endogenous antioxidant that is a part of an extensive defence system against peroxidation in tissues and in prevention of oxidative stress (Hamre *et al.*, 2016). GSH is oxidized to GSSG in a glutathione peroxidase (GPx) catalyzed reaction (Formula 1.1). Glutathione reductase (GR) recycles GSSG to GSH with simultaneous oxidation of lyophilized β- nicotinamide adenine dinucleotide phosphate (NADPH) (Formula 1.2).

$$1.1. H_2O_2 + 2GSH \xrightarrow{GP_X} GSSG + 2H_2O$$

$$1.2. GSSG + NADPH + H^+ \xrightarrow{GR} 2GSH + NADP^+$$

The redox status of GSH can be expressed by its half- cell redox potential (GSH/GSSG E_h), calculated with the Nernst equation based on the concentrations of GSH and the oxidized form GSSG, and the standard electrode potential of the half- cell reaction (Degroote *et al.*, 2019).The GSH/GSSG ratio is thus used as an indicator of the redox state of the GSH pool (Liao *et al.*, 2018), and since GSH/GSSG is present in all cells in high concentrations it may therefore be the most important redox couple (Hamre *et al.*, 2016). Previous studies on oxidative stress in Atlantic salmon focused on the liver and muscle since these are the main tissues involved in lipid deposition (Kjær *et al.*, 2008; Olsvik *et al.*, 2011; Castro *et al.*, 2016; Hamre *et al.*, 2016). The intestine, however, is also highly susceptible to oxidative stress as it has a high cell turnover (Castro *et al.*, 2016).

Including alternative feed ingredient can potentially, due to the nutrient and antioxidative composition, affect growth and metabolism in salmonids (Olsvik *et al.*, 2011). In addition, the dietary nutrient composition has the potential to influence the cellular composition which has been seen as altered fatty composition of the liver cells in Atlantic salmon fed vegetable oil blend (Jordal, Lie and Torstensen, 2007). It is also suggested that altered metabolism due to dietary imbalance may induce increased ROS generation and lead to increased oxidative stress in fish (Olsvik *et al.*, 2011). Existing literature suggests that *S. latissima* (Ferreira *et al.*, 2020) and other kelp species (Laminaria sp.) (Kamunde, Sappal and Melegy, 2019) hold a potential

as a protective ingredient against oxidative stress in salmonids, but the evidence is limited. The role and potential of fermented seaweeds in terms of oxidative stress in fish is to the authors knowledge unknown. An evaluation of the GSH/GSSG pool in the intestine may therefore be useful when evaluating fermented *S. latissima* as a new feed ingredient for Atlantic salmon to get an indication of how the redox status is affected by the dietary modulation.

2. Aim and Objectives

The aim of this master project was to assess whether inclusion of fermented sugar kelp (*Saccharina latissima*) (FSK) in feed for Atlantic salmon post- smolt would affect any gut morphological parameters and the redox status in the first segment of the mid intestine compared with fish fed a commercially relevant diet.

This master thesis objectives were to:

- investigate whether inclusion of up to 4% FSK would affect growth performance indicators and feed utilization in Atlantic salmon.
- investigate whether inclusion of up to 4% FSK would affect the mid- intestinal morphology in Atlantic salmon.
- investigate whether inclusion of up to 4% FSK would affect the mucous cell density and size in the mid intestine of Atlantic salmon.
- investigate whether inclusion of up to 4% FSK would affect the redox status (GSH/GSSG ratio) in the mid intestine of Atlantic salmon.

The experiment was based on the following hypotheses:

$H0_1$: Inclusion of fermented sugar kelp in the feed for Atlantic salmon does not affect growth performance and feed utilization.

$H1_1$: Inclusion of fermented sugar kelp in the feed for Atlantic salmon does affect growth performance and feed utilization.

$H0_2$: Inclusion of fermented sugar kelp in the feed for Atlantic salmon does not affect any gut morphological parameters in the first segment of the mid intestine.

$H1_2$: Inclusion of fermented sugar kelp in the feed for Atlantic salmon does affect gut morphological parameters in the first segment of the mid intestine.

$H0_3$: Inclusion of fermented sugar kelp in the feed for Atlantic salmon does not affect the redox status (GSH/GSSG ratio) in the first segment of the mid intestine.

$H1_3$: Inclusion of fermented sugar kelp in the feed for Atlantic salmon does affect the redox status (GSH/GSSG ratio) in the first segment of the mid intestine.

3. Materials and Method

3.1 Experimental facilities and species

The experiment was conducted in a land- based, indoor flow- through facility at Matre Research station, Norway, over 10 weeks. Sixty- five Atlantic salmon (*Salmo salar*) post- smolts with an initial weight of 206 ±11g were kept in each of 15 quadrangular 1.5m^3 glass fiber tanks. The fish were a mixed population of female and male individuals. The tanks were supplied with seawater at a salinity of 34 ppt, water temperature was 8-9 ^oC and continuous light (24:0) during the experimental period. The fish were acclimatized to the tanks and meals for three weeks prior to experimental start. The fish were given two meals per day, where the diet was given in excess at each meal to ensure enough feed, and uneaten feed was collected to estimate feed intake.

3.2 Experimental design and diets

The fish were given either a reference diet or one of four experimental diets containing increasing levels of fermented sugar kelp (FSK), 1, 2, 3, and 4% FSK, respectively. A commercially relevant diet with 25% FM and 20% SPC was formulated as a reference (Table 3.1).

The fermented sugar kelp was provided by Lerøy seafood group (Bergen, Norway). In brief, the fermentation process was done on fresh material (not heat treated) at ambient temperature (8-14^oC) in closed containers with reduced oxygen supply. 10 grams of dry inoculum per 1000kg finely chopped seaweed (wet weight (WW)) was added to the material and the pH dropped to below 4.0 within 2 weeks. The inoculum was a commercial blend of *Lactobacilius* bacteria produced by European protein (EP199). The experimental diets were produced by Cargill (Dirdal, Norway). The formulation and proximate composition of the experimental diets are shown in Table 3.1.

Table 3.1: Formulation and proximate composition of the experimental diets containing different levels of fermented sugar kelp

	Reference	FSK 1%	FSK 2%	FSK 3%	FSK 4%
Fish oil	10.2	10.3	10.4	10.5	10.6
Rapeseed oil	13.9	13.6	13.4	13.2	12.9
Fishmeal LT	25.0	23.3	21.6	19.9	18.2
Soy protein concentrate (SPC)	20.0	20.0	20.0	20.0	20.0
Raw wheat	11.0	11.0	10.9	10.5	10.0
Other plant proteins[1]	16.8	17.5	18.3	19.4	20.6
Micro- ingredients	3.17	3.29	3.40	3.51	3.60
Yttrium oxide	0.02	0.02	0.02	0.02	0.02
Fermented *S. lattissima*	-	1	2	3	4
Proximate composition (as is)					
Protein (g/100g WW)	48	48	46	46	50
Lipid (g/100g WW)	27	32	28	24	21
Ash (g/100g WW)	7.4	7.6	7.5	7.8	8.2
Energy (J/100g WW)	23700	22800	23300	22600	21800
Dry matter (g/100g WW)	95.28	92.81	93.57	94.80	92.15
Carbohydrate (g/100g DM)					
NDF[2]	16.92	15.31	13.53	13.27	13.94
ADF[3]	2.12	2.07	2.03	2.20	2.28
ADL[4]	0.31	0.28	0.26	0.30	0.29
Hemicellulose	14.80	13.24	11.50	11.07	11.67
Cellulose	1.81	1.78	1.78	1.90	1.99

[1]Wheat gluten meal, pea protein concentrate- and guar meal.
FSK: Fermented sugar kelp**.** WW=wet weight.
NDF[2]= Neutral Detergent Fiber, ADF[3] = Acid Detergent Fiber, ADL[4] = Acid Detergent Lignin
Notes: Ingredients are listed as percentages of whole feed.
The feed composition analyses are conducted by technicians at IMR, Bergen, Norway.
The carbohydrate analyses are conducted by Århus University, Denmark.

3.3 Sampling procedure

At the start of the feeding trial, weight and length was recorded on 30 individual fish, and the total biomass in each tank was determined. At the end of the trial, weight and length was recorded on all individual fish in the tanks. The fish were starved for 24 hours prior to sampling. Sampled fish were euthanized with an overdose of tricaine methane sulfonate (FINQUEL MS-222).

For histological analysis, approximately 1cm sections of mid intestine (section immediately after pylorus caeca, section 3 Figure 1.2) from 3 fish per tank were sampled. The tissue sample were put in an embedding cassette and fixated in 10% buffered formalin before further

processing. For the GSH/GSSG analysis a separate sample of approximately 1cm from the mid intestine, immediately posterior of where the sample for histology, was taken (5 fish per tank). These were flash frozen in liquid nitrogen and stored at -80 ^{o}C for further analysis.

3.4 Histology

3.4.1 Dehydration and infiltration of tissue

After 48 hours fixation in buffered formalin, the samples where further transferred to 70% ethanol before processing. The processing was done in a Leica TP 1020 histokinette (Leica Microsystems Nussloch GmbH, Nussloch, Germany). The histokinette provides fully automatic infiltration of formalin- fixed tissue with paraffin/ HISTOWAX® (Histolab Products AB, Gothenburg, Sweden). The program is shown in Table 3.2.

Table 3.2: Histokinette program for dehydration and infiltration of tissue

Bath number	Bath	Time
1	4 % phosphate buffered formalin	1 hour
2	50 % ethanol	1 hour
3	70 % ethanol	1 hour
4	80 % ethanol	1 hour
5	96 % ethanol	2 hours
6	96 % ethanol	2 hours
7	100 % ethanol	2 hours
8	100 % ethanol	2 hours
9	Xylene	2 hours
10	Xylene	2 hours
11	HISTOWAX® paraffin 56-58^{o}C	2 hours
12	HISTOWAX® paraffin 56-58^{o}C	2 hours

3.4.2 Embedding and sectioning of tissue

Following dehydration, the tissue samples were brought through the embedding process in HISTOWAX® paraffin using Kunz instruments embedding machine (Kunz Instruments, Stockholm, Sweden). Molding trays were filled with hot paraffin wax, and tissue samples were orientated longitudinal in the middle of the tray at the cooling plate. The histology plate was placed on top, more paraffin wax was added, and the samples were brought to the freezer (-20 ^{o}C) for 10-20 minutes before sectioning.

The sectioning of the embedded samples was done with Thermo Scientific™ Automatic Microtome Microm HM 355S (Thermo Fisher Scientific, Braunschweig, Germany). Each of the samples were cut in three sections in the transverse plane with a thickness of 3µm. Each

section was individually placed on microscope slides (SuperFrost®Menzel Gläser, Thermo Fisher Scientific, Braunschweig, Germany).

3.4.3 Colouring

Before colouring, the samples were dried in a Mini Incubator (Labnet, US) for 20 minutes at 60ºC. Two sections from each of the fish were coloured with HES (Hematoxylin- eosin-saffron) and Alcian Blue PAS (Periodic Acid Schiff), mounted with Histokitt and covered with VWR® microscope cover glasses 24x32mm (VWR International, Luven, Belgium) for further histological analysis.

3.5 Analysis of histological sections

Micrographs of intestinal sections of nine fish in each dietary group were captured using a Hamamatsu NanoZoomer S60. The images were saved as high- resolution digital images in NDPI- format, and the viewing software NDP. view2 (version:2.9.25, Hamamatsu, Hamamatsu City, Japan) was used for further processing. For the semi- quantitative analysis of histological sections, the mentioned viewing software was used. For both the morphometric analysis and the mucosal quantitative analysis of histological sections, images were exported as .jpg files in 10x magnification to be imported into the ImageJ software (version: 1.53m). All the histological analyses described in section 3.5 were done without knowledge of the treatment group, e.g. blindly, except from the FSK 2% group which was included at a later time due to other results emerging from the project, as this study is part of a larger project.

3.5.1 Semi- quantitative scoring of histological sections

A semi- quantitative scoring was conducted on the HES- stained histological slides from the fish fed the reference diet, the fish fed FSK 1%, the fish fed FSK 2%, and the fish fed FSK 4%. A semi- quantitative scoring system modified from Knudsen *et al*. 2007 by Hanne Johnsen at Nofima for Johansson (2014) was used for histological evaluation to evaluate inflammatory reactions in the MI. This is an unpublished scoring system developed for mass screening of histological slides from MI based on morphologic appearance that allows for statistical analysis on evaluation of histological slides that has previously been used in studies conducted by Nofima.

The criteria evaluated are the following: **Criterion 1** is the level of vacuolization, **criterion 2** is the appearance of connective tissue in the lamina propria, **criterion 3** is the amount of connective tissue between the base of the mucosal folds and stratum compactum, and **criterion 4** is the appearance of the mucosal folds. This system has a graded criteria (1-5) assessing

different stages of changes in the morphology of the intestine. A score of 1 and 2 are characterized as no- or mild changes, and thus being what is considered a "normal" morphology. Scores from 3 to 5 represent moderate, - distinct, - and severe changes respectively, indicating a more damaged morphology. A more detailed explanation of the scoring of each criterion is described in 3.5.1.1-3.5.1.4.

Each tissue slide went through a minimum of 4 scorings with 5 days between each scoring. Two and two scorings were compared to verify similar results between scoring timepoints. This led to 3 comparisons conducted per slide, as seen in Figure 3.1. In cases where there were disagreements between two scores in the first two rounds of scorings, one new scoring of that tissue slide was conducted and that score was brought to the final comparision. The score achieved after the final comparision, and the subsequent new scoring when there were disagreements betweeen scores, is the final score used for further numerical analysis.

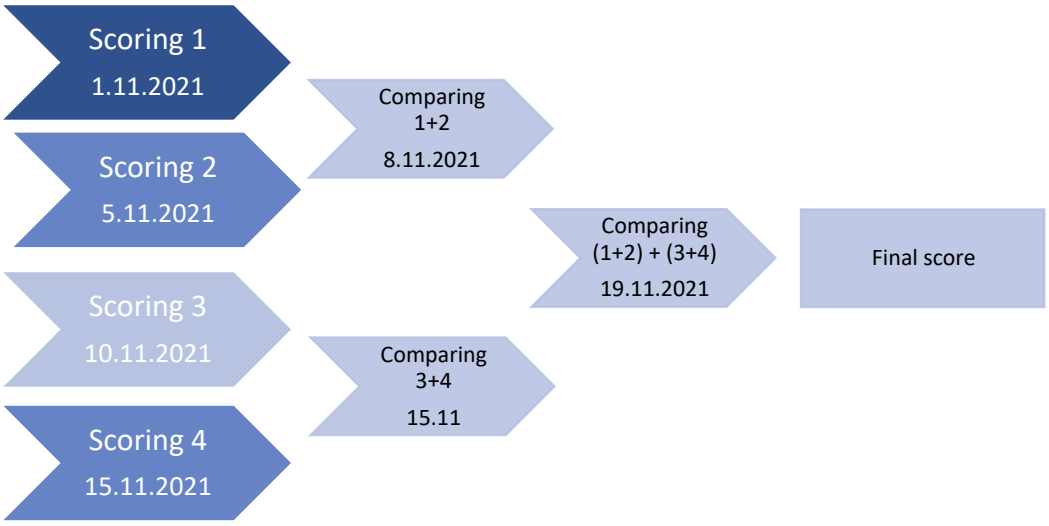

Figure 3.1: Schematic overview of the qualitative scoring process of histological sections. The scoring of the histological sections was initially conducted four times. The scores from scoring 1+2 and scoring 3+4 were then compared. Further, the results from comparing 1+2 and comparing 3+4 were once again compared to result in a final score that were used for further evaluation and statistical analysis. Illustration: I.L. Øverbø

The results from the final scoring of the semi- quantitative scoring were calculated as percentages based on occurrence of scores within each diet group (described in Appendix table 10-13). An overall mid intestine health score for each diet group based on the scores from all four criteria was also calculated (described in Appendix table 14).

3.5.1.1 Criterion 1- Vacuolization of the enterocytes

Criterion 1 is scored due to the degree of vacuolization in the enterocytes. A score of 1 indicates no to very little vacuolization of the enterocytes while a score of 5 indicates a severe increase in vacuolization of the enterocytes (Table 3.3). Examples of criterion 1 are for illustration purposes shown in Figure 3.2.

Table 3.3: Scoring system for criterion 1

Criterion 1: Level of vacuolization of the enterocytes	Score	Grade
No to very little vacuolization in the enterocytes	1	No
A slightly increase in vacuolization of the enterocytes	2	Mild
A clear increase in vacuolization of the enterocytes	3	Moderate
A clear increase in vacuolization that affects most of the enterocytes	4	Distinct
A clear increase in vacuolization that affects all the enterocytes	5	Severe

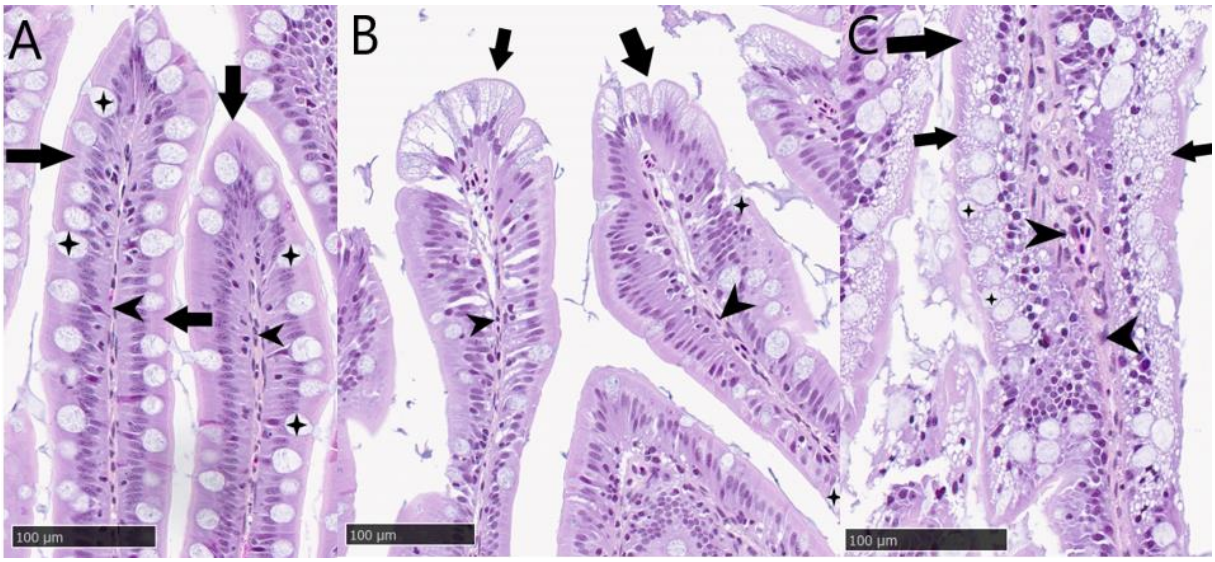

Figure 3.2: Criterion 1- Vacuolization of the enterocytes. A) displays a score of 1 with no vacuolization of the enterocytes (arrow). B) shows a score of 3 with a clear increase in vacuolization of the enterocytes (arrow). C) shows a score of 5 with a clear increase in vacuolization that affects all the enterocytes(arrow). Arrowhead indicates lamina propria. Asterix indicates mucous cells. Scalebar: 100 µm. Illustration pictures: I.L. Øverbø.

3.5.1.2 Criterion 2- Lamina propria of the mucosal folds

Criterion 2 is scored due to the appearance of connective tissue in the lamina propria. A score of 1 indicates a very thin and delicate core of connective tissue in all mucosal folds, while a score of 5 indicates a severe increase of lamina propria in many folds (Table 3.4). Examples of criterion 2 are for illustration purposes shown in figure 3.3.

Table 3.4: Scoring system for criterion 2

Criterion 2: Lamina propria of folds	Score	Grade
There is a very thin and delicate core of connective tissue in all folds	1	No
The lamina propria appears slightly more distinct and robust in some of the folds	2	Mild
There is a clear increase of lamina propria in most of the folds	3	Moderate
There is a thick lamina propria in many folds	4	Distinct
There is a very thick lamina propria in many folds	5	Severe

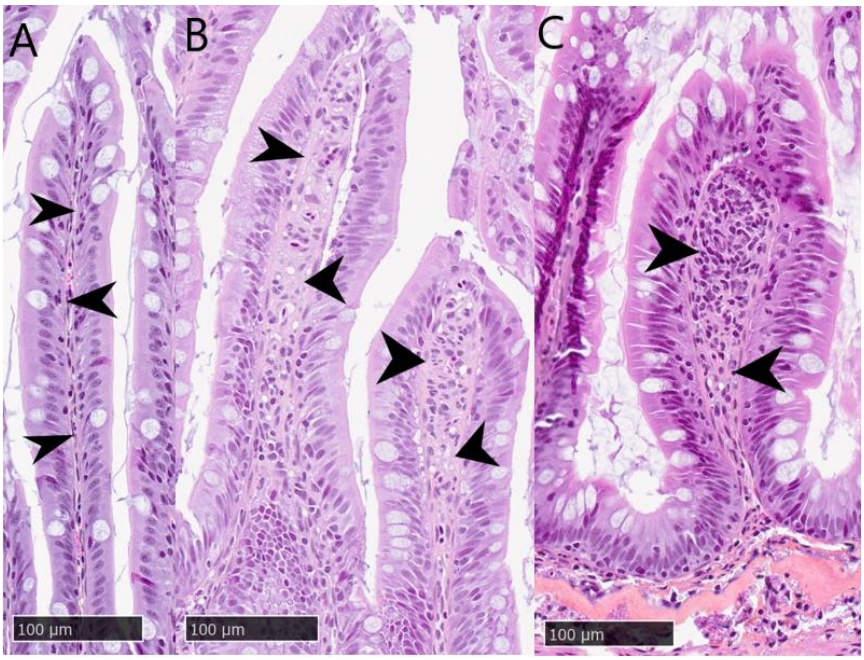

Figure 3.3: Criterion 2- Lamina propria of the mucosal folds. A) displays a typical score of 1 with a very thin and delicate core of connective tissue in the fold (arrowhead). B) shows a typical score of 3 with a thick lamina propria (arrowhead). C) shows a score of 5 with a very thick lamina propria. Scale bar: 100 µm. Illustration pictures: I. L. Øverbø.

3.5.1.3 Criterion 3- Connective tissue between the base of folds and stratum compactum

Criterion 3 is scored due to the amount of connective tissue between the base of the mucosal folds and stratum compactum. A score of 1 indicates a very thin layer of connective tissue, while a score of 5 indicates an extremely thick layer of connective tissue beneath most of the folds (Table 3.5). Examples of criterion 3 are for illustration purposes shown in Figure 3.4.

Table 3.5: Scoring system for criterion 3

Criterion 3: Connective tissue between the base of folds and stratum compactum	Score	Grade
There is a very thin layer of connective tissue between the base of folds and the stratum compactum	1	No
There is a slightly increased amount of connective tissue beneath some of the mucosal folds	2	Mild
There is a clear increase of connective tissue beneath most of the mucosal folds	3	Moderate
A thick layer of connective tissue is beneath many folds	4	Distinct
An extremely thick layer of connective tissue is beneath some folds	5	Severe

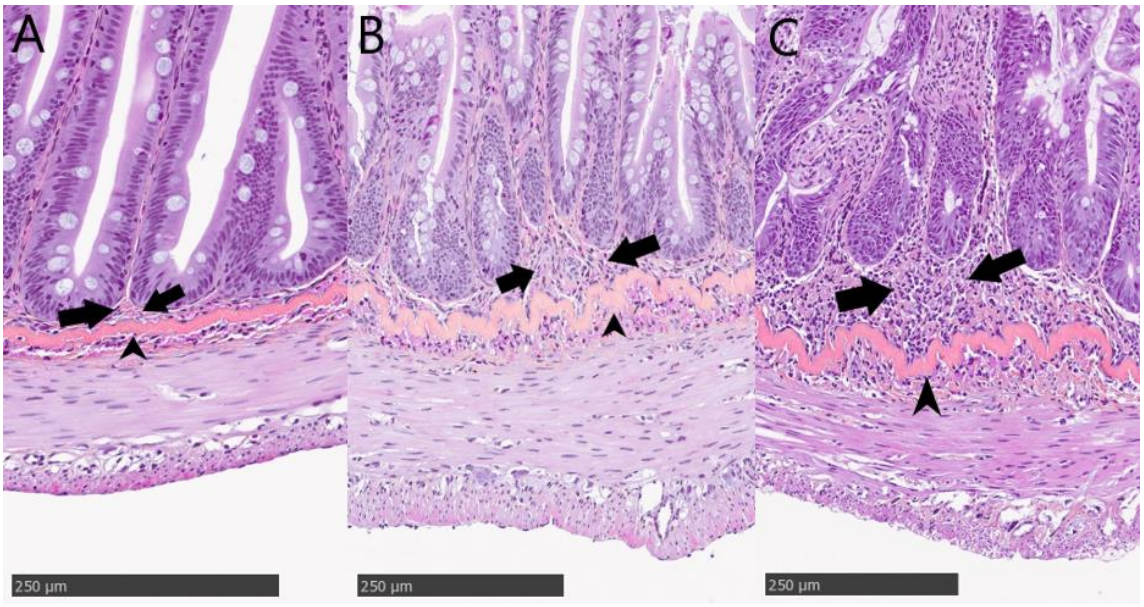

Figure 3.4: Criterion 3- Connective tissue between base of folds and stratum compactum. A) displays a score of 1 with a thin layer of connective tissue (arrow). B) shows a typical score of 3 with a clear increase of connective tissue (arrow). C) shows a typical score of 4 with a thick layer of connective tissue (arrow) Arrowhead indicates stratum compactum. Scale bar: 250 µm. Illustration pictures: I.L. Øverbø.

3.5.1.4 Criterion 4- Mucosal folds

Criterion 4 is scored due to the length and width of the mucosal folds. A score of 1 indicates appearance of mucosal folds as long and thin, while a score of 5 indicates short and stubby mucosal folds (Table 3.6). Examples of criterion 4 are for illustration purposes shown in Figure 3.5.

Table 3.6: Scoring system for criterion 4

Criterion 4: Mucosal folds	Score	Grade
Mucosal folds appear long and thin	1	No
Mucosal folds appear long to medium and are slightly thicker	2	Mild
Mucosal folds have short to medium length	3	Moderate
Mucosal folds are short	4	Distinct
Mucosal folds appear very stubby	5	Severe

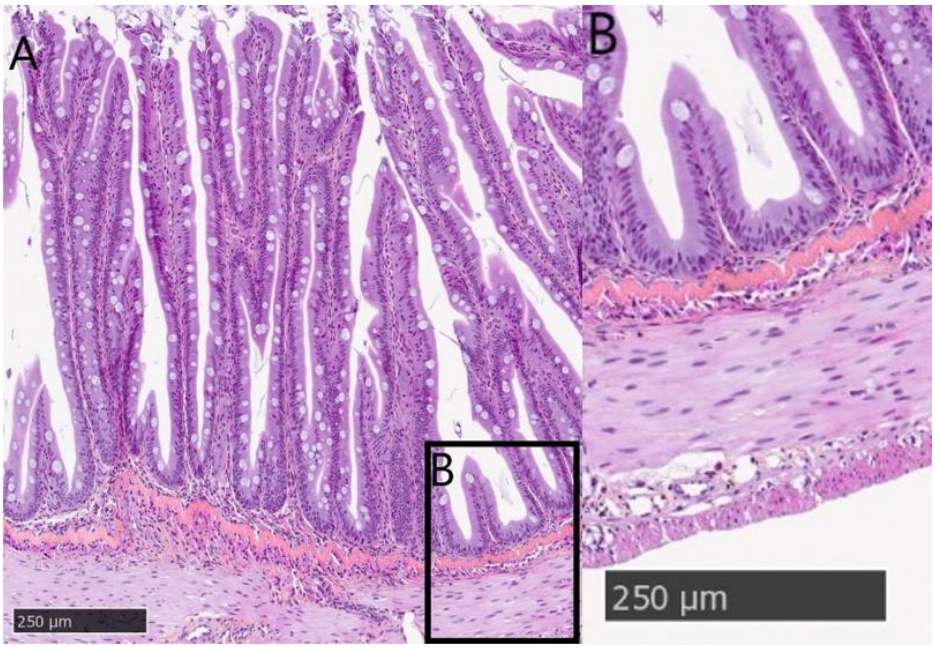

Figure 3.5: Criterion 4- Mucosal folds: A) displays a score of 1, with long and thin mucosal folds. B) shows a short mucosal fold, which indicates a score of 4. Scalebar: 250µm. Illustration pictures: I. L. Øverbø.

3.5.2 Morphometric analysis

The morphometric analysis was performed on the Alcian Blue PAS-stained histological slides from the fish fed reference diet, the fish fed FSK 1%, the fish fed FSK 2%, and the fish fed FSK 4%. For these measurements, the ObjectJ (Version: 1.05h) plugin for ImageJ was used. Morphometric analysis of length of folds, thickness of folds, and the thickness of the intestinal wall was measured as previously described by Moldal *et al*., (2014). The length of the folds (orange line, Figure 3.6) was measured from the fold apex to the bottom of the epithelium at the base of the folds, and the thickness of the folds (red and green line, Figure 3.6) was assessed at two points in every fold. The thickness of the intestinal wall (yellow line, Figure 3.6) was measured from beneath the epithelium at the base of the folds to the serosa (Figure 3.6). Every other fold was measured. If the selected fold was not measurable, due to a torn mucosal fold, inconsistent intestinal wall, or artefacts, the next possible fold was measured, and then continuing again with every other fold. For this reason, n is varying from min n=5 to max n=21 per fish.

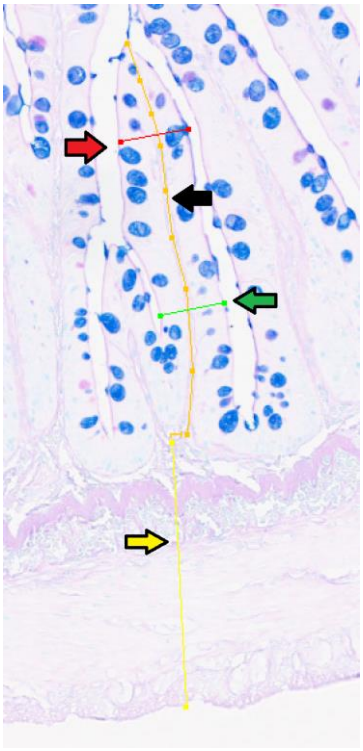

Figure 3.6: Micrograph showing measurement lines for the morphometric analysis. Length of folds (black arrow), thickness of folds (red and green arrow), and thickness of the intestinal wall (yellow arrow). Illustration picture: I.L. Øverbø.

3.5.3 Quantifiable mucosal analysis

The quantifiable mucosal analysis was performed on the Alcian Blue PAS- stained histological sections from the fish fed the reference diet, the fish fed FSK 2%, and the fish fed FSK 4%. The samples from the fish fed FSK 1% had to be excluded from this analysis due to some thicker histological sections, because of the importance of the histological sections to be of even size. Using ImageJ (Version: 1.53m), the spatial scale of the active image was sat to micrometres (µm). To measure the size of epithelium region of interest (ROI), the ImageJ tool "freehand section" was used. First, the histological sections were outlined from stratum compactum (Figure 3.7A) and the inner area was measured. Secondly, lumen was outlined (Figure 3.7B) using the same ImageJ tool and the inner area was measured. Subsequently, the measured area from Figure 3.7B was subtracted from the measured area from Figure 3.7A. The obtained epithelium area was the ROI.

Using the ImageJ tool "rectangle", the previously decided ROI was subdivided into 12 square subsections with varying size as demonstrated with one square subsection in Figure 3.7A. The

size and orientation of the square subsections were actively adjusted to cover as much of the area within the ROI as achievable. To avoid duplication of area measured, as only one square section can be visible in the active image at one time, the subsections were added to the "ROI manager" in ImageJ that allows for working with multiple sections. The epithelium area covered by each of the 12 subsections was measured. The area of ROI covered by each of the 12 subsections ranged from minimum 1.5% to maximum 11% for all samples measured. All 12 subsections in total covered from 40% to 73% of the ROI for all samples measured.

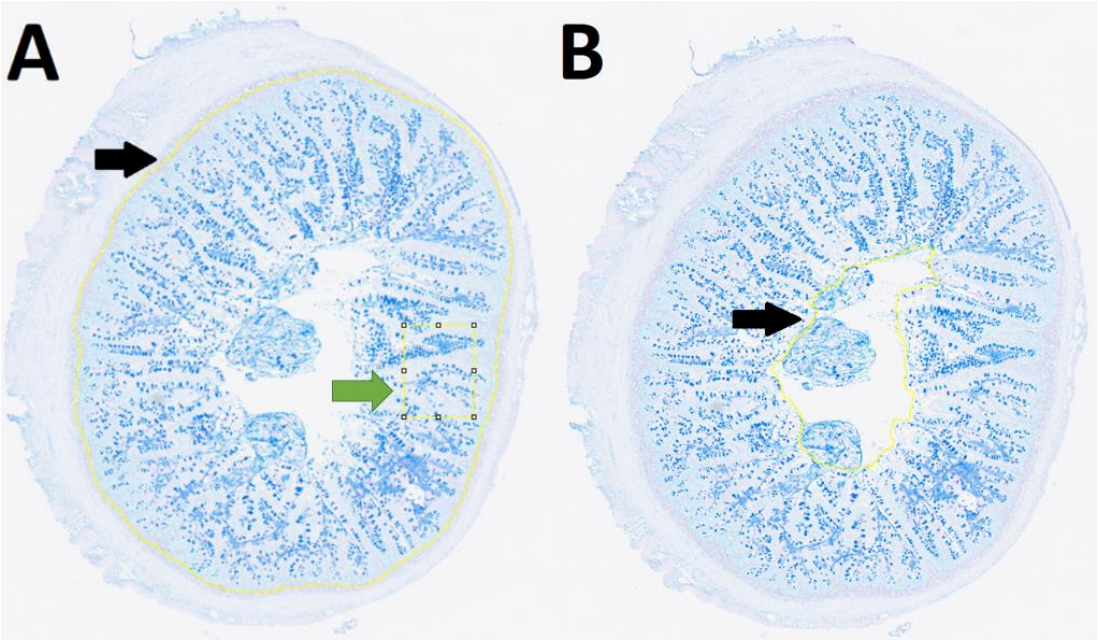

Figure 3.7: Outlining of ROI. Each of the Alcian Blue PAS- stained histological slides were outlined from A) (black arrow) stratum compactum, and the inner area was measured and B) (black arrow) the lumen area was measured. The area from B) was subtracted from the area in A), resulting in the ROI for further measurements. An example of one of the 12 subsection squares is for illustrational purposes noted in A) with a green arrow. Due to how the function of ImageJ "rectangle" and the "ROI manager", not all 12 subsections are shown here. Illustration pictures I.L. Øverbø.

By actively adjusting the colours on a red, - green, - blue (RGB) scale in ImageJ for each of the 12 subsections, the mucous cells (dark blue areas, Figure 3.8A) were selected. When properly selected (red areas, Figure 3.8B), the mucous cells were quantified using the "analyze particle" function. This function gave the percentage of area covered by mucous cells within each subsection (%), and the mean size of the mucous cells measured within each subsection (μm^2). The mean size of mucous cells was calculated as the arithmetic mean based on the number and size of measured particles (mucous cells) within each subsection, ranging from 20 to 630 counted mucous cells.

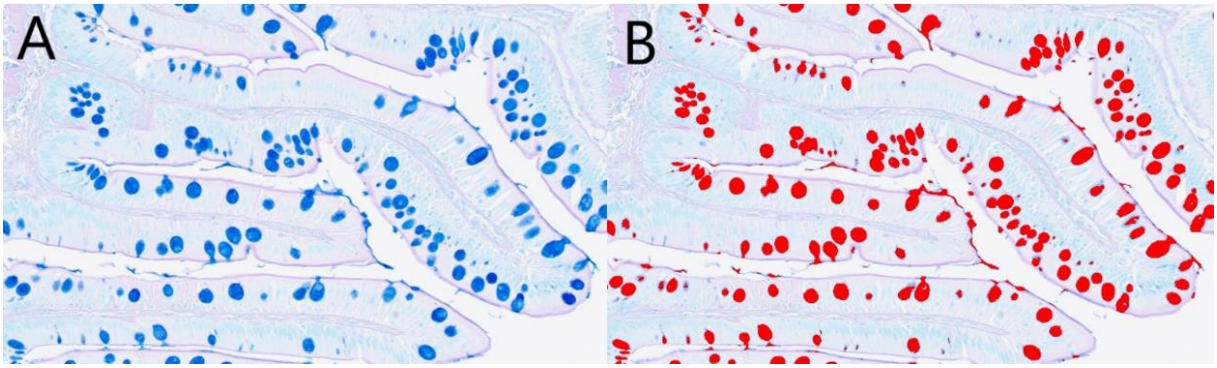

Figure 3.8: Selection of mucous cells. A) shows the original Alcian Blue PAS-stained histological slide before adjusting of threshold colours on a RGB scale in ImageJ, while B) shows the mucous cells coloured in red by adjusting of threshold colours, and thus the mucous cells measured. Illustration pictures: I.L. Øverbø

3.6 GSH/GSSG analysis

3.6.1 General sample preparation

The GSH/GSSG analysis was performed on mid intestine samples from all diet groups (reference, FSK 1%, FSK 2%, FSK 3% and FSK 4%), and a commercial kit was used (Prod. No. GT40, Oxford Biomedical Research, Oxford, UK). Before weighing, mid- intestinal samples from fish were pooled together two-and-two (from same diet and tank) to ensure enough material to conduct the GSH/GSGG analysis. The samples were taken from 12 fish per diet (4 fish per tank, triplicate tanks) and thus pooled to 6 samples per diet. For the fish in the FSK 4% group, samples were taken only from 8 fish in total (4 fish from 2 tanks), and it was thus 4 pooled samples. On a tray of dry ice to avoid de- freezing, the frozen samples were pooled- and homogenized together by use of a hammer and transferred to two 2ml Eppendorf tubes containing two 5mm metal balls: one tube for GSH and one for GSSG. Samples of 80-120mg for GSH and 50-80mg for GSSG were weighted in, and they were stored at -80 ^{o}C until further analysis.

3.6.2 Sample preparations for total GSH

The samples were kept on ice to avoid de-freezing, and 4 parts of 0.9% NaCl were added to the samples (1:5 dilution). They were further homogenized in a Retsch homogenizer (Model: MM301, Germany) at 25 RPM for 90 seconds. The metal balls were removed with a magnet stick and the samples were centrifuged at 20 000G at 4 ^{o}C for 10 minutes. The supernatant was transferred to 1.5mL Eppendorf tubes, and 100µl supernatant was added to 300µl ice cold 5% MPA (metaphosphoric acid solution). The samples were vortexed before centrifugation at 20 000G at 4 ^{o}C for 10 minutes. Dilution was done with assay buffer (general buffer), 5µl sample + 595µl buffer (total dilution = 1:2000). The diluted samples were kept at -80 ^{o}C until further analysis.

3.6.2 Sample preparations for GSSG

The samples were kept on ice to avoid de- freezing, and 2 parts of 0.9% NaCl/Scavanger (thiol scavenger) was added (1:3 dilution). They were further homogenized in Retsch homogenizer (Model: MM301, Germany) at 25 RPM for 90 seconds. The metal balls were removed with a magnet stick and the samples were centrifuged at 20 000G at 4 ^{o}C for 10 minutes. The supernatant was transferred to 1.5mL Eppendorf tubes, and 80µl supernatant was added to 160µl ice cold 5% MPA. The samples were vortexed before centrifugation at 20 000G at 4 ^{o}C for 10 minutes. Dilution was done with assay buffer, 50µl sample + 115µL buffer (total dilution = 1:30). The diluted samples were kept at -80 ^{o}C until further analysis.

3.6.3 450nm absorbance procedure

3.6.3.1 Reagent preparation

For the NADPH (Lyophilized β- nicotinamide adenine dinucleotide phosphate) reagent preparation, the vial was reconstituted with 600 µL Assay buffer. This was added to 6.0mL Assay buffer, vortexed and kept on ice. For the Reductase (recombinant glutathione reductase) reagent, 30 µL Reductase was added to 6mL Assay buffer and kept on ice. The vial of the DTNB (lypholized 5,5'- dithiobis- 2 nitrobenzoic acid) was reconstituted with 600µL Assay buffer. This was added to 6.0mL Assay buffer, vortexed and left at room temperature until further use.

3.6.3.2 Standard curve preparation

A standard curve of Assay buffer and 10µM GSSG Standard Stock was prepared according to Table 3.7.

Table 3.7: Standard curve preparation

Standard	Assay buffer(µL)	Volume of 10µM GSSG Standard Stock(µL)
S7	850	150
S6	900	100
S5	925	75
S4	950	50
S3	975	25
S2	987.5	12.5
S1	995	5
B0	1000	-

3.6.3.3 Assay procedure

Into the corresponding wells on the microplate (well B0, and S1 to S7), 50µL of standards were added in duplicates (Figure 3.9). The GSH samples were placed in the wells from U1 to U20 in duplicates, and the GSSG samples were placed in the wells from U21 to U40 in duplicates. Then 50µL of DTNB solution followed by 50µL of reductase solution were added to each well. The plate was placed on an orbital shaker and incubated at room temperature for 5 minutes. After adding 50µL NADPH solution to each well, the plate was placed in a kinetic microplate reader (iEMS Reader Ms, Labsystems, Finland). The change of absorbance at 405nm was recorded by taking readings every minute for 10 minutes (in total 11 readings).

Figure 3.9: **Microplate for GSH/GSSG analysis.** Well B0 and S1 to S7 are for the standard samples, well U1 to U20 are for the GSH samples, and well U21 to U40 are for the GSSG samples.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	B0	B0	U1	U1	U9	U9	U17	U17	U25	U25	U33	U33
B	S1	S1	U2	U2	U10	U10	U18	U18	U26	U26	U34	U34
C	S2	S2	U3	U3	U11	U11	U19	U19	U27	U27	U35	U35
D	S3	S3	U4	U4	U12	U12	U20	U20	U28	U28	U36	U36
E	S4	S4	U5	U5	U13	U13	U21	U21	U29	U29	U37	U37
F	S5	S5	U6	U6	U14	U14	U22	U22	U30	U30	U38	U38
G	S6	S6	U7	U7	U15	U15	U23	U23	U31	U31	U39	U39
H	S7	S7	U8	U8	U16	U16	U24	U24	U32	U32	U40	U40

3.6.4 GSH/GSSG calculations

The general regression equation describing the calibration curve to determine the concentration of GSH and GSSG is:

$$Net\ Rate\ =\ Slope\ \times\ GSH\ +\ Intercept$$

Total GSH and GSSG concentration were calculated using following equations:

$$Total\ GSH\ =\ \frac{Net\ Rate\ -\ Intercept}{Slope}\times Dilution\ factor$$

$$GSSG\ =\ \frac{Net\ Rate\ -\ Intercept}{Slope}\times Dilution\ factor$$

The GSH/GSSG ratio was calculated by dividing the difference in concentration between total GSH (GSH_t) and GSSG by the concentration of GSSG:

$$Ratio = \frac{GSH_t - 2GSSG}{GSSG}$$

3.7 Data management and statistical analysis

For statistical analysis the software Rstudio (version 2021.09.0, RStudio, PBC, Boston, MA, USA) and R (version 4.1.1, Vienna, Austria) were used. All graphs were made using GraphPad Prism (version 9.3.1 for Windows, GraphPad Software, La Jolla, CA, USA). To evaluate the correlation between two parameters on the parametric data from the morphometric analysis and the mucosal quantitative analysis, a correlation test in R was performed and Kendall rank correlation coefficient was calculated.

For analysing the normality of the datasets, Shapiro- Wilk's test was used (Appendix Table 1). Using the package *car*, Levene's test was used to check the datasets for homogeneity of variance (Fox and Weisberg, 2019) (Appendix Table 2). For data that were non- normal and/or with non- homogeneous residuals, log10, inverse and/or box- cox transformations was performed prior to further analysis. For box cox transformation, the library *MASS* was used (Venables and Ripley, 2002). All the transformation of data that have been performed to make the data suit the prerequisites are presented in Appendix Table 3 and 4.

For all parametric data, nested one- way ANOVA was used to determine statistical differences between diet groups. Tank and/or fish number was added as random factors. This was performed using the *nlme* package (Pinheiro *et al.*, 2022). For statistically significant differences according to ANOVA, a Tukey's Honest Significant Difference (HSD) was used as a post hoc test. This was done using the *multcomp* package (Hothorn, Brezt and Westfall, 2008). For the non- parametric data from the semi- quantitative analysis, a Kruskal- Wallis rank sum test (KWt) was performed. A Student Newman Keuls (SNK) test from the *agricolae* package was used as a post- hoc test (de Mendiburu and Yaseen, 2020).

To evaluate dose- dependent responses, a simple linear regression (LR) was performed on the data from the morphometric analysis, the mucosal analysis, and on data for the GSH/GSSG ratio. For the total GSH and GSSG concentration, a nonlinear regression (NLRM) was performed. These analyses were done with the respective fermented seaweed inclusion percentage from each analysis on the x- axis (0 to 4) using GraphPad Prism (version 9.3.1 for Windows, GraphPad Software, La Jolla, CA, USA).

All data were checked for the effect of the random factors “tank effect” and “fish number” (where applicable). The results of this are presented in Appendix Table 5. All data is presented as arithmetic mean±SD. For all tests, the level of significance was set at $p<0.05$.

3.8 Methodological considerations

3.8.1 Limitations of the semi- quantitative analysis

As with all histological examinations, the semi- quantitative analysis is based upon a subjective impression by the researcher. The researchers experience and skills in histological examination may affect the results ability for comparison across other studies. Moreover, this method requires good planning and randomization of samples to ensure viable results. Although differences between the diet groups perchance will be detected, it is unknown whether this method is the choicest for evaluating the potential of seaweeds in aquafeeds with respect to mid- intestinal health as there is no standardized method for this.

3.8.2 Limitations of the morphometric analysis

The morphometric analysis method was chosen based on well- established methods for assessing morphologic changes correlated with dietary modulations in all three (PC, MI, DI) segments of the intestine (Gu *et al.*, 2014; Moldal *et al.*, 2014; Yarahmadi, Kolangi Miandare and Hoseinifar, 2016). The variety in how the measurements in morphometric analysis of the intestinal wall previously have been assessed in studies with seaweeds in aquafeeds (Silva *et al.*, 2015; Sotoudeh and Mardani, 2018; Granby *et al.*, 2020) limits its ability for comparison across other studies. Further, this method requires good planning with randomization of samples to avoid biased results. The prerequisites for which fold was measurable in the present study were sat by the researcher and thus, subjective decisions that may influence the result were taken. There is also a risk that human errors may occur when conducting the measurements with this method.

3.8.3 Limitations of quantifiable mucosal analysis

The histological sections were sliced in transverse sections because of the need for a clear view of the structures for the other analysis performed in this study. A limitation of this is that relatively few mucous cells are viewable for analysis in one transverse section, while tangential sections could cover considerably more of the tissue analyzed (Pittman *et al.*, 2011, 2013).

Counting of mucous cells from two- dimensional histological sections from the intestine have been used in previous studies with dietary modulations with seaweeds in feed for fish (Moutinho *et al.*, 2018; Sotoudeh and Mardani, 2018; Granby *et al.*, 2020), and in a study with

fermented poultry by- product meal in common carp (*Cyprinus Carpio*) (Dawood *et al.*, 2020). The lack of one standardized method is seen as high variations in how the measurements of mucous cells are conducted in previous studies and should be kept in mind when comparing across studies.

A novel stereology- based method for quantification of mucous cells in salmonids have been described by Pittman *et al.*, (2011), named mucosal mapping. Studies in gills have reported the average mucous cell size retrieved from traditional histology measurements to be about half of the size obtained by mucosal mapping with stereology (Dang *et al.*, 2020). Stereology and mucosal mapping have been used in studies with dietary modulations to evaluate mucosal tissue from intestine (Torrecillas *et al.*, 2015).

Mucosal mapping with stereology was not an available method for the present study. Thus, the examination was done on two- dimensional histological sections. While traditional methods often only assess the number of mucous cells present, the mucous cells density (percentage of area covered by mucous cells) and mean mucous cell area (μm^2) are assessed with stereology (Pittman *et al.*, 2011; Torrecillas *et al.*, 2015; Dang *et al.*, 2020). These two parameters were assessed with the tools available in the current study. Although the method used in this study can reveal differences between diet groups, caution should be taken as this is an unstandardized method.

3.8.4 Limitations of the GSH/GSSG analysis

Evaluation of the GSH/GSSG pool is frequently used to evaluate oxidative stress and redox status in biological tissues of fish (Hamre *et al.*, 2016, 2022; Remø *et al.*, 2017), and have also been used to assess antioxidant capacity in brown seaweed supplemented Atlantic salmon (Kamunde, Sappal and Melegy, 2019). The method used in the present study is a standardized method, but errors can occur during handling. To ensure accurate determination of GSH/GSSG, caution needs to be taken during sampling, storage, preparation, and analysis (Hamad *et al.*, 2021). GSH is susceptible for artifactual oxidation, and both GSH and GSSG concentrations may potentially change *ex vivo* as described by Enomoto *et al.*, (2020). Thus, when methodological artifacts are avoided, GSH/GSSG can be a powerful biomarker for detection of oxidative stress (Giustarini *et al.*, 2011; Hamre *et al.*, 2016).

3.8.5 Statistics

In order to get representative and homogenous samples it is suggested that the sample analyzed is large enough, but this is not always attainable. It should be taken into consideration that there

was a large variation in the dataset for the morphometric analysis of length of mucosal folds, and the chosen box cox transformation ensured homogeneous residuals but not normal distribution of data (Appendix table 3). It was thus selected as the most suitable transformation (Appendix table 4), although the data still did not fulfil the prerequisites for ANOVA.

4. Results

4.1 Proximate composition of feed

Proximate compositions for all diets are presented in Table 3.1. The FSK 1% diet contained 32% lipid while the FSK 4% diet contained 21% lipid, and it was thus a difference of 52% between the two diets with the highest and lowest lipid contents, respectively. The difference in energy content was approximately 9% between the diets reference, which contained the highest energy, and the FSK 4% diet which contained the lowest energy. The protein content was lowest in the FSK 2% and FSK 3% diets at 46%, while the protein content was approximately 9% higher in the FSK 4% diet which contained the highest protein content between the experimental diets with 50% protein.

The NDF content was highest in the reference diet at 16.92%, while the lowest NDF was seen in the FSK 3% diet at 13.27%. The highest portion of ADF were seen in the FSK 4% diet at 2.28%, while the lowest portion of ADF were seen in the FSK 2% diet at 2.03%. The ADL content was highest in the reference diet at 0.31%, and lowest in the FSK 2% diet at 0.26%. Hemicellulose was highest in the reference diet at 14.80%, while it was lowest in the FSK 3% diet at 11.07%. The cellulose content increased with increasing FSK inclusions. The lowest cellulose content was seen in the FSK 1% and the FSK 2% diets at 1.78%, while the highest cellulose content was seen in the FSK 4% diet at 1.99%. There was further a cellulose content of 1.81% and 1.90% in the reference diet and the FSK 3% diet, respectively.

4.2 Growth Performance Indicators

The growth performance indicators are presented in Table 4.1. There was no mortality in this trial. The mean initial body weight (IBW) of the reference group was 210±3g. The IBW of the fish allocated to the reference group was not statistically different from the fish allocated to the experimental diets ($p=0.28$, ANOVA). The mean final body weight (FBW) of the reference group was 485±7g. The FBW was numerically lower in the groups fed the experimental diets compared to the reference group, although not statistically so ($p=0.37$, ANOVA). The numerically lowest mean FBW was seen in the fish fed FSK 3% (451±13g).

The highest weight gain (WG) was seen in the reference group at 275±4g, while the fish fed FSK 3% had the lowest weight gain at 248±11g between the experimental groups. There was further a WG of 267±7g, 267±4g and 250±15g in the fish fed FSK 1%, FSK 2% and FSK 4%, respectively. There was no statistically significant difference between the diet groups ($p=0.25$, ANOVA), but WG decreased linearly in fish fed increasing levels of FSK ($p<0.02$, $R^2=0.3$,

LR). The fish given a 3 and 4% inclusion of FSK weighed around 25 grams less than the reference group, which corresponds to approximately 10% lower WG than the reference group.

The specific growth rate (SGR) of the reference groups was 1.20±0.01, and the same SGR applies for both the fish fed FSK 1% and the fish fed FSK 2%. In the fish fed FSK 3%, the SGR was 1.10±0.03, while it was 1.10±0.05 in the fish fed FSK 4%. There was no statistically significant difference between the diet groups in SGR ($p=0.25$, ANOVA), but SGR decreased linearly in fish fed increasing levels of FSK ($p<0.02$, $R^2=0.3$, LR). The fish given a 3 and 4% inclusion of FSK had approximately 10% lower SGR than the other diet groups. Neither the condition factor (CF), the total feed intake (TFI), the feed conversion ratio (FCR) nor the daily feed intake as percentage of biomass (DFI) were affected by diet, see Table 4.1.

Table 4.1: Growth Performance Indicators of Atlantic salmon post smolt fed graded inclusion of fermented sugar kelp. 30 fish were measured at the beginning of the feeding trial, while all 65 fishes per tank were measured at the end of the feeding trial. FCR applies for each tank, n=3.

	Reference	**FSK 1%**	**FSK 2%**	**FSK 3%**	**FSK 4%**	**ANOVA**	**Regression**
IBW (g)	210±3	203±1	209±3	204±2	209±0.4	n.s.	n.s.
FBW (g)	485±7	470±5	476±10	451±13	459±15	n.s.	n.s.
WG (g)	275±4	267±4	267±7	248±11	250±15	n.s	Y=-7.190x+275.4, $p<0.04$, $R^2=0.3$
SGR	1.20±0.01	1.20±0.01	1.20±0.01	1.10±0.03	1.10±0.05	n.s.	Y=-0.0212x+1.207, $p<0.02$, $R^2=0.3$
CF	1.26±0.01	1.10±0.10	1.25±0.003	1.24±0.003	1.23±0.01	n.s.	n.s.
TFI (g)	11920±159	12670±517	12860±115	12310±718	11906±135	n.s.	n.s.
DFI (%)	0.60±0.13	0.80±0.03	0.70±0.09	0.70±0.08	0.80±0.01	n.s.	n.s.
FCR	0.70±0.01	0.70±0.04	0.70±0.02	0.80±0.04	0.70±0.04	n.s.	n.s.

Notes: IBW=initial body weight (g), FBW=final body weight (g), WG=weight gain (g), SGR=specific growth rate, CF=condition factor, TFI=total feed intake (g), DFI (%) =daily feed intake as percentage of biomass, FCR=feed conversion ratio.
n.s. = not significant. Data is presented as mean±SE.
SGR, CF, DFI (% of biomass) and FCR are calculated as described by Kamunde, Sappal and Melegy (2019). TFI is calculated as described by Helland, Grisdale-Helland and Nerland (1996).

4.3 Semi- quantitative scoring of histological sections

Criterion 1- Vacuolization

The percentagewise distribution of each score for each diet group on criterion 1 is shown in Figure 4.1A. The fish fed FSK 4% displayed the lowest score on criterion 1 (2.3±1.1), while the fish fed FSK 2% showed the highest score on criterion 1 (3.1±1.1). The fish fed the reference diet was scored as 2.8±0.7, while the fish fed FSK 1% was scored as 2.8±1.0. The highest proportion scored as "no" were seen in the fish fed FSK 4 % at 33%, while the fish fed FSK 2% is the only group who displayed "severe" vacuolization (11%, one fish). No statistically significant difference was detected between the diet groups (p=0.59, KWt).

Criterion 2- Lamina propria

The percentagewise distribution of each score for each diet group on criterion 2 is shown in Figure 4.1B. The fish fed the FSK 1 % displayed the lowest score on criterion 2 (2.6±0.5), while the fish fed FSK 4% displayed a slightly higher score at 2.6±0.7. The fish fed the reference diet showed the highest score on criterion 2 (2.9±0.8), while the fish fed FSK 2% was scored as 2.8±0.7. The fish fed FSK 1% was only scored as "mild" and "moderate" while 22% of the fish in the reference group, and 11% of the fish fed FSK 2% and FSK 4%, were scored as "distinct". No statistically significant difference was detected between the diet groups (p=0.67, KWt).

Criterion 3- Connective tissue

The percentagewise distribution of each score for each diet group on criterion 3 is shown in Figure 4.1C. The fish fed FSK 1% displayed the lowest score on criterion 3 (2.1±0.3), while the fish fed FSK 2% showed the highest score on criterion 3 (3.0±0.7). The fish fed the reference diet was scored as 2.6±0.7, while the fish fed FSK 4% was scored as 2.2±07. The fish fed FSK 1% was only scored as "mild" and "moderate" while 22% of the fish in the FSK 2%, and 11% of the fish fed the reference diet and FSK 4%, were scored as "distinct". A statistically significant difference was detected between the diet groups (p=0.014, KWt). The score on criterion 3 in the fish fed FSK 2% was statistically significantly higher than that of the fish fed FSK 1% (p=0.026, SNK) and that of the fish fed FSK 4% (p=0.035, SNK).

Criterion 4- Mucosal folds

The percentagewise distribution of each score for each diet group on criterion 4 is shown in Figure 4.1D. All diet groups were scored mild and moderate, where the fish fed FSK 4% had the highest proportion scored as "mild" at 89%. The fish fed FSK 4% displayed the lowest score on criterion 4 (2.1±0.3), while the fish fed FSK 2% showed the highest score on criterion 4

(2.6±0.5). The fish fed the reference diet was scored as 2.2±0.4, while the fish fed FSK 1% was scored as 2.2±0.4. No statistically significant difference was detected between the diet groups (p=0.18, KWt).

Overall mid intestine health

The percentagewise distribution of all scores for each diet group on all four criteria are shown in Figure 4.1E. The fish fed FSK 4% displayed the highest proportion scored as "no" with 8%, while the only diet group scored as "severe" was FSK 2% with 3%. The fish fed FSK 4% displayed the lowest overall score (2.3±0.5), while the fish fed FSK 2% showed the highest overall score (2.9±0.5). The fish fed the reference diet was scored as 2.6±0.5, while the fish fed FSK 1% was scored as 2.4±0.4. No statistically significant difference was detected between the diet groups (p=0.08, KWt).

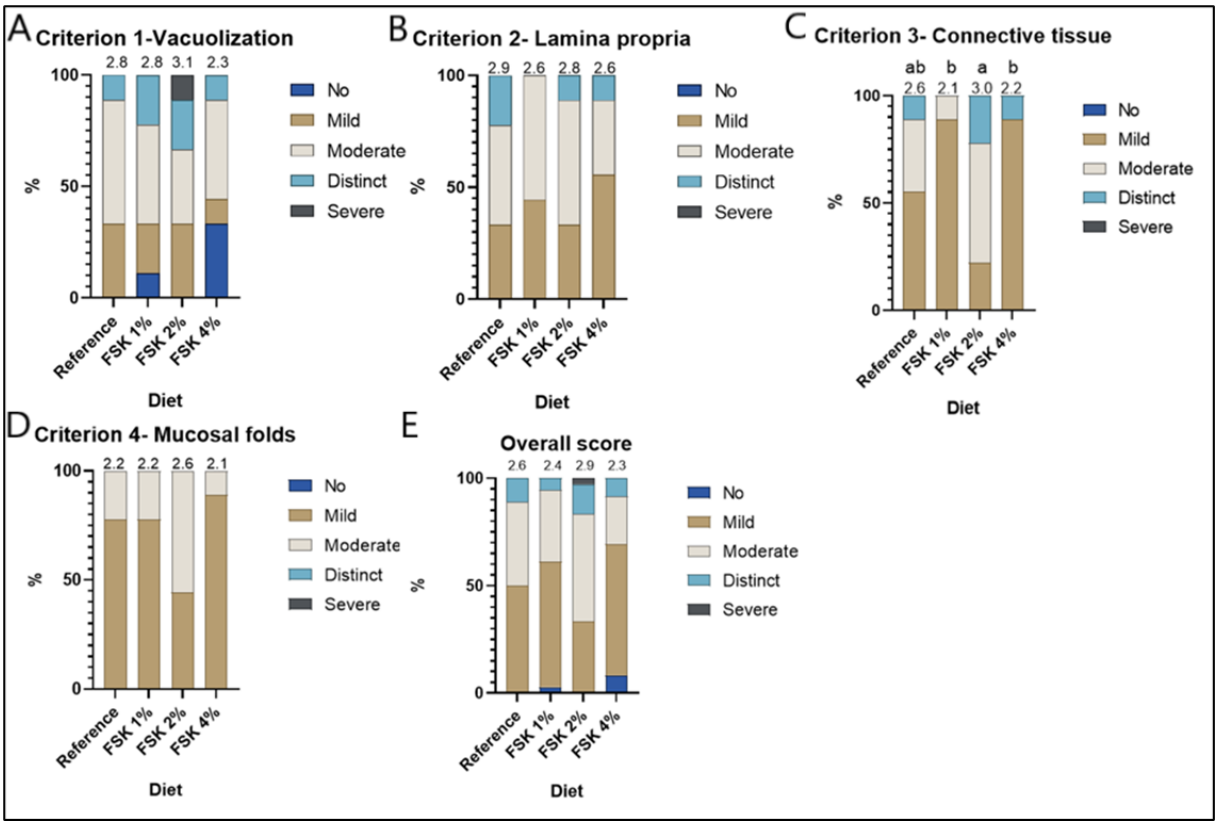

Figure 4.1: Distribution of scores on semi- quantitative criteria. The columns of the contingency charts show the percentagewise distribution of the scores from 1 (no) to 5 (severe) within each diet group (n=9) on A) Criterion 1, B) Criterion 2, C) Criterion 3, D) Criterion 4, and E) the overall score from all four criteria. Numbers above the bars indicates the mean score within each diet group. Groups annotated with different letters are statistically significantly different after KWt and SNK post- hoc, $p<0.05$.

4.4 Morphometric analysis

The main results presented by statistical analysis below are conducted on the whole dataset including the individually measured values per fish on the three parameters: length of mucosal folds, thickness of mucosal folds (2 measurements per fold), and thickness of the intestinal wall. Because of the variable number of observations per individual from the morphometric analysis (min n=5, max n=21) on the three mentioned parameters, a subsequent analysis, using the mean value per fish, was conducted. The results from ANOVA on this analysis are presented in Appendix Table 7, and the results from linear regression using mean values per fish are presented in Appendix Table 8. Since the results coincide with the main results presented below, they are not further considered in this thesis.

Length of mucosal folds

The fish fed FSK 4% had the highest mean length of mucosal folds (632±300 µm), while the fish fed FSK 1% had the lowest mean length of mucosal folds (563±229 µm) (Figure 4.2A). The mean length of the mucosal folds in the fish fed the reference diet and the fish fed FSK 2% were 581±208µm and 592±235µm, respectively. No statistically significant difference was detected between the diet groups (p=0.50, ANOVA). Further, no dose- dependent response was observed (p=0.09, LR).

Thickness of mucosal folds

The fish fed the reference diet had the highest mean thickness of mucosal folds (105±22 µm), while the fish fed FSK 1% had the lowest mean thickness of mucosal folds (92±27 µm) (Figure 4.2B). The mean thickness of the mucosal folds in the fish fed FSK 2% and the fish fed FSK 4% were 98±31µm and 103±32 µm, respectively. No statistically significant difference was detected between the diet groups (p=0.57, ANOVA). Further, no dose- dependent response was observed (p=0.26, LR).

Thickness of the intestinal wall

The fish fed the reference diet had the highest mean thickness of the intestinal wall (371±97 µm), while the fish fed FSK 2% had the lowest mean thickness of the intestinal wall (306±100 µm). The mean thickness of the intestinal wall in the fish fed FSK 1% and the fish fed FSK 4% were 333±96µm and 328±112µm, respectively. No statistically significant difference was detected between the diet groups (p=0.23, ANOVA), but thickness of the intestinal wall decreased linearly in fish fed increasing levels of FSK (R^2=0.013, p=0.037, LR) (Figure 4.2C).

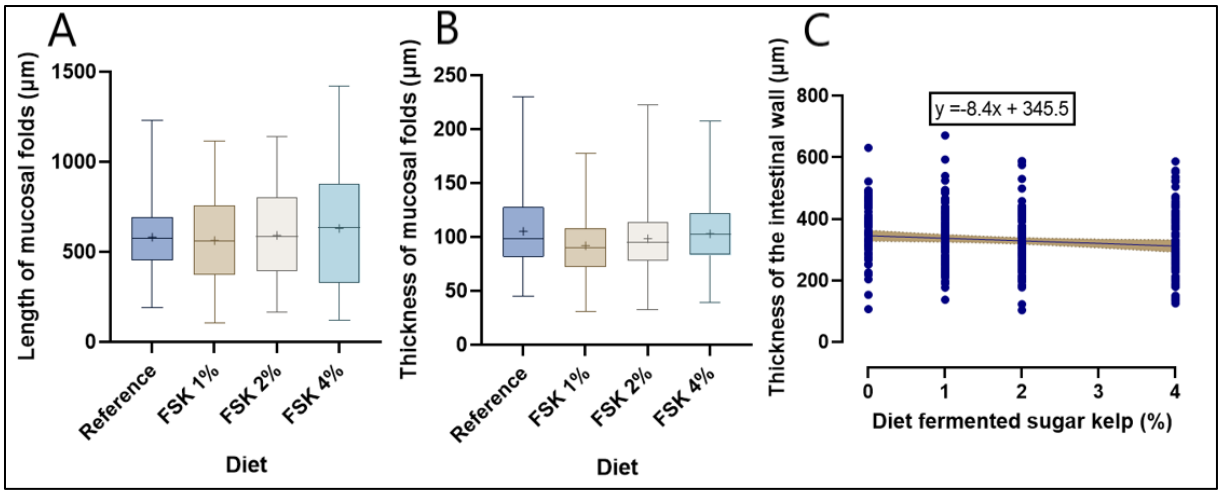

Figure 4.2: Length and thickness of the mucosal folds, and thickness of the intestinal wall. A) Length of the mucosal folds (µm), B) thickness of the mucosal folds (µm), and C) thickness of the intestinal wall in the diet groups reference, FSK 1%, FSK 2%, and FSK 4% at the end of the feeding trial (n=9 fish per diet groups). All values in A), B) and C) are from min n=5, max n=21 individually morphometric measurements within each fish. The boxes in A) and B) range from the first quartile (Q1) to the third quartile (Q3) and show the interquartile range (IQR). The lines across the boxes indicate the median, and "+" indicates the mean. The extended lines range from Q1 and Q3 to the minimum and maximum value, respectively. C) linear regression with 95% confidence interval (FSK inclusion level on the x-axis: 0,1,2,4). No statistically significant differences were detected between the diet groups, $p>0.05$, but a dose- dependent decline in C) wall thickness was observed upon linear regression, $p<0.05$.

Correlation between morphometric parameters

A positive and significant correlation was found between length of mucosal folds and thickness of the intestinal wall (r=0.10, p=0.009) (Figure 4.3A), and between length of mucosal folds and thickness of mucosal folds (r=0.29, p=1.3e-15) (Figure 4.3B). No other significant correlations were found between the parameters.

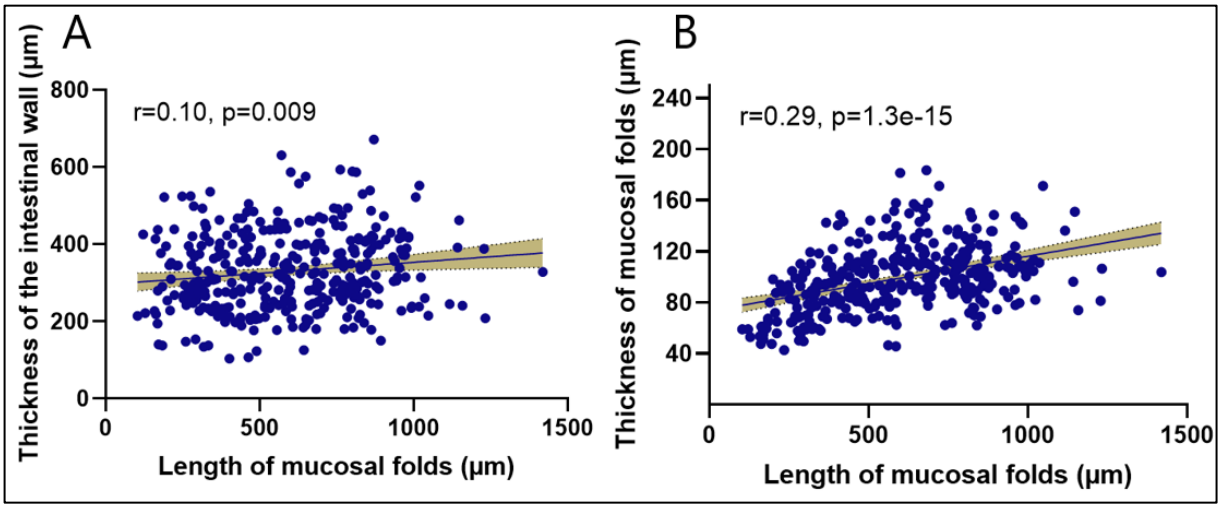

Figure 4.3: Correlation between morphometric parameters. A) the positive and significant correlation between the length of mucosal folds (µm) and the thickness of the intestinal wall(µm), and B) the positive and significant correlation between the length of mucosal folds (µm) and the thickness of the mucosal folds (µm). Respective correlation coefficient r and p- values are denoted for each plot.

4.5 Quantifiable mucosal analysis

Percentage of area covered by mucous cells

The fish fed the reference diet had the lowest percentage of area covered by mucous cells (6.0±3.7%), while the fish fed FSK 4% had the highest percentage of area covered by mucous cells (8.0±3.8%). The percentage of area covered by mucous cells in the fish fed FSK 2% was 6.6±2.9%. No statistically significant difference was detected between the diet groups (p=0.63, ANOVA), but the percentage of area covered by mucous cells increased linearly in fish fed increasing levels of FSK (R^2=0.05, p=<0.0001, LR) (Figure 4.4A).

Mean size of mucous cells

The fish fed FSK 2% had the highest mean size of mucous cells (107±27 µm^2), while the fish fed the reference diet had the lowest mean size of mucous cells (92±39 µm^2). The mean size of mucous cells was 103±28 µm^2 in fish fed the FSK 4% diet. No statistically significant difference was detected between the diet groups (p=0.52, ANOVA). Compared to the reference group, the mean size of mucous cells increased in the fish fed 2% FSK inclusion, while a slight decrease compared to the fish fed FSK 2% was seen in the fish fed FSK 4% (R^2=0.03, p=0.02, NLRM) (Figure 4.4B).

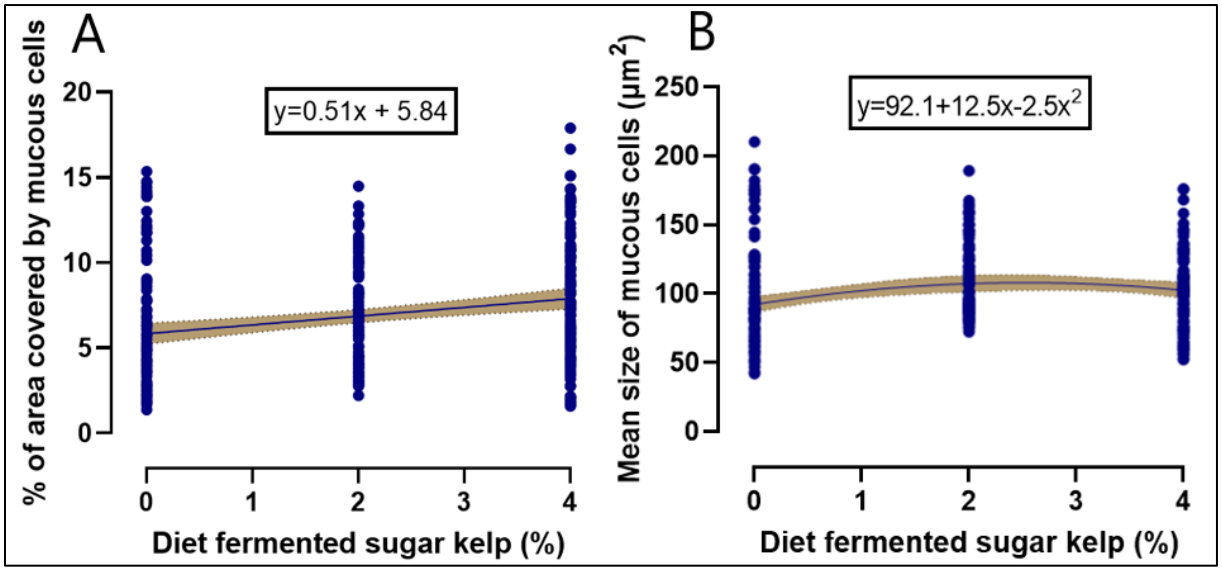

Figure 4.4: Percentage of area covered by mucous cells and the mean size of mucous cells. Data points are values from A) the percentage of area covered in mucous cells within each of the 12 subsections in each fish and B) the mean size of mucous cells within each of the 12 subsections in each fish. (FSK inclusion level on the x-axis: 0,1,2,4). A) linear regression with 95% confidence interval. No statistically significant difference was detected between the diet groups, p>0.05, but a dose- dependent increase was observed upon linear regression, p<0.05. B) second quadratic regression line with 95% confidence interval. No statistically significant difference was detected between the diet groups, p>0.05, but a statistically significant dose-dependent response was observed upon nonlinear regression p<0.05.

Correlations between mucosal qualitative parameters

A statistically significant and positive correlation between the mean size of mucous cells and the percentage of area covered by mucous cells were found in all diet groups, r=0.74, p<2.2e-16, r=0.55, p<2.2e-16, and r=0.61, p<2.2e-16 for the fish fed the reference diet, the fish fed FSK 2%, and the fish fed FSK 4%, respectively (Figure 4.5A, Figure 4.5B, Figure 4.5C).

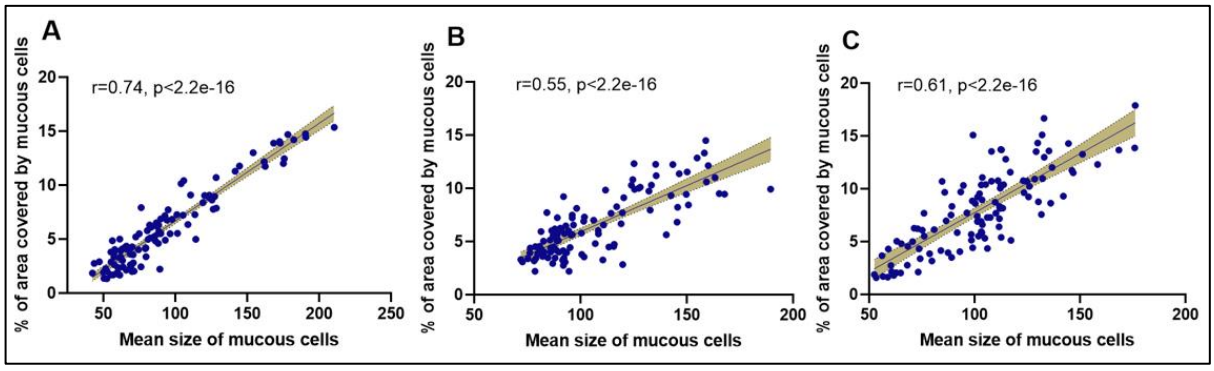

Figure 4.5: Correlations between mucosal parameters. The figure shows the correlation between mucosal parameters with 95% CI for A) the fish fed reference diet, B) the fish fed FSK 2%, and C) the fish fed FSK 4%. Respective correlation coefficient r and p- values are denoted for each plot.

4.6 GSH/GSSG analysis

Total GSH concentration

The mean total GSH concentration in mid intestine samples were 529±184µM, 1068±209µM, 729±124µM, 729±164 µM, and 520±98 µM in the fish fed the reference diet, FSK 1%, FSK 2%, FSK 3% and FSK 4%, respectively. Thus, the highest mean total GSH concentration was seen in the fish fed FSK 1% while the lowest concentration was seen in the fish fed FSK 4%. A statistically significant difference was observed between the diet groups (p=0.01, ANOVA). Total GSH concentration was significantly higher in the fish fed FSK 1% than in the fish fed the reference diet (p=0.0001, HSD), the fish fed FSK 3% (p=0.042. HSD) and the fish fed FSK 4% (p=0.0005, HSD). A dose- dependent response was also observed when performing the non-linear regression (R^2=0.35, p=0.002) (Figure 4.6A).

GSSG concentration

The mean GSSG concentration in the mid intestine samples were 0.91±0.56µM, 1.16±0.44µM, 1.71±0.85µM, 1.61±1.0, and 0.90±0.23 µM in the fish fed the reference diet, FSK 1%, FSK 2%, FSK 3% and FSK 4%, respectively. Thus, the highest mean GSSG concentration was seen in the fish fed FSK 1% while the lowest concentration was seen in the fish fed FSK 4%. No statistically significant difference was detected between the diet groups (p=0.14, ANOVA).

Further, no dose- dependent effect was observed when performing the nonlinear regression (p=0.08) (Figure 4.6B).

GSH/GSSG ratio

The mean GSH/GSSG ratio in the mid intestine samples were 671±266, 1055±505, 623±450, 552±244, and 591±141 in the fish fed the reference diet, FSK 1%, FSK 2%, FSK 3% and FSK 4%, respectively. Thus, the highest mean GSH/GSSG ratio was seen in the fish fed FSK 1% while lowest mean GSH/GSSG ratio was seen in the fish fed FSK 3%. No statistically significant difference was detected between the diet groups (p=0.45. ANOVA). Further, no dose- dependent effect was observed when performing the linear regression (p=0.21) (Figure 4.6C).

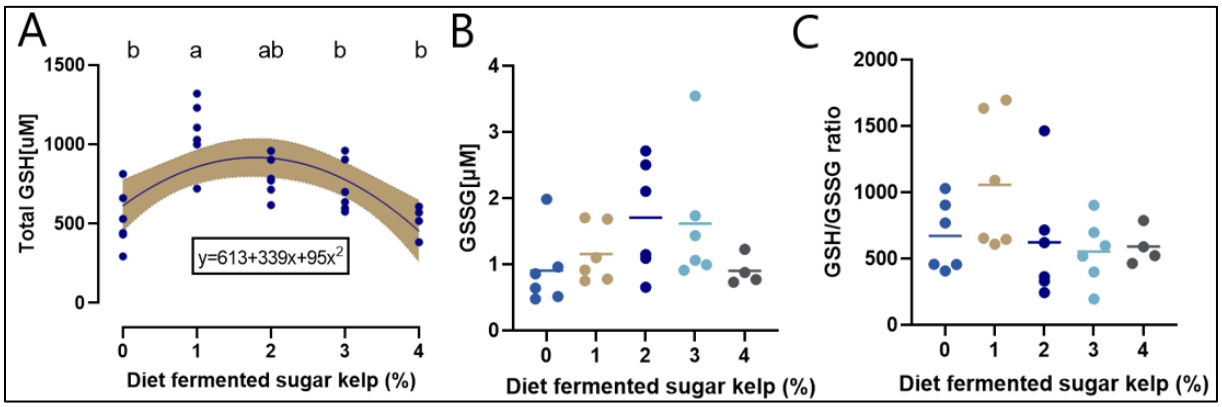

Figure 4.6: Total GSH concentration, GSSG concentration, and GSH/GSSG ratio. Total GSH [µM], GSSG concentration [µM], and GSH/GSSG ratio in MI of Atlantic salmon fed reference diet, FSK 1%, FSK 2%, FSK 3% and FSK 4% (FSK inclusion level on the x-axis: 0,1,2,3,4). Data points in FSK 4% are values from four pooled samples within each group, remaining groups are values from 6 pooled samples. A) nonlinear regression with 95% confidence interval. Groups assigned with different letters are statistically different after one- way ANOVA and Tukey HSD test. A dose- dependent effect was observed when performing the non-linear regression, $p<0.05$. For B) and C), no statistically significant differences were detected between the diet groups, and no-dose dependent responses were observed, $p>0.05$. The horizontal lines indicate the mean.

5. Discussion

The aim of this master project was to assess whether inclusion of fermented sugar kelp (*Saccharina latissima*) (FSK) in feed for Atlantic salmon post- smolt would affect any gut morphological parameters or redox status in the first segment of the mid intestine compared with fish fed a commercially relevant diet. Although studies have been conducted with sugar kelp in feed for salmonids, no previous studies have evaluated the potential of fermented sugar kelp in aquafeeds.

Growth performance and feed utilization

In the current study, FSK supplemented groups up to 2% experienced better growth performance than FSK supplemented groups up to 4% based on WG and SGR. This result is in accordance with a study by Granby *et al.* (2020) in rainbow trout fed 2% *S. latissima* that experienced normal growth with no negative health effect, while higher inclusion levels of *S. latissima* (up to 4%) significantly decreased final weight. Growth impairments in rainbow trout when fed 4% *S.latissima* have also been reported by Ferreira *et al.* (2020). In the current study, despite of a lower growth performance in the 4% FSK supplemented group, feed intake was comparable with the reference group and was not affected by a higher inclusion level of FSK. Breakdown of the polysaccharides and oligosaccharides chains by fermentation (Zhang *et al.*, 2018) may be a rational reason for these findings.

However, the reason for this slightly decreased growth is still questionable. The differences in diet composition, other than the inclusion of FSK, may be an explanation. Due to the low dry matter of the FSK, some practical challenges occurred during manufacturing for it to be incorporate into pellets. This was solved by pumping the FSK directly into the extruder. Thus, lipid and energy decreased with higher FSK inclusions due to the energy dilution when using sugar kelp with low fat- and protein content leading to an increase in protein-to-lipid ratio. Previous studies have linked high- fat diets to have a beneficial effect on growth in Atlantic salmon (Hillestad and Johnsen, 1994; Karalazos, Bendiksen and Bell, 2011; Huyben *et al.*, 2020), but improved growth performance have in contrast also been reported when increasing the protein- to- lipid ratio (Weihe *et al.*, 2018). However, the fish responds to the energy in the feed (Hillestad and Johnsen, 1994), which in the present study decreased with increased FSK inclusions due to lower lipid content. It is thus possible that the reduced growth observed not is due to higher FSK inclusions, but the energy dilution due to manufacturing difficulties.

Another possible explanation for the observed reduced growth is salmonids limited ability to digest kelp (Ferreira *et al.*, 2020). The complexity of the cell walls in algal species has been suggested to limit the ability of higher level trophic carnivorous fish to extract nutrients (Batista *et al.*, 2020). The non- starch polysaccharides (NSPs), which are present in *S.latissima* in high contents as laminarin, fucoidan, alginate, and cellulose, have been suggested to negatively affect fish due to their binding to bile acids, or their impairment of digestive enzymes and movement in the intestine (Francis, Makkar and Becker, 2001). The NSPs may thus act as ANFs. In the current study, ADF and cellulose increased in the 3 and 4% FSK supplemented diets, but the cellulose content still only made up <2% of the DM feed. Previous studies in rainbow trout have however reported that the fish was able to sustain up to 30% inclusion of α-cellulose as a filler without inhibiting growth (Bromley and Adkins, 1984). A clear influence on growth and digestibility of main nutrients in Atlantic salmon have also lacked to be seen when adding 10% cellulose as a filler in a newer publication by Kraugerud *et al.*, 2007. It is thus more likely that the decrease in growth possibly is due to some of the other NSPs present.

The sugar kelp used in the current study was fermented, but Laminaria sp. have been reported to contain 38.3% of non- fermentable sugars in alginate form (Hwang *et al.*, 2011), which is a major cell wall polysaccharide in brown seaweeds (Manns *et al.*, 2016). Alginate, which is a NSP, have previously been shown to reduce the apparent digestibility of protein and fat, and reduce the feed intake when used as a binder in feed for rainbow trout at 10% inclusion, but the final growth was not affected (Storebakken, 1985). Inclusion of sugar kelp meal in rainbow trout has previously showed a small but significant decrease in apparent digestibility of protein, while fat digestibility not was affected by dietary sugar kelp (Granby *et al.*, 2020). Both the other NSPs laminarin (Campbell *et al.*, 2020) and fucoidan (Sørensen *et al.*, 2021a) are reported fermentable, and it is thus likely that, if so, it might be the unfermentable alginate that may have affected the digestibility of other nutrients leading to a decrease in growth when fed higher inclusions. In the current study, it remains unknown if the digestibility was affected by higher dietary FSK and thus is the reason for the observed decrease in growth, so this should be further studied.

Another reported reason for growth impairment, seen together with enteritis signs in the intestine, is “spit outs” by Atlantic cod finding the 10% *A. nodosum* supplemented diet unpalatable (Keating *et al.*, 2021), but the biomass adjusted feed intake in the current study was not significantly affected by diet and extinguishing palatability as a reason for the observed reduced growth. Therefore, more studies are needed to figure out the rational and certain reason

for this slight decrease in growth with higher inclusion levels of FSK in the Atlantic salmon diet.

Mid- intestinal gut morphology

While the second segment of the intestine normally has supranuclear vacuoles in the enterocytes, such vacuolization is not expected to be seen in the PC and the first segment of the MI (Olsen *et al.*, 1999, 2000; Caballero *et al.*, 2002; Bjørgen *et al.*, 2020; Li *et al.*, 2020). In this study, some vacuolization was detected in the first segment of the MI in all diet groups when assessing the semi- quantitative analysis of vacuolization, but there was no significant effect of diets on the level of vacuolization in the enterocytes. Since vacuolization was seen in fish fed all diets, the current study contrast with what seen previously with inclusions of *S. latissima* were the vacuolization in the MI only were seen in fish fed the highest inclusion at 4% (Granby *et al.*, 2020).

Hyper-vacuolization of enterocytes has been observed in the PC of Atlantic salmon fed both black soldier fly (BSF) meal and in fish fed the FM and SPC based control diets (Li *et al.*, 2020), while deficiency of certain fatty acids has been suggested as a plausible reason for lipid accumulation in the enterocytes of PC for Artic charr fed higher dietary linseed oil compared to a FO based control diet (Olsen *et al.*, 2000).Vacuolization of the enterocytes in studies with seaweeds in aquafeeds has to the author's knowledge not been assessed to a wider extent than what conducted by Granby et al., (2020). Findings from experiments with microalgae in feed may be relevant in comparison. Studies with dietary probiotics- and microalgae inclusions in gilthead seabream as immunostimulants reported enterocyte vacuolization together with altered gut morphology in the anterior intestine of all experimental diet groups, while this was not present in the fish fed a FM based control diet (Cerezuela *et al.*, 2012). In the distal intestine of Atlantic salmon, where vacuolization is expected to be observed, inclusions of microalgae resulted in less prominent enterocyte vacuolization in the experimental groups compared to the control group fed a FM based diet (Sørensen *et al.*, 2021b). According to Sørensen *et al.*, (2021b), the lack of vacoulozation was the only sign of gut inflammation observed.

An interesting observation, although not significantly, is that the highest proportion of samples without any vacuolization were seen in the fish fed FSK 4% in the current trial and thus in the group fed the highest inclusion of fermented sugar kelp. The reason for the observed vacuolization in the MI of all diet groups is therefore more likely to be due to other factors than the dietary inclusion of fermented sugar kelp. The vacuolization in the current study may be

explained by digestive status as the fish only were starved for 24 hours. Vacuolization with lipid droplets in the MI have been observed in sea bass (*Dicentrarchus labrax*) 48 hours postprandial, while the droplets disappeared both in the proximal intestine and the distal intestine after three weeks of fasting (Alix *et al.*, 2017). Considering that the intestinal absorption of dietary triglycerides is much slower in salmonids than in mammals (Sire, Lutton and Vernier, 1981), it is thus possible that the vacuolization observed is ongoing absorption.

In aquatic animals, mucosal fold length is regarded a sign of absorption ability (Cerezuela *et al.*, 2012). Both thickening of mucosal folds due to infiltration with inflammatory cells in the lamina propria (Stone *et al.*, 2018) and reduced length of mucosal folds (Moldal *et al.*, 2014) are both well documented negative effects of dietary modulations on the intestinal morphology. There were no statistically significant differences detected between the diet groups in the length of mucosal folds nor in the thickness of mucosal folds in the MI from the morphometric analysis. This is in alignment with the results obtained from the corresponding semi-quantitative analysis of mucosal folds and lamina propria, where no statistically significant differences were detected between the diet groups. No compromising on length of mucosal folds nor thickness of mucosal folds in the MI confirms findings in previous studies in the MI of rainbow trout fed inclusions of *S. latissima* up to 4% (Granby *et al.*, 2020), and in the anterior intestine and PC of rainbow trout fed inclusions of the red seaweed *G. pygmaea* up to 6 % (Sotoudeh and Mardani, 2018). It is however unclear why there in this study is a significant positive correlation between the length of mucosal folds and thickness of mucosal folds, which is discordant with what is usually seen with inflammatory responses in the intestine of salmonids with shorter and thicker folds (Bjørgen *et al.*, 2020), and with healthy long and densely packed mucosal folds (Djordjevic *et al.*, 2021). Not many studies have conducted morphometric measurements of the length and thickness of mucosal folds in the first segment of the MI in Atlantic salmon with the same fish size as in the current study, but the mucosal folds measured in this study were slightly lower and slightly thicker than the folds measured in rainbow trout (Granby *et al.*, 2020) with approximately the same FBW. Considering that no compromising of lamina propria thickness nor appearance of mucosal folds were observed through the morphometric or the semi- quantitative analysis when compared to the control fed group, one probable explanation for this correlation is that the shortest folds measured in this study were small in general, thus also thinner, possibly due to the fish size.

Even though not significantly, the mucosal folds in the fish fed FSK 4% appeared to be the highest from both the semi- quantitative- and the morphometric analysis. A study with 10%

inclusion of the dietary fermentable fibre Vitacel® in rainbow trout reported increased mucosal fold length in the proximal intestine compared to the control fed fish (Yarahmadi, Kolangi Miandare and Hoseinifar, 2016), but the control diet used the mentioned study contained SBM which may be a rational reason for these findings. Studies done in pigs have shown that supplementation with the purified bioactive compound laminarin found in sugar kelp increased the villi length in the duodenum (Walsh *et al.*, 2013a), but this has to the authors knowledge not been observed in fish. On the contrary, Keating et al. (2021) reported a significant reduced length of mucosal folds in combination with increased thickness of intestinal folds in the hindgut of Atlantic cod fed a 10% inclusion of the brown seaweed *A. nodusum*. Considering that unfermented sugar kelp meal inclusions up to 4% previously have demonstrated to not alter the length or thickness of mucosal folds in the first segment of the MI in rainbow trout (Granby *et al.*, 2020), it coincide that both unfermented and fermented dietary sugar kelp up to 4% in salmonids is satisfactory in this respect.

Cellular infiltration with leucocytes of submucosa in the second segment of the MI is a typical inflammatory morphological change seen with SBMIE in Atlantic salmon (Baeverfjord and Krogdahl, 1996), but such cellular infiltration of submucosa have previously also been observed in the proximal intestine with pyloric caeca in Atlantic salmon fed BSF meal (Li *et al.*, 2020). The semi- quantitative analysis of connective tissue in the current study revealed a significant increase in the amount of submucosal connective tissue in the fish fed FSK 2% compared to the fish fed FSK 1% and the fish fed FSK 4%. It has to the authors knowledge not been assessed whether cellular infiltration of intestinal submucosa is affected by dietary seaweeds. Thus, studies where other immunostimulants have been included in aquafeed could in this context be useful in comparison. Studies in the anterior intestine of the mainly carnivorous gilthead sea bream fed microalgae and the probiotic *Bacillus subtilis* reported observations of infiltrated leucocytes in submucosa, as well as in the lamina propria, compared to the control group fed a FM- based diet, and recommended further research on how immunostimulants affect intestinal leucocyte infiltration (Cerezuela *et al.*, 2012). Further, the appearance of the submucosa has also been assessed in Nile tilapia fed *Aspergillus oryzae* fermented date palm seed meal, where both the fish fed the experimental diet, and the fish fed a fish- and SBM based control diet, displayed normal structured submucosa in the anterior, middle and posterior part of the intestine (Dawood *et al.*, 2020). However, Nile tilapia contrasts with Atlantic salmon as a primarily herbivorous fish which may explain why the intestinal submucosa morphology was not affected by the SBM in the control diet nor the dietary fermented palm seed meal.

It is thus important to keep in mind that the intestinal wall is built up by a substantial amount of tunica muscularis that can be affected by dietary modulations (Kihara, Ohba and Sakata, 1995; Yarahmadi, Kolangi Miandare and Hoseinifar, 2016; Granby *et al.*, 2020). While the semi- quantitative analysis conducted in the current study only assessed the amount of submucosal connective tissue, the morphometric analysis of the thickness of the intestinal wall included both tunica muscularis and the submucosa. The obtained result from the morphometric analysis of the thickness of the intestinal wall revealed no significant differences between the diet groups. However, a weak but significant decrease in the morphometric measured thickness of the intestinal wall was seen with increased fermented sugar kelp inclusions. Although not significantly, the fish fed FSK 2% obtained the lowest measured thickness of the intestinal wall from the morphometric analysis. This is an interesting finding considering that the FSK 2% showed a significant increase in the amount of submucosal connective tissue in the intestinal wall from the semi- quantitative analysis, although this was not detected with the morphometric analysis and there are contradicting results between the two measurements.

A decrease in tunica muscularis can decrease intestine strength and motility (Granby *et al.*, 2020). According to Granby *et al.*, (2020), rainbow trout fed a 4% inclusion of *S. latissima* had a significantly lower tunica muscularis thickness in the MI than both the fish fed *S. latissima* inclusions up to 2%, and the fish fed a FM- and SPC based control diet. On the other hand, increased tunica muscularis thickness has been reported in the proximal intestine of rainbow trout fed a 1% inclusion of fermentable fibre (Vitacel®) but it is unclear how these measurements were performed (Yarahmadi, Kolangi Miandare and Hoseinifar, 2016). In the study conducted by Granby *et al.*, (2020), the morphometric measurement of the intestinal wall was done from serosa to the end of muscularis. Hence, the submucosal connective tissue was excluded when considering tunica muscularis thickness in the intestinal wall (Granby *et al.*, 2020), and it remains unknown if the amount of submucosal connective tissue was simultaneous affected by the observed decrease in tunica muscularis. Thus, the inconsistency in how the morphometric measurments are assessed in the study by Granby *et al.*, (2020) and how they are conducted in the present study makes it difficult to compare the results. Further research where tunica muscularis thickness and submucosal connective tissue are assessed separately is needed to establish how fermented sugar kelp affects the thickness of the intestinal wall.

The reliability of the obtained result from the semi- quantitative analysis is thus arguable since the analysis of FSK 2% group, which showed a significantly higher amount of submucosal connective tissue, was done at a later stage and not blindly. It should be noted that the cellular

infiltration of submucosa associated with SBMIE in Atlantic salmon is seen together with lamina propria infiltration (Baeverfjord and Krogdahl, 1996). Considering that the appearance of mucosal folds and lamina propria in the current study were not significantly affected by diet neither from the semi- quantitative analysis or the morphometric analysis, and the contradicting results retrieved from the morphometric analysis and the semi- quantitative analysis, the significant increase in submucosal connective tissue observed in FSK 2% may be biased.

Density and size of mucous cells in the mid intestine

The mucus excreted by the mucous cells is able to bind to organic and inorganic materials and thus remove them by constant secretion (Dang *et al.*, 2019), and an increased mucus secretion is thus an effective defense mechanism (Torrecillas, Montero and Izquierdo, 2014). None of the quantifiable mucosal parameters varied significantly between the diet groups, but dose-dependent responses were seen for both parameters measured in this study (the percentage of area covered by mucous cells and the mean size of mucous cells in the first segment of the MI). The fit of the R^2 of these models are poor so these should be interpreted with caution. Both parameters were the lowest, although not significantly so, in the fish fed the reference diet. There is little available literature describing the effects of dietary seaweeds on mucous cells in salmonid intestines. One article does, however, report that inclusion of *S. latissima* up to 4% in rainbow trout had no influence on the number of mucous cells present in the mid- intestine (Granby *et al.*, 2020). Unaffected number of mucous cells present is in alignment with studies in the distal intestine of Senegalese sole (*Solea senegalensis*) fed 10% inclusions of the green alga *Ulva rigida* and the kelp *U. pinnatifida* (Moutinho *et al.*, 2018).

An increased mucosal barrier in the intestine may improve the immune response against infectious diseases (Dawood *et al.*, 2020). An interesting finding in the current study is the strong positive correlation observed between the mean size of mucous cells and the percentage of area covered by mucous cells in all diet groups, with the strongest correlation seen in the fish fed the reference diet and the weakest correlation seen in the fish fed FSK 2%. Although little is known about how dietary modulations affects the mucosal barrier in the intestine of fish, a recent publication suggests that the gut health is impacted by either reduced or over- activated mucosal protection (Sørensen *et al.*, 2021b). According to Sørensen *et al.* (2021b), decreased mucous cell size and low mucosal barrier status was seen in the distal intestine of fish fed SBM, while increased mucous cell- size and abnormal barrier status was seen in fish fed a diet with a combination of other normal plant- protein sources. In the current study, there are indicators that larger mucous cells are correlated with an increased coverage of mucous cells in the

intestine, but that this correlation is weaker when fed fermented sugar kelp. How the barrier status is affected remains unknown with the analysis performed.

Previous work has suggested that altering of the microbiota can alter the composition of mucus and mucous cell function (Deplancke and Gaskins, 2001; Cerezuela *et al.*, 2012). As the *S. latissima* used in the present study is fermented, findings from other fermented products and probiotics may thus be relevant in comparison. Increased number of mucous cells has been seen in the MI of common carp (*Cyprinus Carpio*) fed 20% yeast fermented poultry by- product meal for 90 days compared to fish fed the FM- and SBM based control diet (Dawood *et al.*, 2020b). Further, increased number of goblet cells have also been reported in the MI of channel catfish (*Ictalurus punctatus*) fed yeast polysaccharide at 0.1 and 0.3% inclusions, but the duration of this trial was only two weeks (Zhu *et al.*, 2012). No such modulation of mucous cell presence can be reported from the current study. Since the differences detected between the diets were not significant and the fit of the dose- dependent responses are weak, there is little reason to believe that the dietary inclusion of fermented sugar kelp is the reason for the observed effects. Both significant tank effects and fish effects were detected, and it is more likely that the variety between tanks and within fishes are the reason for the observed effects. There are thus indicators of something affecting the mucosal tissue, but if this is due to the dietary inclusion of fermented sugar kelp remains to be further studied.

Oxidative stress and redox status

Glutathione is the major endogenous antioxidant acting as a free radical scavenger in animal cells (Degroote *et al.*, 2019). As the total GSH represents the state of glutathione, the sum of both reduced and oxidized form (GSH and GSSG), its level is related to antioxidant depletion and recovery of GSSG (Eroglu *et al.*, 2015; Peixoto *et al.*, 2016). The differences between the diet groups in the current study were seen in the total GSH concentrations, as this was significantly higher in the fish fed FSK 1% than in the fish fed the reference diet, FSK 3%, and FSK 4%. The concentration of GSH is regulated in two ways: either by *de novo* synthesis of GSH where the key enzyme is glytamyl cysteine ligase, or by reduction of GSSG by glutathione reductase (GR) (Hamre *et al.*, 2010). The increase in total GSH levels seen in the fish fed FSK 1% were neither reflected by a significantly higher GSSG concentration nor a significant change in the GSH/GSSG ratio. The observed increase in total GSH must thus be due to an enhance in the reduced glutathione (GSH) available. This result is in accordance with a previous study with dietary brown seaweed supplementation, where increased total GSH was seen in Atlantic salmon liver (Kamunde, Sappal and Melegy, 2019). It was here addressed that dietary

brown seaweed supplementation in Atlantic salmon, due to the bioactive compounds, directly can enhance antioxidant capacity directly by increasing levels of antioxidant compounds like GSH in tissues (Kamunde, Sappal and Melegy, 2019). Thus, the result of the current study indicates that the fish fed FSK 1% experienced an enhanced antioxidant status affected by supplemented FSK in the diet.

No previous studies have evaluated the effect of fermented sugar kelp on GSH/GSSG status in fish intestine, however studies in the liver of rainbow trout have reported a reduction in expression of *gpx1b2* with inclusion of 2% *S. latissima* (Ferreira *et al.*, 2020). The *gpx1b2* translates to glutathione peroxidase (GPx) , key enzyme in the regulation of the oxidative status and protection of cells against lipid peroxidase (Ferreira *et al.*, 2020). A decrease in GPx may indicate a reduced need for removing of ROS from the tissue (Sotoudeh and Mardani, 2018), and decreases the demand for production of endogenous antioxidant enzymes (Ferreira *et al.*, 2020). LAB species, as the bacteria used to ferment the sugar kelp in the present study, are known to produce several ROS- removing enzymes including GPx (Zotta, Parente and Ricciardi, 2017). Gene expression was not assessed in the present study, but it can be speculated whether a decrease in the production of endogenous antioxidant enzymes due to the dietary inclusion of fermented sugar kelp is the mechanism behind the higher total GSH level in the fish fed FSK 1%. It is necessary to mention, the intestine`s ability for GSH synthesis and accumulation have previously been reported to be limited compared to liver (Castro *et al.*, 2016). So, the effect of diet composition on redox status may be highly tissue- related (Castro *et al.*, 2015), and also affected by the interaction between hepatic GPx activity, biliary GSH efflux and GSH homeostasis in the intestine (Degroote *et al.*, 2019). Therefore, further studies should be assessed to fully examine the potential of fermented sugar kelp as an intestinal antioxidant.

6. Conclusion

The main findings of this study shows that Atlantic salmon post- smolts fed fermented sugar kelp were able to utilize the feed comparable to the fish fed the reference diet and obtain a similar growth performance when fed up to 2% FSK, but a decrease in WG and SGR were seen with increasing FSK inclusion up to 4%. It is unsure if the growth reduction was due to FSK inclusion or due to the other unintended changes in the high inclusion feeds. Despite a significant increase of submucosal connective tissue observed by semi- quantitative analysis in the fish fed FSK 2%, the mid intestinal morphology was not significantly affected by dietary inclusions of fermented sugar kelp, indicating that fermented sugar kelp is satisfactory in this respect. There were no significant differences between the diet groups on the mucosal quantitative parameters, but dose- dependent responses were seen indicating some modulation, yet more research is needed. The significant increase in total GSH in the MI of the fish fed FSK 1% indicates an enhanced antioxidant status by dietary fermented sugar kelp. Moreover, total GSH was not altered by FSK up to 4%, and no adverse effect was observed. The GSH/GSSG ratio was not significantly affected by the diets.

$H0_1$: Inclusion of fermented sugar kelp in the feed for Atlantic salmon does not affect growth performance and feed utilization, **is rejected. $H1_1$**: Inclusion of fermented sugar kelp in the feed for Atlantic salmon does affect growth performance and feed utilization, **is accepted.**

$H0_2$: Inclusion of fermented sugar kelp in the feed for Atlantic salmon does not affect any gut morphological parameters in the first segment of the mid intestine, **is accepted.**

$H0_3$: Inclusion of fermented sugar kelp in the feed for Atlantic salmon does not affect the redox status (GSH/GSSG ratio) in the first segment of the mid intestine, **is accepted.**

7. Future perspectives

To evaluate the full potential of fermented sugar kelp as a feed ingredient for Atlantic salmon, further studies are needed. First, the result of this study highlights the need for a better understanding of why growth is affected by the dietary modulation with fermented sugar kelp. Preferably, a more complete description of fermented seaweeds and its possible functional components should be established. Moreover, longer trials with more specific challenges are needed. Additionally, the full potential of fermented sugar kelp as a stimulator of the mucosal barrier in the intestine should be further investigated. The antioxidative potential of fermented sugar kelp should also be further explored, possibly in more commercial relevant conditions, and with additional biomarkers such as the genes coding for oxidative stress related parameters, and the interaction between organs could be included.

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Appendix V

Appendix table 1: Results from Shapiro Wilk's test

Shapiro Wilk's test		
Parameter	**p-value**	**Distribution p>0.05=normal**
Length of mucosal folds	0.0001948	Non- normal
Thickness of intestinal wall	0.001695	Non- normal
Thickness of mucosal folds	1634e-10	Non- normal
Length of mucosal folds mean	0.0003825	Non- normal
Thickness of the intestinal wall mean	0.1355	Normal
Thickness of mucosal folds mean	0.639	Normal
Percentage of area covered by mucous cells	5.007e-09	Non- normal
Mean size of mucous cells	1.245e-07	Non- normal
Total GSH	0.7202	Normal
GSSG	0.0007283	Non- normal
GSH/GSSG ratio	0.002946	Non- normal

Appendix table 2: Results from Levene's test

Levene's test		
Parameter	**p-value**	**Variance p>0.05=homogeneous**
Length of mucosal folds	0.0001455	Non- homogeneous
Thickness of intestinal wall	0.084544	Homogeneous
Thickness of mucosal folds	0.1773	Homogeneous
Length of mucosal folds mean	0.3594	Homogeneous
Thickness of the intestinal wall mean	0.8	Homogeneous
Thickness of mucosal folds mean	0.9672	Homogeneous
Percentahe of area covered by mucous cells	0.02467	Non- homogeneous
Mean size of mucous cells	0.01598	Non- homogeneous
Total GSH	0.7006	Homogeneous
GSSG	0.3098	Homogeneous
GSH/GSSG ratio	0.212	Homogeneous

Appendix table 3: Results from Shapiro- Wilk's and Levene's test from box-cox transformed data

Box cox transformation					
Parameter	**Power λ**	**Shapiro-Wilk normality test p-value**	**Distribution p>0.05=normal**	**Levene's Equal Variance test p-value**	**Variance p>0.05=homogeneous**
Length of mucosal folds	0.6666667	0.0008983	Non- normal	0.0085	Non- homogeneous
Thickness of mucosal folds	0.3030303	0.1248	Normal	0.9153	Homogeneous
Perncentage of area covered by mucous cells	0.3030303	0.4237	Normal	0.188	Homogeneous
Mean size of mucous cells	0.1414141	0.07601	Normal	0.1930	Homogeneous

Appendix table 4: Results from Shapiro Wilk's test and Levene's test on transformed data

Transformation of data					
Parameter	**Transformation**	**Shapiro-Wilk's normality test**	**Distribution p>0.05=normal**	**Levene's Equal Variance test**	**Variance p>0.05=homogeneous**
Length of mucosal folds	Sqrt	0.0004645	Non- normal	0.0007593	Non- homogeneous
Length of mucosal folds	Inverse	2.2e-09	Non- normal	0.06301	Homogeneous
Length of mucosal folds	Log10	4.861e-09	Non- normal	0.006937	Non- homogeneous
Thickness of the intestinal wall	Sqrt	0.5089	Normal	0.05454	Homogeneous
Length of mucosal folds mean	Inverse	0.4965	Normal	0.3419	Homogeneous
GSSG	Log10	0.4871	Normal	0.4452	Homogeneous
GSH/GSSG ratio	Log10	0.8971	Normal	0.3645	Homogeneous

Appendix table 5: Significance of random factors

Random factors				
Parameter	**Random factor «Tank number»**	**p<0.05= significant**	**Random factor: «Fish number»**	**p<0.05= significant**
Length of mucosal folds	0.675	Not significant	0.2124	Not significant
Thickness of the intestinal wall	4.123e-06	Significant	1.469e-13	Significant
Thickness of mucosal folds	2.31e-13	Significant	9.334e-12	Significant
Percentage of area covered by mucous cells	2.2e-16	Significant	2.2e-16	Significant
Mean size of mucous cells	2.2e-16	Significant	2.2e-16	Significant
Length of mucosal folds mean	0.6428	Not significant	NA	NA
Thickness of mucosal folds mean	0.01939	Significant	NA	NA
Thickness of the intestinal wall mean	0.1506	Not significant	NA	NA
Criteria 1	0.08457	Not significant	NA	NA
Criteria 2	0.2054	Not significant	NA	NA
Criteria 3	0.03239	Significant	NA	NA
Criteria 4	0.12	Not significant	NA	NA
Overall score	0.04858	Significant	NA	NA
Total GSH	0.0004132	Significant	NA	NA
GSSG	0.05221	Significant	NA	NA
GSH/GSSG ratio	0.01157	Significant	NA	NA

Appendix table 6: Results from ANOVA

One- way ANOVA					
Parameter	**Transformation of dataset**	**Without random factor(s)**	**p<0.05=significant**	**With random factor(s)**	**p<0.05=significant**
Length of mucosal folds	Box cox	0.492	Not significant	0.504	Not significant
Thickness of the intestinal wall	Sqrt	0.001159	Significant	0.2366	Not significant
Thickness of mucosal folds	Box cox	0.0005088	Significant	0.579	Not significant
Percentage of area	Box cox	5.167e-05	Significant	0.630	Not significant

covered by mucus cells					
Mean size of mucous cells	Box cox	2.411e-06	Significant	0.521	Not significant
Total GSH	None	6.433e-05	Significant	0.0104	Significant
GSSG	Log10	0.1935	Not significant	0.1357	Not significant
GSH/GSSG ratio	Log10	0.1885	Not significant	0.4537	Not significant

Appendix table 7: Results from one- way ANOVA on mean values from morphometric analysis

One- way ANOVA					
Parameter	**Transformation of dataset**	**Without random factor(s)**	**p<0.05=significant**	**With random factor(s)**	**p<0.05=significant**
Length of mucosal folds mean	Inverse	0.359	Not significant	0.399	Not significant
Thickness of mucosal folds mean	None	0.356	Not significant	0.662	Not significant
Thickness of intestinal wall mean	None	0.099	Not significant	0.2174	Not significant

Appendix table 8: Results from linear regression for mean values from morphometric analysis

Linear regression				
Parameter	**Equation**	**R^2**	**p-value**	**Significance(p<0.05)**
Length of mucosal folds mean	Y = 16.49*X + 565.9	0.06253	0.1474	Not significant
Thickness of intestinal wall mean	Y = -14.73*X + 356.6	0.1124	0.0490	Significant
Thickness of mucosal folds mean	Y = 0.5307*X + 99.92	0.003387	0.7398	Not significant

Appendix table 9: Results from Kruskal- Wallis rank sum test

Kruskal- Wallis rank sum test			
Criteria	**Chi- squared**	**p- value**	**Significance(p<0.05)**
Criteria 1	1.902	0.593	Not significant
Criteria 2	1.5357	0.6741	Not significant
Criteria 3	10.62	0.01397	Significant
Criteria 4	4.8462	0.1834	Not significant
Overall score	6.7493	0.08033	Not significant

Appendix table 10: Percentagewise distribution of scores on criterion 1- vacuolization

Vacuolizaton								
	Reference		**FSK 1%**		**FSK 2%**		**FSK 4%**	
	Score	**%**	**Score**	**%**	**Score**	**%**	**Score**	**%**
No	0	0.00	1	11.11	0	0.00	3	33.33
Mild	3	33.33	2	22.22	3	33.33	1	11.11
Moderate	5	55.56	4	44.44	3	33.33	4	44.44
Distinct	1	11.11	2	22.22	2	22.22	1	11.11
Severe	0	0.00	0	0.00	1	11.11	0	0.00

Appendix table 11: Percentagewise distribution of scores on criterion 2- Vacuolization

Lamina propria								
	Reference		**FSK 1%**		**FSK 2%**		**FSK 4%**	
	Score	**%**	**Score**	**%**	**Score**	**%**	**Score**	**%**
No	0	0.00	0	0.00	0	0.00	0	0.00
Mild	3	33.33	4	44.44	3	33.33	5	55.56
Moderate	4	44.44	5	55.56	5	55.56	3	33.33
Distinct	2	22.22	0	0.00	1	11.11	1	11.11
Severe	0	0.00	0	0.00	0	0.00	0	0.00

Appendix table 12: Percentagewise distribution of scores on criterion 3- Connective tissue

Connective tissue								
	Reference		**FSK 1%**		**FSK 2%**		**FSK 4%**	
	Score	**%**	**Score**	**%**	**Score**	**%**	**Score**	**%**
No	0	0.00	0	0.00	0	0.00	0	0.00
Mild	5	55.56	8	88.89	2	22.22	8	88.89
Moderate	3	33.33	1	11.11	5	55.56	0	0.00
Distinct	1	11.11	0	0.00	2	22.22	1	11.11
Severe	0	0.00	0	0.00	0	0.00	0	0.00

Appendix table 13: Percentagewise distribution of scores on criterion 4- Mucosal folds

Mucosal folds								
	Reference		**FSK 1%**		**FSK 2%**		**FSK 4%**	
	Score	**%**	**Score**	**%**	**Score**	**%**	**Score**	**%**
No	0	0.00	0	0.00	0	0.00	0	0.00
Mild	7	77.78	7	77.78	4	44.44	8	88.89
Moderate	2	22.22	2	22.22	5	55.56	1	11.11
Distinct	0	0.00	0	0.00	0	0.00	0	0.00
Severe	0	0.00	0	0.00	0	0.00	0	0.00

Appendix table 14: Percentagewise distribution of scores on scores from all four criteria as "overall mid intestine health score"

Overall mid intestine health score								
	Reference		**FSK 1%**		**FSK 2%**		**FSK 4%**	
	Score	**%**	**Score**	**%**	**Score**	**%**	**Score**	**%**
No	0	0	1	2.78	0	0.00	3	8.33
Mild	18	50.00	21	58.33	12	33.33	22	61.11
Moderate	14	38.89	12	33.33	18	50.00	8	22.22
Distinct	4	11.11	2	5.56	5	13.89	3	8.33
Severe	0	0.00	0	0.00	1	2.78	0	0.00