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EFFECTS OF THE DOWNSTREAM PROCESSING OF YEAST ON THE GASTROINTESTINAL HEALTH OF ATLANTIC SALMON DURING SEAWATER TRANSFER

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Master of Science Feed Manufacturing Technology

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ABSTRACT

Aquaculture intensification and sustainability concerns have prompted research into new feed ingredients, notably microbial ingredients such as yeast. *Cyberlindnera jadinii* (CJ) and *Wickerhamomyces anomalus* (WA) are among the functional protein sources with immunostimulatory effects that are currently explored as novel ingredients for fish feeds. In this study, the two yeast species were grown on sugars from hydrolyzed wood and nitrogen from hydrolyzed chicken offal and subjected to two forms of downstream processing of either autolysis (16 hrs, 50^0C) or direct inactivation by spray drying. The objective of this research was to determine how two yeast species with different downstream processing counteract soyabean meal induced enteritis (SBMIE) in Atlantic salmon (*Salmon salar* L.) during seawater transfer using morphometry, histomorphology and immunohistochemical techniques. The experiment was conducted on post-smolt of Atlantic salmon fed in triplicates a fishmeal (FM) control diet (negative control), a diet containing 30% SBM (positive control) and four treatment diets containing 30% SBM supplemented with 10% yeast either inactivated or autolyzed. The results indicate that 10% inclusion of both *C. jadinii* and *W. anomalus* (when inactivated or autolyzed) had no negative effect on growth and feed conversion. Histological observations on the submucosa and lamina propria cellularity also indicate that inactivated *C. Jadinii* has the potential to prevent SBMIE in Atlantic salmon. No differences in the CD3ε $^+$ population was observed across diets but similarity between lamina propria CD8α$^+$ labelled cells between the FM and the yeast containing diets was observed. This indicates that the yeast had some immunostimulatory effect. Crude lipids were better digested in *C. Jadinii* while inactivation resulted in the release of more β-glucans. To conclude, the potential of yeast to alleviate SBMIE depends on the yeast strain and the downstream processing.

1.0: INTRODUCTION

1.1: Overview of the Global Fisheries and Aquaculture

As the fastest growing animal food production sector in the world (Troell et al., 2014) (7.5% p.a. since 1970), fish farming is expected to bridge the gap between fish protein demand and supply and contribute to economic growth (Tacon et al., 2011). A total of 46% of all fish produced in 2018 was obtained from aquaculture which accounted for 52% of all food fish and this proportion is projected to reach 58% by 2028 (FAO, 2020; OECD & Nations, 2020). In contrast, fish outputs from capture fisheries have declined over the years not until around 2016 when it started increasing, reaching a historical peak of 96.4 million tons (MT) in 2018 (FAO, 2020). This increase was largely driven by the increase in capture of anchoveta and other small pelagic fish in South America (FAO, 2020; OECD & Nations, 2020), coupled with some strict global fishery's management programs. With estimated 9.8 billion people on earth by 2050, current food production will have to increase by 70% to meet demand (FAO, 2020). Better standards of living and increased per capita income over the years have subsequently increased the per capita fish & meat consumption, especially in Asia. As a result, fish consumption has also increased at an annual rate of 1.5%, from 9 kg per capita in 1961 to 20.5 kg in 2018, despite the increased human population and declining capture fisheries (FAO, 2020).

The rapid growth in this sector has led to several challenges including diseases, sustainability concerns, and limited access to high-quality protein sources for fish feed. From the onset, aquaculture has been reliant on fishmeal (FM) and fish oils (FO) as the primary source of proteins and fat/oils especially for carnivorous fishes such as Atlantic salmon and trout (Fry et al., 2016; Tacon & Metian, 2008). Fishmeal and FO are considered the 'golden standard' due to their balanced amino acid composition, high energy level, high levels of long chain fatty acids, as well as their content of nucleotides, minerals and vitamins (Naylor et al., 2009; Tacon & Metian, 2015). A larger percentage of the non-food fish is used for FM and FO processing for use in the aquacultural industry. For example, 68% and 74% of the total FM and FO produced in 2012 solely used for aquaculture production (FAO, 2020). However, it is noteworthy that 25-35% of the FM and FO were derived from trimmings and discards which cannot directly serve as human food, but the remaining fraction was obtained from fish that is

edible and termed ‘pristine’ fishmeal and fish oil. Aside the fact that these ingredients are non-sustainable, their supply depends heavily on climate driven events (Naylor et al., 2011). Sardines and anchovies are largely harvested in the South Pacific and their abundance depends on the climate-induced El-Nino southern oscillation (Boissy et al., 2011; Naylor et al., 2009) making their supply unreliable. For this reason, the market prices of these ingredients have increased.

Over the last decade, FM and FO supply has been constant but there is not enough to meet demand due to the rapid expansion of the aquaculture sector. Therefore, reliance on FM and FO have reduced significantly (Tacon & Metian, 2008; Bostock et al., 2010; Ytrestøyl et al., 2015) due to declining capture fisheries, market price volatility, potential sources of contaminants, sustainability concerns and growing interests in alternative feed ingredients, notably plants source ingredients. For example, marine sourced proteins and oils constituted 90% of the total salmon feed composition in 1990 which has since reduced to 24 % in 2019 (Ytrestøyl et al., 2015; MOWI, 2020). Fishmeal and FO production rate has been reduced by 1.7% and 2.6% respectively since 1995 (Tacon et al., 2011). Plant proteins as of 2013 made up 37% of the total proteins used in the feed of Atlantic salmon from a record low of 0% in 1990 with a subsequent reduction in the Fish-in-Fish – Out ratio (FIFO) for Atlantic salmon (Ytrestøyl et al., 2015).

As a sign of commitment, efforts are still being made to further reduce the dependency on FM and FO and to fully replace them with alternative feed ingredients. Most prominent alternatives are terrestrial plants such as soybeans and other leguminous crops such as field pea, faba bean, as well maize and wheat gluten due to their high protein content and favorable amino acid profile (Krogdahl et al., 2015). Soy is used as food for humans but about 75% is used as a protein source for animal feed production or as a source of oil (Masuda & Goldsmith, 2009; Abraham et al., 2020). Both livestock and fish depend on soy as feed ingredients, however, as of 2014, approximately 4% of total soy used in animal feeds was used for fish feed production (Troell et al., 2014). Therefore, as aquaculture intensification continues, so as the demand for these ingredients for use in aquafeeds will intensify crop production, resulting in pressure on land use, water usage, energy and biodiversity (Fry et al., 2016; Pelletier et al., 2018) and/or disproportionately affect direct human usage for food (Ytrestøyl et al., 2015; Abraham et al., 2020). World soyabean production has increased

exponentially from 28.6 million metric tons (MMT) from 1961-1965 to 217 MMT in 2005-2007 (Masuda & Goldsmith, 2009). Consequently, land usage for such purposes quadrupled from 24 M ha to 94 M ha at the same time. Soy production is predicted to grow at a rate of 2.2% p.a to 371 MMT in 2030 (Masuda & Goldsmith, 2009), land usage will continue to increase especially in Argentina and Brazil. This will increase encroachment especially on the Amazon Forest, which is still threatened from climate change.

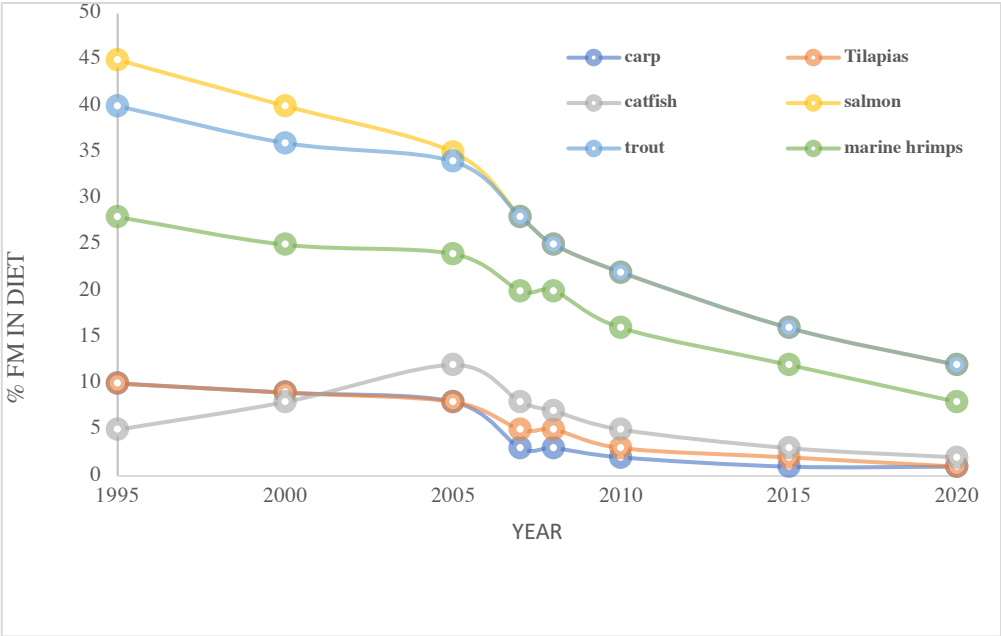


Fig 1. Estimated percentage FM inclusion in diet of cultured fish and marine shrimps from 1995 to 2020. Data obtained from (Tacon et al., 2011). From the figure, there is a gradual reduction in the degree of inclusion of FM in the diet of almost all cultured fish species.

1.2.0: Overview of the Norwegian Salmon Industry

Global salmon production has increased at the rate similar to Norway (6% p.a) since 2000, thereby contributing 4.5% of the total seafood supply in 2018 (FAO, 2020; MOWI, 2020). Norway is the largest producer of salmon in the world. For example, 27.3% of all Atlantic salmon produced in 2018 was from Norway and the rest from Chile, the UK, North America and Iceland. Salmon aquaculture is dependent solely on commercial feeds. The feeds have changed from being mostly dependent on FM as a protein source to alternative protein sources predominated by plant protein sources like soy protein in the form of soy protein concentrates (SPC) following the increased growth in the salmon industry. The SPC constituted 21% (approx. 350, 000 tons) of the salmon feed in 2012 .

Furthermore, a large proportion of the feed ingredients used in the salmon industry are imported such as soybean meal and SPC from Brazil, corn gluten from USA, and FM from Peru (Boissy et al., 2011) which further increases the carbon footprint of salmon feed production.

1.2.1: Alternative protein ingredients in Norwegian salmon feed

According to Aas et al., (2019), SPC, wheat gluten, corn gluten, Faba beans, and pea protein concentrates (PPC) constituted 19%, 9%, 3.6%, 3.4% and 1.3% respectively of the total proteins used in the Norwegian salmon industry in 2016.

1.2.2: Corn gluten meal (CGM): CGM is a by-product after extracting corn starch from corn kernel and consists predominantly of gluten (Ayadi et al., 2012; Sauvant et al., 2002). On a dry matter basis, it has a higher crude protein content (66.7-74.7%) but a low level of lysine (1.0-2.1%), and methionine (0.9-1.8%) (Ayadi et al., 2012).

1.2.3: Wheat gluten meal (WGM): wheat gluten has a protein content between 75%-85% which is higher than FM and soy proteins. Their mode of production is similar to corn gluten meal from wheat flour. Similarly, lysine is the first limiting amino acid as well tryptophan and arginine. Supplementing CGM and WGM with limiting amino acids (AA) can facilitate their usage as alternative protein sources in fish feed.

1.2.4: Soy proteins (SBM, SPC)

Soy proteins are available as SPC, SBM and soy protein isolates (SPIs). The SPIs are the purest available form of soy proteins with the highest content of crude proteins (88.5% - 92.6%), lysine (4.5-5.7%) and methionine (1.1%-1.3%) (Ayadi et al., 2012). They undergo further processing to remove insoluble fiber through alkaline extraction and soluble sugars through acid precipitation. Their inclusion in aquafeeds is limited due to poor palatability and too high price. Soy proteins, like other leguminous plants, are abundant and cheap and have a high nutritional value with a favorable AA profile. However, the use of soybean meal in diets for carnivorous fishes such as salmon is limited mainly due to the high level of antinutrients, but also due to a low/medium protein content (47% crude protein) and a relatively low concentration of essential AA such as methionine compared with fishmeal (Pelletier et al., 2018; Gyan et al., 2019).

Table 1: Amino Acid composition of Soybean meal, Soy protein concentrates and fishmeal (g/16 g N)

Amino acid	SBM[a]	SPC[a]	FM[b]
Arginine	6.7	6.4	6.5
Histidine	2.4	2.5	2.2
Isoleucine	4	4.1	4.8
Leucine	6.7	6.6	8.2
Lysine	5.1	5.5	2.6
Methionine	1.1	1.2	2.7
Phenylalanine	4.6	4.5	4.2
Threonine	3.7	3.5	4.2
Tryptophan	1.5	1.3	1.2
Valine	4.1	4.1	5.5

SBM = soybean meal, SPC= soy protein concentrates, FM = Fishmeal

Source: [a](Gyan et al., 2019), [b](Gómez-Requeni et al., 2004). The AA profile of SBM and SPC is comparable and impressive than FM in some instances aside their low concentrations of Sulphur-containing AA.

The production of SBM involves crushing soybean to extract their oils. Soybean oil is extracted from dehulled soybeans flakes with hexane. The defatted white flakes are roasted to inactivate their proteins and milled to produce the SBM. The defatted flakes could also be heated mildly, and the proteins extracted in hot water and ethanol to produce SPC (Fig. 1). This method of extracting SBM results in remnants of all heat stable antinutrients such as phytic acids in the meal. However, all protein-like antinutrients such as lectins and protease inhibitors such as Kunitz and Bowman Birch are expected to be removed. The heat application also leads to inactivation of proteins which increases their digestion and assimilation.

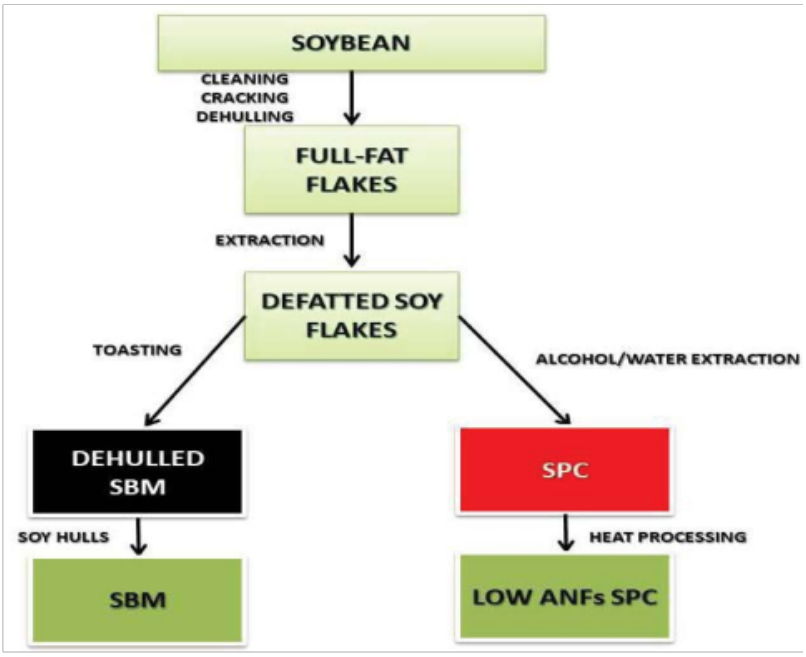


Fig. 2: Flow chart illustrating the extraction of Soy Protein Concentrates and Soybean meal. Adapted from (Gyan et al., 2019)

The SPC on the other hand have less antinutritional factors (ANFs) due to the additional treatment these ingredients go through. This makes them favorable to most fish species. The crude protein content of SPC is higher (approx. 62% - 75%) than SBM (Hardy, 2010; Pelletier et al., 2018) but they have a higher accumulation of phytic acid which reduces the availability of divalent cations such as Zn^{2+}, Ca^{2+}, Mg^{2+}. Therefore, increased use of SPC may be detrimental to bone formation in fishes (Helland et al., 2006). Due to the high cost of producing SPC, it is mostly used for high-value aquaculture fish (Hardy, 2010; Pelletier et al., 2018), while SBM is the most prominent source of plant protein in compound aquafeeds. The SBM can make up 60% of the proteins in tilapias, 25% in carps, up to 35% in trout and 12% in Atlantic salmon (Tacon et al., 2011). Increasing the proportion of SBM in fish diet exposes the fish to ANFs which results in intestinal disorders (enteropathies), especially in sensitive fishes such as salmonids which have shown inflammation in the distal intestines (DI), commonly referred to as soybean meal induced enteritis (SBMIE) (van den Ingh et al., 1991; Baeverfjord & Krogdahl, 1996; Hardy, 2010; Krogdahl et al., 2010; Venold et al., 2012). The alcohol-soluble fractions are removed during the processing and thus the use of SPC do not cause SBMIE in salmonids. The SPC replace up to 75% of FM without any negative effects on growth (Refstie et al., 2000). For this reason, SBMIE induced by feeding high levels of SBM is

often used as a model to study the gut health in Atlantic salmon as well as the efficacy of other feed ingredients in reducing or preventing this enteropathy.

1.3.0: Soybean meal induced enteritis (SBMIE)

Plant protein sources also such as soybean meal contain a wide range of antinutritional factors (ANFs). ANFs such as lectins, protease inhibitors, phytic acids, saponins, glycosinolates, soluble non-starch polysaccharides (NSPs) and allergens can compromise the gut health of fish, reduce nutrient absorption, leading to reduced growth performance (Krogdahl et al., 2010; Pelletier et al., 2018). The precise causes of SBMIE are still not fully understood even though several studies have attributed this phenomenon to the presence of alcohol soluble ANFs in SBM as the causative agents. Particularly saponins is highly suspected to be the main cause of this enteropathy (van den Ingh et al., 1996; Krogdahl et al., 2010; Hedrera et al., 2013; Krogdahl et al., 2015). Saponins are amphiphilic phytochemicals present in several terrestrial and marine plants as well as some microorganisms such as bacteria (Knudsen et al., 2008) as well as in the animal class Echinodermata (Anisimov, 1987). Soy saponins particularly are sugars or oligosaccharide moieties linked to a steroid or triterpenoid aglycone (Knudsen et al., 2008; Neacsu et al., 2020). Studies have revealed that soy saponins may reduce the risk of colon cancer in humans through intestinal membrane interaction as well as reduce the risk of cholesterol accumulation by binding with bile acids and cholesterol in the lumen of the intestines (Yoshiki et al., 1998; Savage, 2016; Neacsu et al., 2020). For this reason, saponins have been considered suitable candidates for the development of functional feed and nutraceuticals for defense against chronic diseases. They are therefore, suggested for use in food as antimicrobial and antifungal agents (Tamer et al., 2019). The role of saponins in growth depression is related to their activity on trypsin and chymotrypsin, therefore, affecting the absorption of proteins (Savage, 2016). Although the following may vary, soy saponins are in the range of 2.4 g/kg and 6.4 g/kg depending on the environment, strain, maturity and location (Oakenfull, 2001; Neacsu et al., 2020). Two main groups, group A and group B soy saponins exists depending on the position of glycosylation to the sapogenin. Group A soy saponins are glycosylated at the C-3 and C-22 position of soya sapogenol A, while group B soy saponins, are glycosylated at the C-3 position of soya sapogenol B (Knudsen et al., 2008; Neacsu et al., 2020). While saponins may be

generally less harmful to most mammals (Tamer et al., 2019), fishes and especially carnivorous fishes are more susceptible to saponins.

Krogdahl et al., (2015) documented a dose dependent increase in the degree of inflammation and alteration in the digestive function in the distal intestine (DI) of Atlantic salmon. According to the author, supplementing 95% purified soy saponins at 0, 2, 4, 6 or 10 mg/kg to either FM or 25% lupin meal revealed that saponins alone can cause inflammation independent of the presence of other ANFs. In another experiment, soy molasses was fractionated with different proportion of soy saponins, soy oligosaccharides and soy proteins and fed to Atlantic salmon. Results showed that the fraction containing high saponins caused inflammation in the DI of salmon with characteristics similar to SBMIE except alterations in the mucosal folds (Knudsen et al., 2007). Considering that feeding pure soy oligosaccharides or soy proteins did not cause enteritis in the same experiment, further enforces the fact that soy saponins are the potential causes of this enteropathy. In a similar study, FM based diet containing soy saponins did not trigger enteritis in Atlantic salmon, but lupin kernel diet containing 25% soy saponins triggered significant enteritis (Knudsen et al., 2008).

SBMIE is not exclusive to salmonids as similar observations were observed in common carp when 20% of the protein was replaced with SBM (Urán et al., 2008). Contrary to salmonids, recovery from this pathology in common carp was spontaneous with the intestine function and morphology returning to normal after four weeks of feeding. The Atlantic salmon on the other hand recovered from the SBMIE only after the diet was changed to FM (Baeverfjord & Krogdahl, 1996). Extreme case of this pathology was reported by Dale et al. (2009), who observed that feeding salmon brood stock with SBM for four years resulted in severe intestinal tumors similar to human colorectal cancers associated with inflammatory bowel disorder (IBD). Differential tolerance and severity may exist among different salmonids. By including 20% SBM in the feed triggered enteritis in Atlantic and chinook salmon after one week of feeding which became more severe in the chinook salmon after 3 weeks, but mild symptoms were instead observed in pink salmon (Booman et al., 2018). Similar observations were made with different strains of rainbow trout (Venold et al., 2012), zebrafish (Hedrera et al., 2013) and turbot (Gu et al., 2016) in each case a 26% FM or more was substituted.

Although the severity of SBMIE and reactivity may be different between species, the histopathological characterization of SBMIE is almost similar in all species. Reduced growth is a common characteristic due to reduced feed intake and digestion resulting from the changes in the digestive tract was observed in all fish species under SBMIE distress. Several observations in fish suffering from SBMIE have been observed including shortening of the heights of the mucosal folding, loss of supranuclear vacuolization of the enterocytes in the intestinal epithelium, infiltration of the lamina propria with inflammatory cells such as eosinophilic granulocytes & lymphocytes, increased number of goblet cells in the epithelium (Baeverfjord & Krogdahl, 1996; van den Ingh et al., 1996; Hedrera et al., 2013; Krogdahl et al., 2015; Booman et al., 2018) as shown in fig 3 below. Increased number of mucocytes in the epithelium is an indication of tissue regeneration mainly based on secretory cells and cell proliferation coupled with migration from the crypt to the epithelium of the villus. Tissue response to inflammation can be local, suggested by infiltration of inflammatory cells in the site where the SBM antigens have led to hypersensitive reactions (Baeverfjord & Krogdahl, 1996)

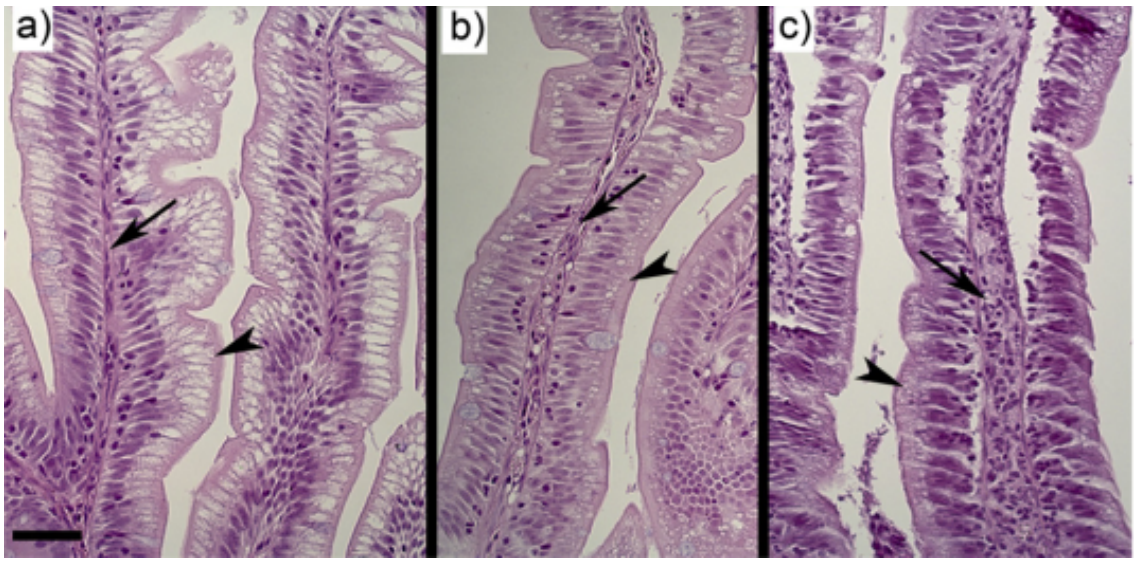


Fig 3: light microscopy showing morphological changes in the DI of Atlantic salmon fed FM and SBM for 7 days. a) Healthy condition: normal lamina propria (LP) (arrow), high degree of supranuclear vacuolization of enterocytes (arrowhead); b) 5 days of feeding: signs of widening LP (arrow) and loss of supranuclear vacuolization (arrowhead); c) 7 days of feeding: showing severe signs of enteritis, widening of LP (arrow), loss of supranuclear vacuolization (arrowhead). Bar = 50mm. Adapted from (Sahlmann et al., 2013).

The underlying mechanism of SBMIE relies on the ability of these bioactive compounds to interact with the components of cells specifically the sterol components of bio-membranes which alters the semi permeability properties of plasma membranes.

Feeding SBM to salmonids increases paracellular and transcellular permeability in the DI which reduced carrier-mediated transport of nutrients as observed by Nordrum et al., (2000) when rainbow trout and Atlantic salmon were fed SBM and using an *in vitro* everted sleeve technique. In a similar study, transepithelial resistance, increase permeability of mannitol and low transepithelial potential difference in salmon was observed in an *in vitro* study by (Knudsen et al., 2008). This author also concluded that saponins increase intestinal epithelial permeability and not necessarily induce enteritis since a diet containing fishmeal and saponins led to increase epithelial permeability but did not trigger enteritis. At the same time, in a contrasting experiment, a diet containing raffinose, stachyose, a combination of both as well as another combination with soy saponins did not cause any visible signs of enteritis in Atlantic salmon (Sørensen et al., 2011). The transepithelial membrane permeability caused by saponins inhibit the active transport of some nutrients, but at the same time, they facilitate the uptake of some materials to which the gut under normal circumstances (homeostasis), would be impermeable (Savage, 2016)

1.3.1: Inflammatory response to SBMIE in DI

Increase in epithelial permeability leads to a series of events which results in immune response and inflammation. Immunohistochemistry, transcriptomics and proteomics have been used to determine molecular pathways activated during SBMIE in fish. Epithelial permeability may lead to an upregulation or a downregulation of genes to compensate for the immune response or to manage the damage following the stimulus or during the healing process. Sahlmann et al. (2013) reported an initial upregulation of genes responsible for lipid metabolism, proteolysis, transport, metabolism and detoxification in Atlantic salmon in the first 3 days of feeding SBM. After three days, genes associated with tissue repair and extracellular tissue remodeling were upregulated and a downregulation of the initial genes signaling an impairment of the digestive and metabolism function following the inflammation.

Immune response can be grouped into cell-mediated or humoral and innate or adaptive. Natural killers and macrophages are considered innate with T cells being adaptive immune response cells. Cell-mediated immune responses are carried out by white blood cells in the form of granulocytes, lymphocytes, dendritic cells and macrophages (Fischer et al., 2013). B and T are the two main types of lymphocytes or cells. B cells make antibodies with each B cell set to make one specific antibody which matches a specific antigen (foreign body) like a key and lock configuration. The T lymphocytes cells can be natural killers (NK) or T cells which compose of the cytotoxic T lymphocytes (CTLs), T helper cells and regulatory T cells (Fischer et al., 2013).

Among the immune-relevant genes associated with inflammation in the DI of Atlantic salmon that have been identified with RT- PCR include transforming growth factor b (TGFb), interleukin 1b (IL-1b), gamma interferon-c inducible lysosomal thiol reductase (GILT) and clusters of differentiation (CD) CD3, CD4, and CD8 and Major Histocompatibility Complex (MHC) class I & II (Lilleeng et al., 2009; Marjara et al., 2012; Fischer et al., 2013). CTLs kill host cells infected with intracellular pathogens by binding of their T cell receptor (TCR) and its co-receptor CD8 to a complex of MHC class I and bound peptide on the infected host cell (Fischer et al., 2013). Antigens outside the host cells are taken up by macrophages, dendritic and B cells so the peptides from those antigens are sent to the $CD4^+$ T-helper cells with the help of MHC II. CD8 is a co-receptor of cytotoxic T cells that interacts with MHC class I during presentation of antigenic peptides of intracellular origin (Fischer et al., 2013).

Enteritis in the DI results in the activity of trypsin and other proteinases which leads to the activation of proteinase activated receptors (PAR), which are key activators in inflammatory response in the gut and airways of animals (Thorsen et al., 2008). Study has confirmed the signaling of PAR-2 in Atlantic salmon fed SBM after one day (Thorsen et al., 2008). Matrix metalloproteins (MMPs) involved are involved in tissue remodeling are also known to induce PAR-2 expression during SBMIE suggesting the degradation of extracellular matrix proteins in the DI where injury and repair takes place (Sahlmann et al., 2013). Transforming growth factor (TGF)-b is anti-inflammatory. According to Urán et al. (2008), upregulation of TGF-b was observed 3 weeks of feeding common carp with SBM at which point the histopathological changes associated with SBMIE was starting to

decline. This implies that TGF-b could serve pro-inflammatory as well as an anti-inflammatory purpose and the signaling of TGF-b might be a beginning of a healing process with regards to the tissue damage caused by the inflammation indicating their multifunctional purpose (Yu & Stamenkovic, 2000). Yu & Stamenkovic, (2000) further reported that TGF-b may stimulate leucocyte infiltration, synthesis, decrease in protein degradation as well as suppress cell proliferation. These authors further suggested that coordination between the receptor hyaluronan receptor CD44, MMPs, and TGF-b may provide a physiological mechanism of tissue remodeling. However, this was not observed in Atlantic salmon, despite an upregulation of TGF-b following SBMIE enteropathy (Marjara et al., 2012) in contrast to report by (Urán et al., (2008) with common carp. The up-regulation of four pro-inflammatory genes IL-1β, IL-8, tumor necrosis factor (tnf)-α and IL-17a/f have been reported in during a SBMIE in salmon (Marjara et al., 2012), turbot (Gu et al., 2016), zebra fish (Hedrera et al., 2013) and common carp (Urán et al., 2008) during the inflammation process while the anti-inflammatory IL-10 was down-regulated after an initial upregulation in the first week of feeding in the common carp.
GILT plays a role in facilitating major histocompatibility complex (MHC) class II restricted antigen processing and a negative regulation of T cells activation as well as immune response to bacterial challenge (Cui et al., 2012). According to Lilleng et al., (2009), there is early-stage downregulation of GILT in Atlantic salmon fed SBM. In contrast, Marjara et al. (2012) also reported a pre- and early SBMIE upregulation of GILT in salmon and down-regulation during late SBMIE.

Both MHC class I and II are expressed in epithelial cells of the intestines and are responsible for directing peptides from proteolysis of antigens to T lymphocytes such as natural killers (NK), $CD8^+$, and $CD4^+$ T cells (Jørgensen et al., 2007; Fischer et al., 2013). In SBMIE in Atlantic salmon, the antigen presentation in the enterocytes might have been altered as a result of mucosal change (Baeverfjord & Krogdahl, 1996) which might lead to cell proliferation in response to tissue repair. Following enteritis and tissue damage, regeneration of cells especially epithelial cells is likely to occur. The process includes cell differentiation leading to proliferation, programmed cell death (apoptosis) and migration of cells from the crypt to the epithelium of villi (Chikwati et al., 2013). Markers used to detect this process is the proliferating cell nuclear antigen (PCNA) (Chikwati et al., 2013; Venold et al., 2013), fatty acid binding protein (fabp2) (Venold et al., 2013) and caspase

(Sahlmann et al., 2013). During a SBMIE in Atlantic salmon, Fabp2 was highly expressed in the intestines and decreased aborally towards the DI (Venold et al., 2013). Leading to up-regulation of Fabp, there is the activation of a ligand dependent transcription factors such as peroxisome proliferator activated receptor (PPAR) and fatty acids activated receptors (FAAR) (Lawrence et al., 2000). Processes that lead PCNA upregulation might be an antithesis of fabp2 upregulation. While fabp2 was downregulated during SBMIE in salmon (Venold et al., 2013), there was an increase in the expression of PCNA in the DI of the fish. Proteins such as the heat proteins (HSPs) are produced in response to stress in organisms (Roberts et al., 2010) are likely to be expressed during tissue healing from damage. HSP family 70 and 90 play a critical role in protein folding, assembly of other cellular proteins and in translocation and assembly of proteins.

1.3.2: Change in intestinal microbiota during SBMIE

There is a longstanding symbiotic relationship between gut microbiota and the host organism where the former derives nutrition from the host and in return play vital roles in disease resistance by regulating colonization of harmful bacteria, digestion and release nutrients such as vitamins, amino acids, enzymes production, immune response regulation (Bakke-McKellep et al., 2007; Merrifield et al., 2011). Reports on the effect of SBM on bacteria population in the gut of fish are inconsistent. For example, Bakke-McKellep et al., (2007) reported an increase in the diversity of the bacterial population in the gut of rainbow trout fed SBM compared to the reference diet while Mansfield et al., (2010) and Desai et al., (2012) reported a decrease in the diversity. In a similar study, Reveco et al., (2014) revealed that the species richness and diversity were strongly reduced by the SBM diet in the DI of Atlantic salmon with the SBM diet producing a higher relative abundance of firmicutes than the FM diet similar to observations by Grammes et al., (2013). However, Bakke-McKellep et al., (2007) observed a higher total number and a more diverse population of bacteria in the DI of Atlantic salmon fed SBM. Similar number of different genera and strains of bacteria were identified in the SBM fed and FM fed salmon, but the number of some isolated lactic acid bacteria was higher in the FM fed despite *Brevibacterium* and *Enterococcus spp*. were detected in the SBM group but not in the FM group (Merrifield et al., 2011). Although the causes for these discrepancies are unknown, Merrifield et al. (2011) suggested that different feeding duration, the different

SBM inclusion level and characteristics and the different culture conditions may be responsible for the observed differences.

It is fair to assume that non-salmonids and specifically omnivorous and herbivorous feeding fishes have better tolerance and less susceptible to plant source feed ingredients such as the enteritis caused by SBM. Although Urán et al. (2008) observed a reversible SBMIE in common carp, there was no report on the effects on the microbiome of the fish. Herbivorous and omnivorous fishes through evolution have developed longer guts with some several times their body lengths to allow enough time to ferment plant material with the help of the gut microflora. In contrast to Atlantic salmon and rainbow trout, no significant effect of dietary SBM (30% inclusion) on the gut microbiota of silver crucian carp (*Carassius auratus gibelio* & *Cyprinus carpio*) after 3 weeks feeding (Cai et al., 2012) was observed. These results indicate SBMIE is associated with changes in the gut microbiota especially salmonids and carnivorous fishes in general. However, the role and effect of the microbiota community change as a result of the SBMIE on fish immune response remains unknown except for the fact that translocation of opportunistic bacteria following the disruption epithelial layer and increased transepithelial permeability is more likely to occur. Bacterial translocation refers to the relocation of bacteria from the gastro-intestinal to extraintestinal sites, such as the mesenteric lymph node complex (MLN), liver, bloodstream, kidney etc. (Berg, 1999). Increased permeability of the intestinal mucosal barrier, and tight junctions reduces host immune defense, disruption of the ecologic gastro-intestinal equilibrium to allow intestinal bacterial overgrowth are the mechanisms by which bacteria is translocated in the gut (Berg, 1999). Due to the reduced density of microvilli enterocytes caused by SBM enteropathy, the exposure of tight junctions to brush border bacterial populations is likely to be increased which may have negative connotations towards defensive barrier function to opportunistic bacterial populations (Merrifield et al., 2011).

1.3.3: Effects of selection on ANFs tolerance in salmonids

Research has shown that rainbow trout (*Oncorhynchus mykiss*) can be selectively improved to grow on plant-based diet regardless of their ANFs content. In an experiment to assess the efficiency of selection and consequences on various nutritional traits, the results suggest that after only three generations of selection, rainbow trout could grow on

full plant-based diet at rate equal to the FM control group compared with a 36.8% body weight reduction before selection (Callet et al., 2017). These authors suggested that the enhanced growth was a result of the higher feed intake with the selected fish. In a similar experiment, 8 strains of rainbow trout were introjected to produce a single strain selected for faster growth when fed a FM-free plant-based diet for four generations. The selected strain was compared with the parental generation that was fed either the plant-based diet or FM for 12 weeks. The results suggested that there was a significant effect of strain and not diet on weight gain and an interaction of strain by diet (Overturf et al., 2013). The selected strain gained more weight than the parental strains across all diets with those fed plant-based diet gaining more weight than those fed FM diet. The author concluded that rainbow trout can be selectively improved to grow on plant-based diet. Both authors only considered the effects of selection on growth and did not report any effects on the general and gut health upon administration of the full plant-based diet. Similar observations were reported by Venold et al., (2012) and Abernathy et al. (2017) who further reported that no enteritis in the distal intestines of selected rainbow trout were observed. Although these reports of tolerance upon genetic selection are very important in this era of plant nutrient-dominated fish feeds, it also important to take into consideration the risk of genetic pollution and probably the loss of parental genetic populations overtime.

In a more relatable way, perhaps the need for aquaculture growth amidst of all the constraints can be likened to the Red Queen's race/dynamics. In evolutionary biology, for a species to survive, it must constantly adapt, evolve and proliferate to stay in place or survive, pitted against ever-evolving opposing species. In that analogy, if aquaculture growth is the species, the opposing species in that context could be the problems associated with protein supply in aquafeeds. Constraints with FM supply, ANFs in feed ingredients etc, are catching up quickly with the purported aquaculture growth. In response, relentless efforts have been made to ensure that protein supply in aquaculture keeps growing and evolving in the phase of all these challenges to keep thriving. Replacement of pristine marine proteins and oils with processing byproducts and vegetable alternatives would improve the sustainability of production of farmed carnivorous fish, such as Atlantic salmon (Tacon & Metian, 2008; Naylor et al., 2009; Hardy, 2010). Therefore, exploring and developing feed ingredients that close the nutrient loop (circular economy) in salmon farming or venturing into resources that are not

mainstream could enhance the resource efficiency and lead to a reduced carbon footprint in a major way. A major non-mainstream category of such ingredients is single cell proteins (SCP) also known as microbial feed ingredients including microalgae, bacteria, but most importantly yeast as a potential protein-source feed ingredient in aquafeeds.

1.4.0: Yeast as a potential protein ingredient in aquafeeds

One of the most important characteristics of yeast as a potential feed ingredient aside their nutritional profile is their ability to convert less valuable, non-edible food by-products as well as forestry and industrial biomass into quality food/feed ingredient with little or no reliance on arable lands, water with a net climate mitigation effects (Øverland et al., 2013; Anwar et al., 2014; Lapeña et al., 2020). With crude protein content of 45-55 % (Akanni et al., 2014; Lapeña et al., 2020b), their methionine, lysine and cysteine content are comparable to FM, but their content of threonine and tryptophan is higher (Skrede et al., 1998). In addition to proteins, yeast also contain free amino acids, nucleic acids, low levels of lipids, carbohydrates, minerals and vitamins (Bajpai, 2017) which makes them interesting alternatives in aquafeeds (Øverland et al., 2013). With a generation time of 10-20 minutes, yeast have a higher specific growth rate and biomass yield, can be produced all year round, and have a more efficient substrate conversion than beef, fish and chicken (Israelidis, 1998). Recent advances in yeast research are more focused on their potential as nutritional supplements and functional properties with beneficial effects on the immune responses and gut health in fish (Agboola et al., 2020). The yeast cell wall is composed of functional components such as mannoproteins; β-1,3 and 1,6 glucans; mannan – oligosaccharides and chitin (Klis et al., 2002; Akanni et al., 2014; Schiavone et al., 2014).

SCP and yeast depend on the bioconversion of low-cost carbon feed stock into biomass with added value that can be used as feed ingredients. Lignocellulosic biomass which contains cellulose and hemicellulose and readily available for SCP bioconversion (Akanni et al., 2014; Anwar et al., 2014). Commercial production and utilization of yeast for food and feed started during in world war I in Germany when baker's yeast or *Saccharomyces cerevisiae* was first cultured and used as a protein supplement (Lipinsky et al., 1970; Litchfield, 1979). *Saccharomyces cerevisiae* cannot utilize hydrocarbons, lactose, pentose sugars for growth and is reliant upon supplementation of AA, B vitamins such as thiamin,

niacin, pyridoxine, pantothenic acid, and inositol usually blended with molasses. During WWII, *Candida utilis* was produced for food by hydrolyzing wood with no AA or vitamins supplementation but required $NH4^+$ salts as a source of nitrogen (Lipinsky et al., 1970; Litchfield, 1979).

1.4.1: Production of yeast from low value, non-food biomasses

Like any other organism, yeast require proteins and polysaccharides for growth. The ideal substrate and nitrogen concentration is adjusted at a C:N ratio of 10:1 or 7:1 for a favorably high protein yield (Litchfield, 1979). Although molasses are the traditional substrates used for yeast production (Agboola et al., 2020), it could be dependent on geographical locations especially where they are scarce or expensive other alternatives like petroleum fractions are used (Lipinsky et al., 1970). Inorganic N sources such as NO_3^-, and ammonium salts are used as N sources. Modern day production of yeast from lignocellulosic biomasses are based on this same theory of cultivating *Candida utilis* during the second world war, except that considerable improvement from the green biotechnologies related to lignocellulose biomass have appeared. Lignocellulosic biomass typically composes of about 10-25% lignin, 20-30% hemicellulose, and 40-50% cellulose and other minor components such as proteins, ash, pectin, minerals (Wyman, 1999; Anwar et al., 2014; Balan, 2014). Their conversion to useful materials is a multistep processing includes (i) a pre-treatment (mechanical, chemical, or biological); (ii) an enzymatic hydrolysis and a (iii) fermentation process (Anwar et al., 2014) as depicted in (Fig 4) below.

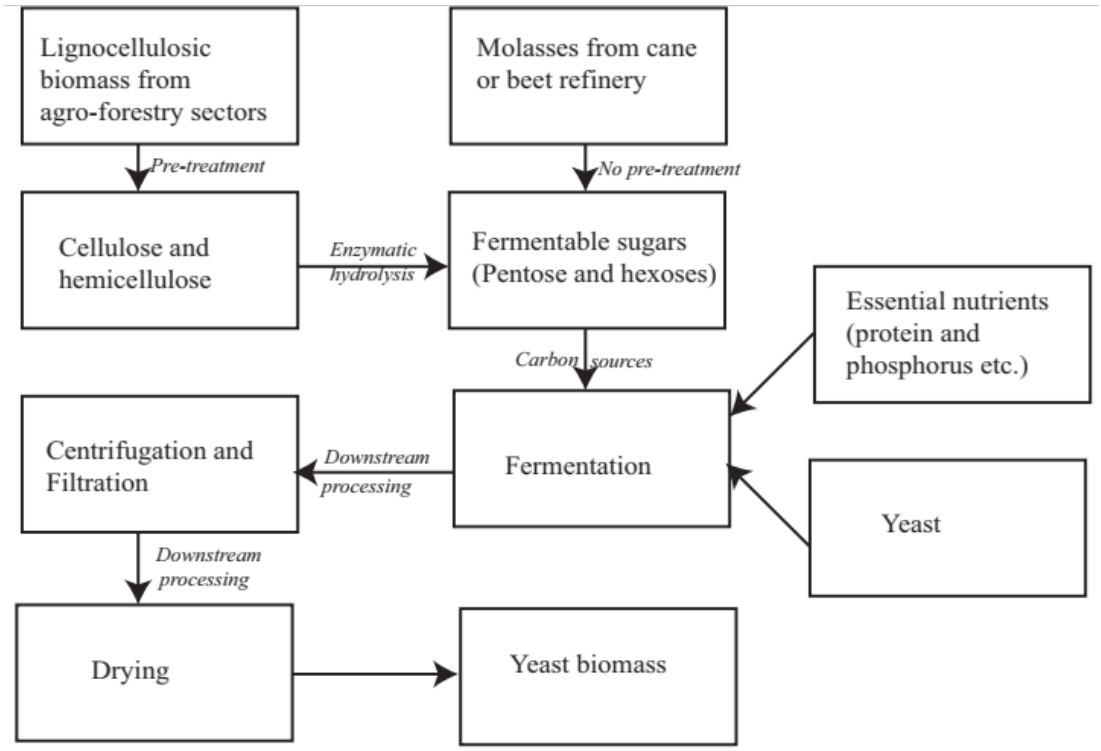


Fig 4: Block flow diagram of converting low value lignocellulosic biomass to high value yeast. Adapted from (Agboola et al., 2020).

First generation biorefineries rely on converting edible foodstuff such as cassava, corn, rye, soybean, sugarcane, beet etc. to biofuels which has drawn a lot of criticisms considering there are several millions of people without food in the world (Balan, 2014). For this reason, second generation refineries rely on non-edible materials mainly urban and agricultural waste that cannot serve food purposes. For instance, 70% of all the total feedstock (341 M tons) was agricultural residue and the 30% from forest residue (Balan, 2014). Feedstocks are usually in the forms of straw from cereals and sorghums, miscanthus, eucalyptus, spruce tree, poplar, willow, pine etc. Due to their recalcitrant nature, their rate of decomposition is low and can take days to break them down. Molecular crosslinks need to be broken to access the lignin, hemicellulose and cellulose complexes.

Pretreatment is carried out to reduce the physical structure of the biomass by physical, chemical, biological, thermal or a combination of methods, which breakdown the hemicellulose-lignin crosslinks in order to separate the lignin and make the cellulose-hemicellulose complex more accessible for later hydrolysis by enzymes to monosaccharides (Balan, 2014; Øverland & Skrede, 2017). The preferred pretreatment

method depends on the nature of the feedstock as some may require rigorous treatment for maximum yield of sugars. The enzymatic treatment involves the use of a cocktail of appropriate enzymes to breakdown these polysaccharides to simple monomeric sugars. The use of appropriate enzymes is crucial in saccharification to ensure efficient breakdown of crystalline polysaccharides to fermentable sugars to ensure economical and sustainable yeast production from lignocellulosic biomass (Øverland & Skrede, 2017). The cocktail usually contains 40-50 different enzymes in the class of cellulases 70-85%, hemicellulase and pectinases 15-30% hydrolyzing enzymes (Balan, 2014). Other forms of enzymes added are in the form of ligninase to hydrolyze lignin. Hexose and pentose sugars which are fermentable main products of the saccharification process affected by temperature, pH, enzyme loading and concentration of the substrate (Litchfield, 1979; Agboola et al., 2020). Yeast is produced by fermentation based on a media containing sugars, nitrogen and minerals. The fermentation process can be carried out in a single step with the enzymatic hydrolysis or separately (Øverland & Skrede, 2017). A downstream process of filtration and centrifugation is used to retrieve the yeast from solution which is dried to obtain the yeast biomass.

1.4.2: Components of the yeast cell

The function of the cell wall is to provide structural support as well as adapt to structural and morphological changes. 25-32% dry weight of the cell is the cell wall which is about 85%-90% polysaccharides and 10-15% proteins (Klis et al., 2002; Schiavone et al., 2014). The polysaccharides content of the cell wall could vary more than 50% with the nature of the carbon source, nitrogen limitation, pH, temperature and aeration, and with the mode of cell cultivation and are determined using chemical and enzymatic methods (Aguilar-Uscanga & François, 2003). Although variability may exist, for *S. cerevisiae*, the polysaccharides content of the cell wall is estimated to be 30-60 % β-1,3-glucan and 1,6-glucan, 25-30% mannan and 5-10% chitin (Schiavone et al., 2014). The 1,3-glucose units which represents ca. 85% of total cell wall b-glucan have long chains, while β-1,6-glucose units accounts for ca. 15% of the β-glucan (Klis et al., 2002). The structural complexity of glucans can further increase when a monosaccharide replace glucose in the polysaccharide called heteroglucans (Ferreira et al., 2015). Mannoproteins refer to mannans that have form complexes with cell wall proteins. β-glucans are widely found in

the cell walls of many plants including rye, barley, wheat oat. β-glucans from oat and barley have 1,3 and 1,4 linkages, mushrooms have shorter β (1,6)-linked branching β-glucans from β (1,3) while yeast β-glucans have β (1, 6) branches further with additional β (1, 3) regions (Meena et al., 2013).

It is worth knowing that the glucans in these cereals and yeast may be different bio-actively due to their differences in solubility. Whereas the β-glucans from yeast are branched, those from cereals such as oat, rye and barley have straight β (1,6) chains and differ in their molecular weights, molecular charge and conformation in solution which affect their immune and health potentials (Volman et al., 2008). Glucans from cereals are soluble non-starch polysaccharides (NSP) which act as ANFs by increasing the viscosity of the digesta in the intestinal lumen, interact with starch and other nutrients and interfere with enzymes and increase the amount of bile salt (Wang & Ellis, 2014). β-glucans from whole oats have less solubility but milled and extruded oat in high temperature results in higher solubility (Wang & Ellis, 2014). Mildly extruded breakfast oatmeal with incorporated oat bran have also shown high solubility. Making up to 7% of the oats, a larger proportion of β-glucans are in the cell wall surrounding the endosperm (Wang & Ellis, 2014). Therefore, milling makes them more readily soluble in water than whole oat. Yeast β-glucans on the other hand are naturally insoluble although may change based on processing (Ishimoto et al., 2018). For example, acid treatment of yeast β-glucans can solubilize them to low molecular b-glucans at 20^{o}C. Yeast β-Glucan can bind to dectin-1 regardless of its solubility, but its activity changes with change in solubility (Ishimoto et al., 2018)

Yeast cells have appreciable amino acid profiles comparable with FM and soy proteins except their deficiency in sulphur-containing amino acids such as methionine and cysteine (Table 2) and may differ among species based on their strains, culture media used, culturing conditions, downstream processing and methods of analysis (Lipinsky et al., 1970; Øverland et al., 2013). *S. cerevisiae* have higher content of methionine and cysteine, but lower content of lysine compared with other species (Agboola et al., 2020).

Table 2: Average amino acid composition (g/16 g nitrogen) of selected yeast species of commercial importance, fishmeal and soybean meal and the nutritional composition (% DM basis) of some common feed ingredients.

	S. Cerevisiae [α β μ]	*C. jadinii* [α β μ]	*W. anomalus* [α]	FM [μ]	SBM [μ]
Arginine	4.35±0.31	4.99±0.28	4.70	5.74	7.38
Histidine	2.1 ±0.34	1.91±0.1	2.60	2.36	2.67
Isoleucine	4.05±0.56	4.20±0.1	5.00	4.53	4.94
Leucine	6.22±0.69	6.44±0.43	6.90	7.06	7.8
Lysine	6.51±0.39	7.19±0.45	6.90	8.18	5.53
Methionine	1.61±0.33	1.18±0.16	1.50	2.87	1.41
Phenylalnine	3.79±0.46	3.80±0.31	3.90	3.84	5.26
Threonine	4.45±0.24	4.69±0.09	4.60	4	4.03
Tryptophan	2.01±1.69	2.36±1.87	NA	1.05	1.41
valine	3.81±2.36	3.72±2.29	4.50	4.87	5.51

Source	crude protein	crude lipid	nucleic acids	Ash
Yeast[Y]	46-53	1-6	6-12	5-10
Bacteria[Y]	72-78	2-3	8-16	3-7
FM[Y]	70-78	8-10	1-2	11-21
SPC[Y]	60-69	1-3	0-1	8-9
SBM[Y]	47-51	1-3	0-1	6-8

Sources: *α* - Agboola et al., (2020); *β*- Øverland et al., (2013); *μ*-Øverland & Skrede (2017); Y- (Sauvant et al., 2002) NA- Not available

Yeast is not only endowed with proteins, lipids and energy, but also important minerals such as calcium, phosphorus and selenium. The lipid content of yeast cells is usually low and tend to form complexes with proteins and RNA (Yamada & Sgarbieri, 2005). Although fish muscle fatty acid profile reflects the dietary FAs inclusion, this may be slightly different in yeast meals. Using feed ingredients with low proportion of FA such as yeast (Brown et al., 1996), may have no or little effect on the muscle FA profile of fish (Øverland & Skrede, 2017). Fatty acids typically comprise 70-90% of the lipids in yeasts

and it is common to find high concentrations of oleic acid [18: 1n-91] but lack the two most important polyunsaturated fatty acids (PUFAs) (20:5n-3 and 22:6n-3) needed in fish and bivalve nutrition (Brown et al., 1996). Since the nutritional composition of yeast depends on their growth media, optimization and product tailoring may lead to a higher accumulation of PUFAs yeast cells. With nucleic acids between 5-12% of the of the cell in yeast (Øverland et al., 2013), Brown et al. (1996) argued that this high level of nucleic acids may limit their use as food for humans due to the lack of uricase: an enzyme for converting harmful uric acid (an intermediate of nucleic acid catabolism) to a less harmful allantoin. However, mollusk bivalves have this enzyme and would pose no problems in their nutrition. Salmonids on the other hand can tolerate high levels of uric acid due to their efficient hepatic uricase activity (Rumsey et al., 1991; Andersen et al., 1992). Although not much is known about this in finfishes, Øverland et al. (2017) suggested that nucleic acids from yeast may have a protein-sparing effect on non-essential amino acid nitrogen through endogenous utilization or be directly incorporated in the body, in this case fishes.

1.4.3: Yeast cell wall components as immunostimulants in aquaculture

Yeast cell wall derivatives have gained the attention of many researchers largely due to their immunostimulatory properties and to some extent the ban on the use of chemotherapeutants in animal feeds. In retrospect, majority of research into polysaccharides as potential immunostimulants were more focused on lipopolysaccharides from bacteria. However, most recent advances in yeast cell wall research are more geared towards polysaccharides lacking the lipid moiety, as they have also been reported to interact with cells of the immune system, as well as with molecules involved in humoral immunity (Ferreira et al., 2015). In vivo studies have shown an enhanced macrophage phagocytic function was induced by treatment with immunostimulatory polysaccharides such as (1 → 4)-d-glucans with side chains of C-3 (1 → 6)-d-Glc units (Zhao et al., 2010; Sun et al., 2015). Immunostimulatory polysaccharides are also reported to stimulate macrophage proliferation, differentiation and enhanced function through the production or reactive oxygen species (ROS) (Meena et al., 2013; Ferreira et al., 2015) and proinflammatory factors (IL-1b, G-CSF, IL-1a, GM-CSF, IL-6, COX-2, TNF-a, IFN-b, CXCL10, CCL2, TNF-b, IL-10), and the genes involved in NF-B signaling pathway (Sun et al., 2015). Macrophages are the primary cells

involved in immunity. Polysaccharide conformation determined by the connection between the sugar units and their branching with other molecules, their molecular weights and structural complexity in solution can influence their contact and interaction with cells and immune system components (Mueller et al., 2000). Binding of immunostimulatory polysaccharides such as glucans to macrophages and neutrophils results in the activation and nuclear binding activity of nuclear factor kB (NF-kB) and Nuclear Factor – Interleukin 6 (NF-IL6) which results in immunostimulatory activity of these polysaccharides (Mueller et al., 2000; Ferreira et al., 2015). Activation of b-glycan receptors generally improves all immune functions by releasing of cytokines which stimulate the production and release of new leukocytes (Meena et al., 2013).

Mueller et al., (2000) suggested that glucans modulate innate immunity by binding to specific receptors on monocytes, neutrophils, and natural killers. Dectin-1 (β -glucan receptors) is a major receptor amongst the several classes of β -glucan receptors. Dectin-1 is a transmembrane protein which binds β-1,3 and β-1,6 glucans and implicated on cells that phagocytose pathogens such as fungi, viruses, pathogenic bacteria (Schorey & Lawrence, 2008; Volman et al., 2008). It is lectin-like carbohydrate recognition domain and possesses an immunoreceptor tyrosine-based activation motif. It recognizes β -1,3 / β -1,6 glucan linkages in polysaccharides and is expressed on immune cells (Schorey & Lawrence, 2008; Meena et al., 2013). According to Volman et al. (2008), when β-glucan binds to the dectin-1 receptor, it activates NF-kB through intracellular signaling which results in cytokine production, phagocytosis and respiratory burst.

Yeast mannan contains an α-(1-6)-linked backbone with α-(1-2)-linked and α-(1-3)-linked branches (Korolenko et al., 2019). The mannose receptor (CD206) is a C-type lectin which is expressed by macrophages, dendritic and endothelial cells and play a role in phagocyting manno-glycoproteins. Mannose receptor is also believed to play an active role in pathogen clearance by binding to mannose-containing and fucose-containing microorganisms via carbohydrate recognition domains (Korolenko et al., 2019). Mannan-oligosaccharides (MOS) are non-digestible short-chain branched carbohydrates composed of up to 10 mannose units with α-(1,3) and α-(1,6) linkage bonds (Tungland, 2018). They are used in farm animals to improve the feed conversion ratio (FCR) and as alternatives to antibiotics to clear opportunistic bacteria in the gut of those animals, improve intestinal morphology and function as well as the innate and acquired immune function (Tungland,

2018), a concept which evolved from the fact that some sugars such as mannose could be used as inhibitors of pathogen adhesion to intestinal cells (Torrecillas et al., 2014).
For this reason, the use of yeast in aquaculture is based on the idea that their cell wall components have the potential to serve antibiotic purposes and enhance the general health of the fish aside their nutritional value. A lot of studies have been conducted to determine the effects of these immunostimulants on the growth, intestinal morphology and general health of fish which have yielded variable results.

1.4.4: Protective effects of β-glucans in fish

In one of such studies, Rainbow trout were fed pellets containing β -1.3/1.6 - glucan at a dose of 0.5 g/100 g of pellets (0.5%) per day. After one week of feeding, the fish were immunized with a vaccine. The results showed that the group supplemented the glucan increased the number of specific antibody secreting cells and specific Ig levels in serum (Siwicki et al., 2004). In a similar study, b-glucans were administered to fry of rainbow through bath at concentration of 0.1mM and 1mM for 45 minutes for four times at 1week interval. Results showed that β-glucan treated fish possessed higher gene expression regarding the proinflammatory cytokines IL-1b, TNF-a, IL-6 and the anti-inflammatory cytokines IL-10 and TGF-b post treatment although IL-17A showed no significant difference from the control group (Zhang et al., 2009). Comparatively, 32% protein commercial diets were supplemented with 0, 50, 100 and 200 mg β-glucan and fed to immunized Nile tilapia for 14 weeks. Although no differences in survival and weight gain was observed, feed efficiency ratio was lower in the 100 and 200mg supplemented diets which also showed lower serum lysozyme activity 14 days post challenge (Whittington et al., 2005). Also, 0g pr 10g per kg of whole wild baker's yeast or fks-1 mutant strain was fed to the gilthead seabream (*Sparus aurata*) for 2, 4 or 6 weeks. Results showed a decrease in serum peroxidase and complement activity and increase in lysozyme activity after 6 and 2 weeks respectively. Phagocytosis was increased to a significant degree with fks-1 strain supplemented diet, while both strains enhanced respiratory burst activity and natural cytotoxicity after 4 and 6 weeks (Rodrı´guez et al., 2003) but neither of the strains changed intracellular content. This may be an indication that different fish may respond differently to different sources of β -glucans or perhaps the dosage and mode of administration of the b-glucans may have varying resulted. Similar results were obtained when shrimps were administered b-glucans and challenged with bacteria.

When post larvae of tiger shrimp (*Panaeus monodon* tiger shrimp) were immersed in aerated β -glucans suspension, enhanced growth was observed in concentrations 0.5, 1 and 2mg/ml but not 0.5mg/ml after 3 hours with immediate shrinkage of gill tissue in the 2mg/ml solution. Immersion of these treatment groups in *Vibrio vulnificus* suspension on 10, 18 and 43 days resulted in death for a month post infection. Protected effect glucan treatment was observed only in treated with 0.5 and 1 mg/ml glucan up to day 18. In vitro beta-glucan treatment enhanced the phenoloxidase activity in shrimp hemocytes (Sung et al., 1994). These authors concluded that beta-glucan may be a short-term immunostimulant for shrimp. From literature, the protective effects of β -glucans are not only observed before fish are challenged with pathogen as shown in the few examples above. b-glucans have also shown to be very effective in immunocompromised fish by restoring tissue and physiological integrity post b-glucans administration (Meena et al., 2013).

1.4.5: Protective effects of MOS in fish

The use of MOS in aquaculture have increased dramatically over the past decade due to their beneficial effects on fish including growth performance, disease resistance, feed efficacy, pathogen protection by potentiation of the systemic and local immune system and the reinforcement of the epithelial barrier structure and functionality among others have been reported (Torrecillas et al., 2014). According to Bavington & Page (2005), adhesion of bacteria to cell surface is a result of the interaction of the bacteria with specific cell surface carbohydrates through specific lectins is the basis for pathogenesis. Therefore, the use of MOS in aquaculture is to mimic those cell surface polysaccharides and to adhere bacteria to themselves and ejected via feces reducing the possibility and severity of bacterial pathogenesis. In other words, serving as natural antibiotics. Torrecilla et al. (2014) further explained that the MOS enhances innate immunity by activating pattern recognition receptors (PRR) and proteins to recognize substances that are not related to the system. This observation is not exclusive to only bacteria as Buentello et al. (2010) reported a higher survival rate of juveniles of red drum (*Sciaenops ocellatus*) fed soybean-base diet supplemented with MOS and challenged with dinoflagellate *Amyloodinium ocellatum*.

Rainbow trout was cultured in either a raceway or net cages and fed commercially extruded diet with or without 2000ppm MOS obtained from cell wall of *S. cerevisiae* for 90 days and 42 days respectively. Improved weight gain, reduced FCR, reduced mortality, and improved indicators of immune status for fish fed the MOS supplement compared with controls in the net cages. Fish cultured in the raceway showed improved weight gain for the MOS-treated groups, lower FCR, reduced mortality compared with the control treatment. In the raceway trials, however, only the indicators of immune status lysozyme concentration, alternate and classical pathways of complement activation were significantly improved by MOS treatment (Staykov et al., 2007).

In another trial, Atlantic salmon smolts were fed either a basal diet containing 52% FM supplemented with 48% plant proteins or the basal diet supplemented with 0.4% MOS for 14 days. Although MOS supplementation did not affect growth, body protein composition and liver glycogen reserves were significantly higher than the basal diet. The MOS supplemented diet resulted in a significantly higher absorptive surface & microvilli density in the anterior & distal intestine than the control diet (Dimitroglou et al., 2011). The author further added that supplementing MOS to the diet significantly reduced sea lice infestation like the report by Buentello et al. (2010) above with the red drum and the dinoflagellates. Similar observation was observed by Micallef et al. (2017) when yeast cell wall extract was fed to Atlantic salmon. Skin mRNA revealed a calreticulin-like protein increased in abundance at both the protein and transcript level in response to dietary yeast cell wall extract. This protein was identified as possible biomarker for yeast derived functional feed since it showed the most consistent change in expression in both the mucus proteome and skin transcriptome.

Torrecillas et al. (2011) reported that inclusion of MOS in the diet of the European seabass (*Dicentrarchus labrax*) reduced lipogenic enzymes in the liver, increased phagocytic activity of head kidney leukocytes with 4-6g/kg MOS supplement diet, and a dose-dependent enhancement in the number of cells secreting acid mucins by unit of area which could be related to the previous improvement in resistance to bacterial infection. In a follow up study by Torrecillas et al. (2012), reported a positive effect of MOS on disease resistance, reduced cumulative mortality against gut inoculated *Vibrio anguillarum* and reduced effects of stress on microbiota diversity in *D. labrax*.

In other studies, MOS diet supplementation did not significantly cause any changes compared with other experimental and control diets. For example, adding 5gkg^{-1} MOS to the diet of Japanese flounder did not affect the phagocytic activity of leucocytes, feeding rate or condition factor although growth rate and some health parameters were improved (Ye et al., 2011). In a similar experiment involving Nile tilapia, commercial diet supplemented with 0, 0.2, 0.4, 0.6, 0.8, and 1% dietary MOS. Results showed no differences in hematological parameters between the MOS supplemented diet and the control diet and decreased feed consumption with increasing inclusion of MOS. MOS did not increase leukocytes count. Dietary MOS did not increase leukocyte count with no significant difference in weight gain (Sado et al., 2008). Therefore, results of dietary MOS supplementation are inconsistent to some degree which may be attributed to the fish species, the source of the MOS, the duration of the trial and rate of addition.

1.5.0: Role of yeast & yeast products on fish health with emphasis on immune system and intestinal integrity

Whole yeast cell wall contains fractions of immunostimulants (β-glucans and MOS), nucleic acids and other components. Therefore, feeding whole yeast cell to fish means exerting the full effect of these components on the fish. Research have shown that supplementing fish feed with yeast meal modulates the gut microbiota, improve growth and restore compromised gut & immune system of fish. Research on the potential of yeast as a possible fish feed ingredient is scanty with a lot of focus on *S. cerevisiae* (Agboola et al., 2020). Majority of the results suggest that moderate level of yeast can be used as a fish feed supplement especially up to 40% of FM or soy protein without imposing any negative effects on growth and health of the fish. In one of such study, 40% of FM was replaced with yeast meal from either *Candida utilis*, *Kluyveromyces marxianus* and Saccharomyces cerevisiae and fed to pre-smolts of Atlantic salmon (Øverland et al., 2013). It was concluded that 40% of *C. utilis* & *K. marxianus* can potentially replace high quality FM with no adverse effect on fish growth performance, nutrient digestibility & performance while *S. cerevisiae* decreased retention of nitrogen and energy.

A down-regulation of stress genes, HSP 70 & pcna was observed in the DI of seabass fed diet containing multi-strain and single strain yeast fractions. At the same time, an upregulation of anti-inflammatory genes; Interlukin 1-b (IL-b) and interlukin 10 (IL-10) were observed in the group fed the multi-strain yeast supplement suggesting that even at

the lowest fraction used (1 g/kg) was potent enough to improve intestinal gut health and growth performance of the European seabass (Rawling et al., 2019). Supplementing the diet of Nile tilapia with 1% hydrolyzed yeast (*Rhodotorula mucilaginosa*) enhanced immune response and antioxidant capacity by increasing villi height and intraepithelial lymphocytes of mid-intestine and increased protection against *Streptococcus iniae* (Chen et al., 2019). Similar observation was made in rainbow trout by Yilmaz et al., (2007) when 1.5 parts per thousand of commercial rainbow trout was replaced with MOS from yeast. According to Gatesoupe (2007), several yeast strains have been isolated in the gut of both freshwater and marine fish and though the relationship defined as commensalism, some intestinal yeast may be opportunistic. Yeast strains of *S. cerevisiae* and *Debaryomyces hansenii* have been found to be dominant in rainbow trout. Accelerated maturation of the digestive system of fry fed yeast and stimulated metabolism and growth is also common in adult fish fed yeast.

The mode by which yeast cell wall components enhance intestinal integrity was best demonstrated by Torrecillas et al. (2013) on European seabass fed 4 g/kg MOS. Transmission electron microscopy observation revealed a better preserved cytoarchitecture of the intestinal epithelial barrier in the posterior gut of fish fed MOS. The fish fed 4 g/kg MOS showed a densely packed non-damaged enterocytes, better preserved tight junctions structure, healthier and more organized microvilli, and a higher presence of infiltrate lymphocytes and granulocytes compared fish fed the control diet (Torrecillas et al., 2013). Enhanced fish performance, protection against pathogen via strengthened systemic and local immune system as the reinforcement of the epithelial barrier structure and function are some means by which MOS supplements in fish diets stimulate the gut health (Torrecilla et al., 2014).

Feeding solvent extracted SBM is known to cause enteritis in the DI of fish especially salmonids. However, adding yeast cell wall extracts or whole yeast cell to SBM diet resulted in the elimination of SBMIE (Refstie et al., 2000). These research observations are usually not consistent with each other. For example, increasing supplements of *Candida utilis* in SBM fed Atlantic salmon did not reduce the severity of SBMIE (Hansen et al., 2019) which contradicts reports by Grammes et al., (2013) who reported that supplementing 20% SBM with *Candida utilis* or microalgae *Chlorella vulgaris* showed

healthy intestines with no signs of inflammation. In the same experiment, addition of *K. marxianus* resulted in a different outcome. According to Hansen et al., (2021), autolysis of yeast resulted secretion of IL-8, while cell crushed yeast induced the secretion of TNFα in the DI of Atlantic salmon and that different down-stream processing of *S. cerevisiae* led to increased protein and β-glucan solubility as well as trigger different immune responses.

Due to this, there is the need to conduct more research in order to reduce the inconsistencies and paucity of knowledge with respect to this discipline. This project will be conducted as part of the Foods of Norway (FoN) project which is aimed at increasing value creation in the Norwegian aquaculture, meat and dairy industries by developing novel feed ingredients from natural bioresources, improving feed utilization through industrial exploitation of cutting-edge research to achieve sustainability especially in a time when consumer demand for sustainability is higher than ever. Within the scope of the project, effort to develop new ingredients such as yeast for animal feed preparation from non-edible lignocellulosic spruce wood and chicken offal as described below (Fig. 5).

1.6.0: Main objective: The overall objective of this research is to determine how two yeast species*: Cyberlindnera jadinii* and *Wickerhamomyces anomalus* with two different downstream processing (16h autolysis or inactivation by spray drying) counteract SBMIE in Atlantic salmon (gastro-intestinal health) during seawater transfer. The experiment was be conducted with post-smolt Atlantic salmon fed a fishmeal control diet (negative control), a diet containing 30% SBM (positive control) and four treatment diets containing 30% SBM supplemented with 10% yeast either inactivated or autolyzed. The experiment will be conducted for a period of six weeks in seawater.

1.6.1: Specific objectives

The specific objectives of the study are to:

- compare the effects of the test diets to the control diets in terms growth rate of fish and feed conversion ratio
- determine the digestibility of test diets in comparison with the control diets
- determine the effect of autolysis and inactivation on composition of cell wall polysaccharides
- determine the effect of inactivated and autolyzed on gut health of the fish.

- determine the histological changes in the DI of the fish in response to the diet.
- use immunohistochemistry techniques to determine immune response following the diet administration

1.7.0: Justification

The health benefits of yeast are well established. However, using microbial feed ingredients come with several challenges. One of such challenges is the rigid cell wall in yeast and bacteria which limits digestibility and nutrient availability if processing is not sufficient. Several downstream processing techniques exist but not all are effective to render nutrients available for use by animals. For example, high pressure application followed by autolysis of bacteria meal did not improve nutrient availability, but ultrafiltration did (Øverland et al., 2010). Information on the effects of downstream processing on digestibility and health implications are still scanty. Hansen et al. (2021) reported that processing of yeast resulted in increased level of soluble protein in the yeast cream and that direct inactivation of yeast resulted in lower protein digestibility while 16 h autolysis of yeast resulted in the highest protein digestibility similar to a control FM diet. This experiment was conducted in freshwater which might have played a significant role in the outcome. Therefore, it is imperative to conduct similar experiment in seawater to determine how that may affect the outcome and for that matter necessitated this research.

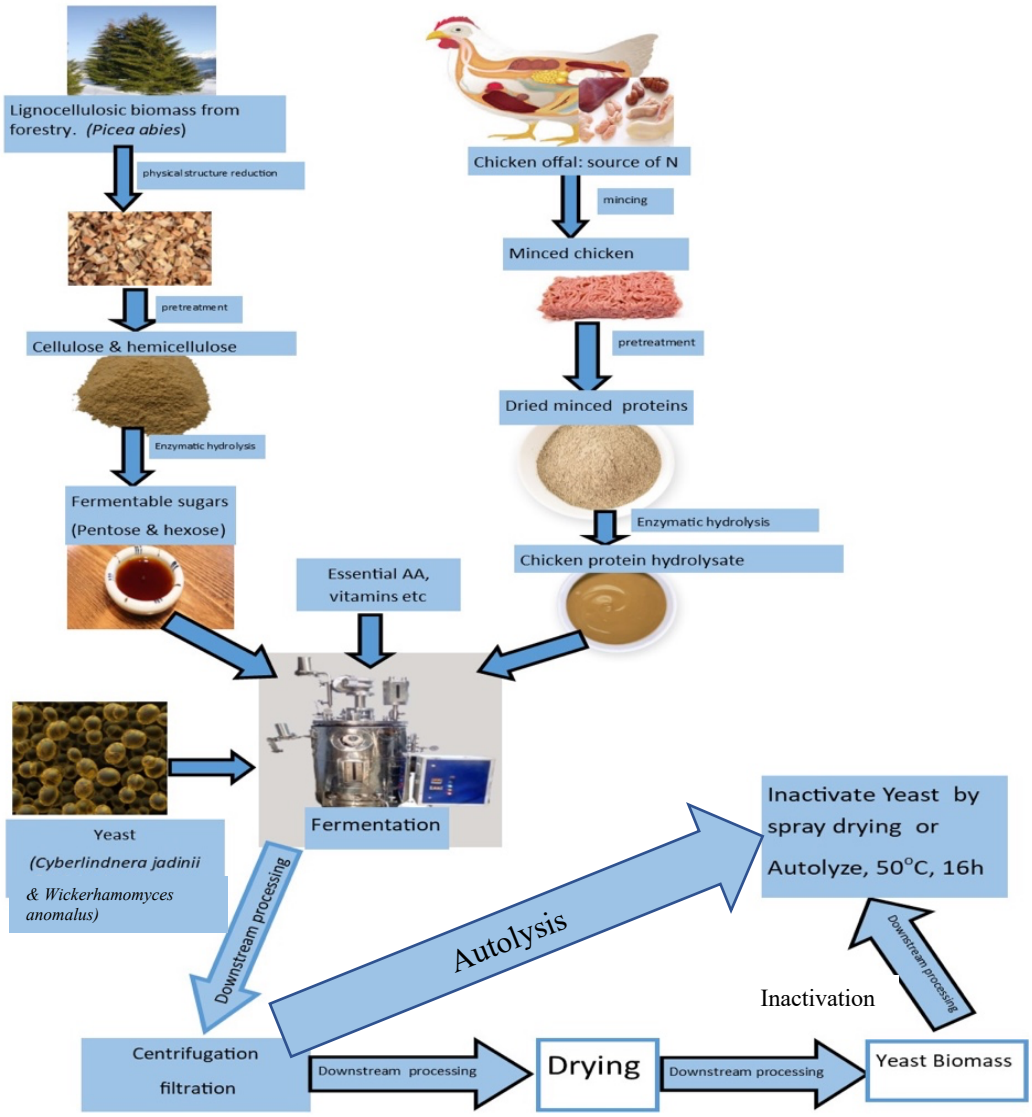


Figure 5: Schematic description of the production and downstream processing of yeast *C. jadinii* & *W. anomalus* for inclusion in test diets

2.0: MATERIALS AND METHODS

2.1: Yeast processing

Strains of two different yeast, *Cyberlindnera jadinii* LYCC 7549 and *Wickerhamomyces anomalus* J121 from the Swedish University of Agricultural sciences (Uppsala, Sweden) were cultured on media composed of enzymatically hydrolysates of spruce wood (Picea abies), hydrolyzed chicken by-products and urea using methods described by (Lapeña et al., 2019, 2020). The chicken hydrolysates were obtained from BIOCO AS (Hærland, Norway), urea from Yara international ASA (Oslo, Norway) and the Enzymatic hydrolysates of BALI™ pretreated spruce were provided by Borregaard AS (Sarpsborg, Norway). The yeast species were cultured in duplicates at 30°C for 48 hours in a repeated fed-batch fermentation process with the addition of KH_2PO_4, $CaCl_2.2H_2O$, $MgSO_4.7H_2O$ and NaCl in a 42L EINAR bioreactor system (Belach Bioteknik, Sweden).At the end of the culture, the yeast broth was centrifuged at a flowrate of 70L/h and discharged in 120 s using GEA Westfalia Separator Easyscale 10.S (GEA, Bönen, Germany). The yeast fraction was resuspended in water and washed at a flowrate of 50L/hr and a discharge of 90 s. With dry matter content of approximately 15%, the yeast was divided in parts and stored as yeast paste. Th yeast biomass was divided into two and either inactivated through spray drying or autolyzed using methods described by (Agboola et al., 2021). The inactivation process was carried out with a spray dryer SPX 150 MSPage 12/21 (SPX Flow Technology, Denmark AS) with inlet and outlet temperatures of 180°C and 80°C respectively with the pump speed set at auto and stabilized at 35%. The end inactivated yeast was stored at 4°C. The other half of the yeast paste was autolyzed at 50°C for 16 hours in a 30 L EINAR bioreactor system and stirred at 50 rpm. The cream was spray-dried using the above-described method and the powdered yeast stored at 4°C as yeast meal until ready for use.

2.2: Experimental diet formulation

Six experimental diets including a fishmeal diet (FM) negative control, 30% SBM positive control and four treatment diets containing 30% SMB and 10% of either inactivated or autolyzed *C. jadinii* (CJ) or *W. anomalus* (WA). The diets were designated as [Inactivated CJ (ICJ), Autolyzed CJ (ACJ), Inactivated WA (IWA), Autolyzed WA (AWA)]. The recipe of each of the diets is summarized in table 3 below.

The digestible protein to the digestible energy (DP:DE) ratio was 22.8 ± 0.3 with the right nutrient balance to meet the specific requirement of Atlantic salmon smolts. Due to the substantial amounts of SBM in some of the diets, the lysine and methionine levels of those diets were corrected by the addition of crystalline forms of these AAs to achieve a balanced AA required by Atlantic salmon. The exact amount of each feed ingredient was weighed and mixed using the Moretti Forni mixer (Spiry 25, Mondolfo, Italy) for about 5 minutes. Moderate amount of cold water was added to the gelatin and microwaved to reach a temperature of 60°C with occasional stirring to ensure equal heat distribution and gelatinization.

The gelatin was added to the meal mix and mixed again for about three minutes after which the FO was also added and mixed for some extra two minutes to obtain a uniform mash. After cooling to room temperature, cold pelleting was carried out using P35A pasta extruder (Italgi, Carasco, Italy) with a die size of 3mm and addition of moderate amount of water to obtain the right amount of moisture in the feed as well as render the extrusion process lenient. The pellets were dried at 60°C with moisture content reduced to about 7%. The feeds were transferred into labelled paper bags and stored at 4°C until ready to be used and throughout the experiment.

Table 3: Proportion of feed ingredients (g/kg) in experimental diets

	Diet 1 (FM-based)	Diet 2 (30% SBM)	Diet 3 (10% ICJ)	Diet 4 (10% ACJ)	Diet 5 (10% IWA)	Diet 6 (10% AWA)
FM	433.4	261.4	208.4	208.4	208.4	208.4
SBM	0	300	300	300	300	300
WGM	170	136	111	111	111	111
ICJ	0	0	100	0	0	0
ACJ	0	0	0	100	0	0
IWA	0	0	0	0	100	0
AWA	0	0	0	0	0	100
GPS	120	90	68	68	68	68
CELLULOSE	80	0	0	0	0	0
FO	130	130	130	130	130	130
MCP	0	10	10	10	10	10
PREMIX	5	5	5	5	5	5
GELATIN	60	60	60	60	60	60
L-lysine	0	3	3	3	3	3
DL-Methionine	0	3	3	3	3	3
Yttrium	0.1	0.1	0.1	0.1	0.1	0.1
Choline Chloride	1.5	1.5	1.5	1.5	1.5	1.5
Proximate Chemical Analysis						
Dry matter (g/kg)	926.5	897.0	889.5	889.0	924.5	914.0
Ash (g/kg)	72.5	69.5	66.5	66.5	68.5	67.0
Crude protein (%)	49.3	48.7	46.1	47.2	48.0	47.7
C (%)	47.2	45.7	44.7	46.1	47.5	46.7
S (%)	0.6	0.6	0.6	0.5	0.6	0.6
Starch (g/kg)	12.3	9.3	8.3	8.3	8.3	8.0
Crude fat (g/kg)	134.0	125.0	164.5	157.5	141.0	151.5
Energy (MJ/kg)	21.6	20.8	20.8	20.7	21.4	21.1

FM = Fishmeal, WGM= wheat gluten meal, MCP = Monocalcium phosphate, FO= fish oil, GPS = Gelatinized potato starch, C = Carbon, S = Sulphur

2.3: Fish husbandry and feeding trials

450 salmon smolts of average weight 136g were transported in oxygenated plastic bags from the Fish lab of the Norwegian University of Life sciences (Ås, Norway) to the research facility of the Norwegian Institute of Water Sciences (NIVA) (Drøbak, Norway) where fish was cultured for six weeks. 18 flow-through fiber glass tanks (300L) were stocked with 25 fish per tank and acclimated for 48 hours before the start of feeding. Each diet was fed in triplicates starting at 1% of fish body weight and gradually increased based on feed intake in each tank. The feed was evenly distributed on fitted automatic feeder and fed to fish over a 6-hour period (supplied for 6 mins, pauses for 12 mins) to ensure a large proportion of the supplied feed was consumed. Fifteen minutes after the feeding cycle, uneaten feed was collected on a perforated screen, weighed and stored. The water salinity(ppt) was increased gradually to full salinity of 34.4ppt over the first 4 weeks into the experiment. Salinity, Water temperature (^{o}C), water flow rate (sec/L) and oxygen concentration (% saturation) were all monitored and recorded daily over the period of the experiment to ensure they are within acceptable ranges optimal for salmon growth. Feed intake was calculated from the dry weight of the given feed and the dry weight of the recovered uneaten feed after adjustment for feed recovery rate from each tank.

2.4: Fish Tissue sampling

At the end of the experiment, the total biomass of fish in each experimental tank was determined after the fish were anaesthetized with metacaine (MS-222, 50mg/l water). Six fishes were randomly selected from each tank and their weight and length determined. Blood sample was drawn from selected fish with heparinize-treated syringe into labelled tubes, centrifuged and the plasma transferred into an Eppendorf tube and kept on ice. Gills of fish were collected, the cartilage was removed, cut into three pieces and added to a tube containing RNAlater. The fish was carefully dissected, the spleen collected and transferred into a cryotube. The DI of the fish was collected and dissected longitudinally. Digesta was carefully collected into cryotubes and immediately stored in liquid nitrogen. The DI was cut into three pieces and the mid-part rolled inside out (Swiss roll) into a bottle containing formalin for histology. The upper part of the DI was cut into three pieces and the whole lower part of the DI transferred into a tube containing RNAlater for RNA/protein analysis. The fish head kidney was gently collected and stored in cryotubes.

2.5.0: Histological processing

The fixed samples were left in formalin for a period of 24 hours after which were transferred into a 70% ethanol until ready to be processed.

2.5.1: Hematoxylin-Eosin (H&E) staining of the DI

During the processing, the tissues were trimmed to obtain the appropriate size as well as get rid of excess tissue. They were passed through graded ethanol, cleared with xylene using standard histological techniques (Histological Laboratory, Norwegian School of Veterinary Science). The tissues embedded in paraffin wax with arrangements to obtain sagittal sections. Serial sections of 4 μm thick were cut with a microtome (ThermoFisher Microm HM 355S) and stained with hematoxylin–eosin, mounted and air dried. The slides were cleaned and observed under a microscope (Olympus BX3) fitted with a camera (UI3260CP-C-HQ 2.3 MP) and Sony IMX249 sensor with installed Micro Visioneer Manual scanning software for any histological alterations. Histological alterations in DI samples were scored based on the following criteria: mucosal fold height, submucosal width, submucosal cellularity (infiltration by inflammatory cells), Lamina propria width, Lamina propria cellularity (infiltration by inflammatory cells), enterocytes supranuclear vacuolization, enterocyte supranuclear vacuole size (hyper-vacuolization). By a blind semi-quantification described by (Baeverfjord & Krogdahl, 1996) was used to classify the degree of change in the DI as Normal (0), mild (1), moderate (2), marked (3) or severe (4).

2.6.0: Immunohistochemistry

The 4 μm microtome sections of the paraffin embedded tissues were dried in an oven at 55^{o}C for 30 mins and deparaffinized. Unmasking was carried out by autoclaving the slides at 121^{o}C for 10 minutes in 0.01M citrate buffer of pH 6. To inhibit endogenous peroxidase activity, slides were treated with phenyl hydrazine (0.05% PBS) for 40 minutes at 37^{o}C. Samples were collected in PBS buffer and tissues delineated using a hydrophobic pen and collected back in PBS.

2.6.1: CD3ε staining

To avoid non-specific binding, tissues were incubated with bovine serum albumin in Tris buffered saline (5% BSA/TBS) with normal goat serum (s-goat) diluted at 1: 50 ratio for 20 minutes. A primary monoclonal antibody (mouse anti-trout CD3ε) was diluted with 1% BSA/TBS at a ratio of 1:600. Slides were sparingly dried with a tissue paper and incubated with the primary antibody for one hour. A negative control was incubated with the 1% BSA/TBS for the same period. The slides were incubated with a secondary antibody kit polymer-HRP anti-mouse (DAKO En Vision+ System-HRP, Dako, Glostrup, Denmark) for 30 mins. Peroxidase activity was detected using 3,3' diaminobenzidine (DAKO En Vision+ System-HRP, Dako, Glostrup, Denmark). Sections were counterstained with hematoxylin for 10 seconds, washed with distilled water and mounted using a mounting agent (Aquatex® Merck KGaA, Damstadt Germany). Between blocking and treatment with primary antibody, slides were washed for 15 mins with PBS between steps.

2.6.2: CD8α staining

For CD8a staining, tissues were blocked using 0.1M Tris saline buffer (pH 7.5) containing blocking agent FP1012 (TSAÔ INDIRECT, PerkinElmer Inc, MA USA) for 30 mins using the manufacturer's recommendation. Without washing, slides were treated with a monoclonal primary antibody (CD8a mouse anti-salmon) diluted with 1% BSA/TBS in a ratio of 1:50 overnight at 4^{o}C with a negative control incubated with 1% BSA/TBS for a similar duration. A Biotinylated Anti-Mouse IgG secondary antibody BA-9200 (Vector lab Inc. CA USA) diluted with BSA/TBS (1:50) was used to treat the tissue for 30 min at room temperature. To increase the signal of the peroxidase activity, a 3-step amplification process was included. In the first step, slides were treated with Streptavidin (FP 1049) diluted with 1% BSA/TBS (1:100) for 30 mins after which they were treated for 5 mins with biotinylated tyramide stock (FP1052) in an amplification diluent in a 1:50 ratio. Step one was repeated for another 30 mins. To determine the peroxidase activity, sections were incubated with 3-Amino-9-EthylCarbazole, SK- 4205 (Vector lab Inc. CA USA) for 5 minutes. Sections were counter stained with hematoxylin for 10s and mounted using a mounting agent (Aquatex® Merck KGaA, Damstadt Germany). Unless stated otherwise, washing was caried out between all steps.

2.7: Image analysis, morphometric measurements and calculation of immune cells quantification

Morphometric measurements and determination of immune cells densities was carried out on the Labelled sections of the immunohistochemically labelled described above. Qupath digital pathology software (v 0.2.3) was used to capture area of the tissue containing at least five simple folds and saved as a Tiff file in mm with a resolution of 1. ImageJ version 1.53c was used to perform the measurements and calculations. The Tiff file was imported into ImageJ and the measurement scale set in pixels and converted to mm. For each tissue, five simple folds with a maximum of one between a pair of complex folds were selected and measured. With a free hand tool, the total fold area and epithelium area were delineated and measured and a segmented line to determine the fold height. The fold area was measured from both sides of the fold from the stratum compactum to the tip of the fold. Fold length was measured along the mid lamina propria from the stratum compactum to the tip of the simple fold. Immune cells within the fold were measured as the percentage of the fold occupied by those cells.

The percentage of the fold occupied by CD3ε^+- or CD8α^+-labelled cells was calculated as [(area of fold occupied by cells/area for the entire fold) X 100] and repeated for the epithelium. The cell % coverage in the lamina propria was determined as the difference of the fold and the lamina propria. For each diet group, 18 measurements were calculated and the mean of the five simple folds from each tissue used as the mean of each measurement. For each tissue, simple folds that are long, regularly shaped with well-defined epithelium was selected for measurements.

2.8: Yeast cell wall compositional analysis

Dry yeast samples were pulverized, homogenized and weighed into an Eppendorf tube. Samples were hydrolyzed with 72% H_2SO_4 and incubated in a water bath for 1 hour at 30^{o}C to release sugar monomers (mannose, N-acetylglucosamine and glucose). Samples were diluted with water to reduce the concentration of the H_2SO_4 to 4%. After a thorough vortexing, needle-size holes were made in the lid of the Eppendorf to allow steam escape from the samples after which were wrapped in aluminum foil and autoclaved at 121^{o}C for an hour. Samples were centrifuged and supernatants subjected to sugar analysis using high-

performance anion exchange chromatography coupled to pulsed amperometry detection (HPAEC-PAD) described by (Hansen et al., 2021). Polysaccharides were quantified using the following formula:

$$C_{strc.carb} = \frac{(C_{HPAEC} \times D \times V_{sample} \times P_{hex/pent})}{(R_f \times mDMload/1000)},$$

where C is the polymeric concentration, $_{CHPAEC}$ is the measured concentration of the monomer, D is the dilution factor, V_{sample} is the sample volume, P is the polymeric conversion factor for Glc/Man), R_f is the recovery due to monosaccharide decomposition during hydrolysis and mDM_{load} is the mass of the dry matter load. This method did not differentiate between 1,6- and 1,3-linkage in β-glucan.

2.9: Data analysis

Feed utilization was quantified using feed conversion ratio (FCR) calculated as; FCR = feed consumed X weight gained^{-1} and specific growth rate (SGR) = SGR = 100 × (ln (final weight) – ln (initial weight)/duration in days). The apparent digestibility coefficients (ADC) of nutrients in feed was calculated using the following equation (Hansen et al., 2021):

$$ADC\ (\%) = 100\ X\ \frac{[(Nutrient\ in\ feed/Yttrium\ in\ feed) - (Nutrient\ in\ feces - Yttrium\ in\ feces)]}{[(Nutrient\ in\ feed/Yttrium\ in\ feed)}$$

Statistical differences with multiple group comparison between non-parametric data from the histological evaluations was calculated using Kruskal-Wallis followed by post hoc Dunn's test. Normal distribution of data for specific growth rate (SGR), feed conversion ratio (FCR), digestibility and morphometric measurements of T-cell density were determined by the Shapiro-Wilk test followed by one-way ANOVA to test treatment effects. Tukey HSD post hoc test was used to determine between mean significant difference for fish growth performance and morphometric data and T-cell percentage coverage. All the statistical analyses were conducted using SPSS statistical software package version 25 (IBM Institute, Armonk, NY, USA) with significance level set at (P = 0.05) for all analysis

3.0: RESULTS

3.1: Fish growth, feed intake, apparent digestibility, and general health

Supplied feed was accepted in all test groups and feed intake increased gradually (Fig.6) over the period of the experiment with no significant differences in both SGR and FCR (table 4). The average initial and final weights were 136g and 179g respectively. Since the average initial and final weights were recorded in bulk, it is assumed that fish in all dietary groups gained weight. No mortalities were observed over the period of this study, suggesting a general good health in fish and culture environment. All water parameters measured were within acceptable ranges and were considered optimal for salmon growth. No significant differences were observed in both SGR and FCR between the control and experimental diets. Similarly, there were no differences in the initial and final weight and weight gain among the test diets. There was no statistical difference in ADC for crude protein in FM and SBM diets, but the FM diet had a significantly higher crude protein digestibility than the other diets. Also, there were no significant differences across diets for ADC of DM and ash. Both sulphur and crude lipids did not significantly differ between FM, SBM and the two forms of CJ. ICJ had a significantly higher ADC of crude lipids than CJ and IWA and AWA.

Table 4: (a) Growth performance of Atlantic salmon smolts fed six different diets for six weeks and (b) apparent digestibility of dietary nutrients on dry matter basis.

	DIETS							
(a) Growth	FM	SBM	ICJ	ACJ	IWA	AWA	P-value	S.E.M
Initial Biomass(g)	136.0	135.9	136.0	136.2	135.7	135.9	0.410	0.06
Final Biomass(g)	184.8	182.4	182.5	174.3	175.5	174.3	0.270	1.71
Weight gained (g)	48.8	46.4	46.5	38.1	39.7	38.4	0.290	1.73
FCR	1.50	1.49	1.51	1.77	1.76	1.81	0.410	0.06
SGR (% Day^{-1})	0.72	0.70	0.70	0.58	0.61	0.59	0.310	0.02
(b) Apparent Digestibility (% on DM)								
DM	67.5	65.1	65.0	65.4	62.6	61.9	0.048	0.6
Ash	7.5	-4.7	-0.1	1.6	-4.8	-5.3	0.061	1.51
Crude protein	86.6^{a}	82.9^{ab}	82.2^{b}	82.1^{b}	79.8^{b}	79.9^{b}	0.001	0.62
Carbon	75.3	74.8	73.2	74.2	70.8	70.9	0.031	0.56
Sulphur	27.6^{a}	23.8^{ab}	15.9^{ac}	15.0^{abc}	9.7^{bc}	2.0^{c}	0.004	2.42
Crude lipids	92.6^{ab}	92.2^{abc}	94.2^{a}	92.5^{abc}	90.3^{c}	91.8^{bc}	0.003	0.32

FCR = Feed conversion ratio, SGR = Specific growth rate, MSE = Standard Error Mean, BW = Body weight, DM= Dry matter. Values are presented as the mean (n = 3) for all groups. Values on the same row with different superscripts are significantly different (p = 0.05).

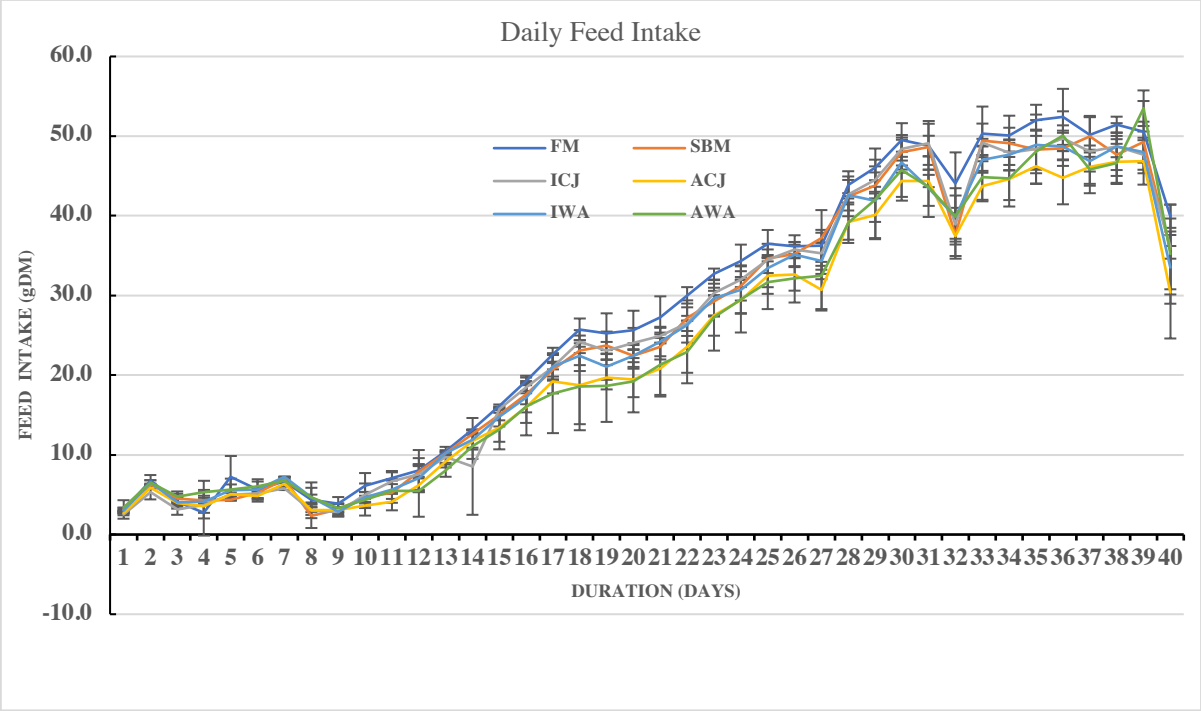


Figure 6: Average daily feed intake (corrected for recovery) of salmon smolts fed six experimental diets in 42 days. Each data point is the mean of the feed intake ± standard error. Feed intake increased gradually across diets from day 7 with some observed fluctuations.

3.2: Histological observations in the DI

The DI of fish fed the FM control diet showed normal histology with long simple folds with well-defined epithelium and slender lamina propria. The gut health status of fish from SBM, ICJ, ACJ, IWA and AWA were sub-optimal characterized by infiltration of eosinophilic granulocytes and immune cells in the lamina propria and submucosa, total loss of supranuclear vacuolization in SBM, ACJ, IWA and AWA and the shortening of mucosal fold height in some individuals (figure 6).

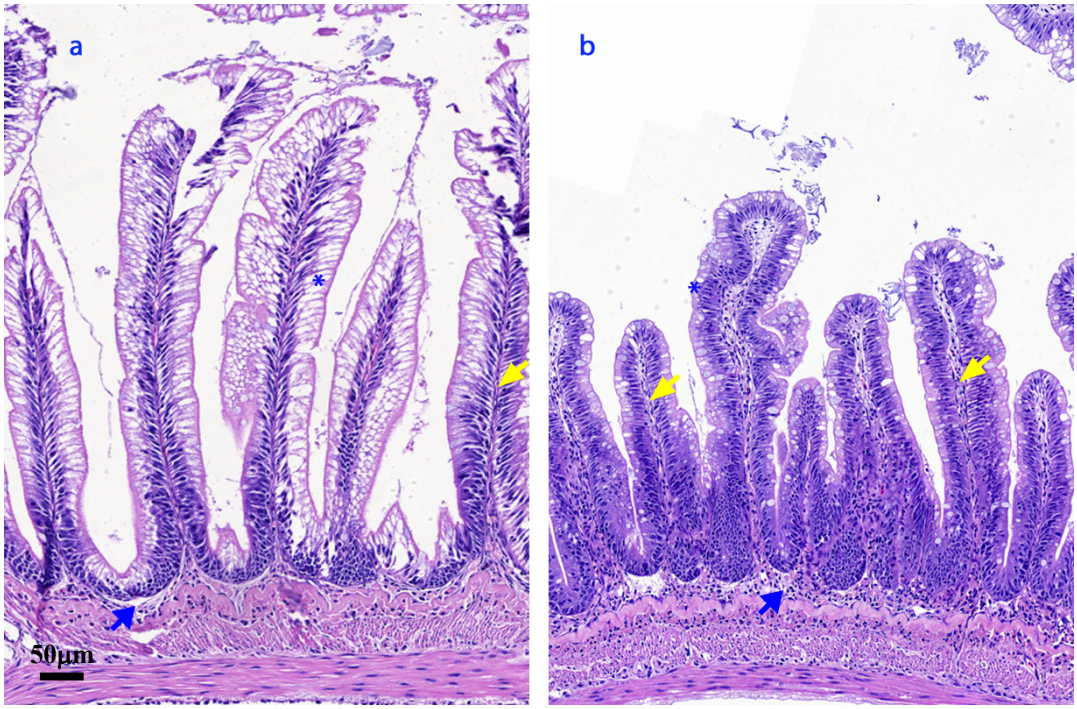


Fig 7. Histological staining of the Distal Intestines of Atlantic salmon fed six different diets for six weeks. (a) normal morphology and (b) signs of inflammation. In (a), simple folds are characterized by slender lamina propria (yellow arrowhead) and highly endowed with supranuclear vacuoles (blue asterisk) and few eosinophilic granulocytes (blue arrowhead) whereas in (b), lamina propria is widened (yellow arrowhead), loss of supranuclear vacuoles (blue asterisk) and infiltration of several eosinophilic granulocytes (blue arrow). From observation, simple folds are long and slender in (a) and shortened and irregular in (b) which are signs of an inflamed DI. Magnification = x20

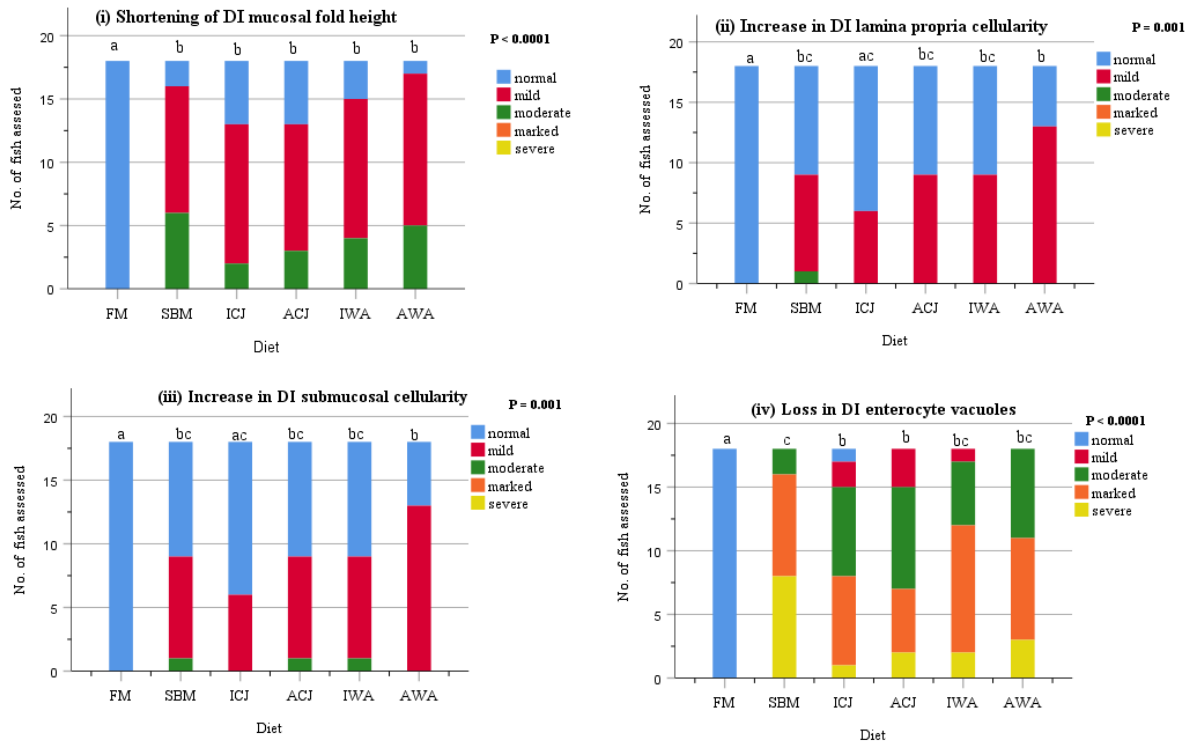


Fig. 8: Histological evaluation of the DI of Atlantic salmon morphological characteristics by (i) shortening of the mucosal fold height, increase in the inflammatory cells in (ii)lamina propria and (iii) submucosa as well as (iv)the loss in enterocyte supranuclear vacuolization. Bars with different alphabets indicate statistically significant differences (P = 0.05) in the overall morphological alterations in the DI of the fish. Significant differences were observed between the FM diet and all the other diets except for ICJ in terms of the increase in cellularity of the lamina propria and the submucosa. ICJ differed significantly from AWA in terms of increase in the cellularity of the LP and submucosa while SBM was different from ICJ and ACJ in terms of the loss in supranuclear vacuolization of the enterocytes. Changes in these parameters are scored from 0 to 4, with 0 being normal morphology, 1= mild changes, 2= moderate changes, 3 = marked changes, 4 = severe changes. N = 18 for all groups.

3.3: Yeast cell wall composition

In this study, the cell wall composition of the two different down-stream processed yeast species primarily is made up of β -glucans and mannans making up 23% in the inactivated *C. jadinii*, 16% in the autolyzed *C. jadinii*, 25% in the inactivated *W. anomalus* and 21% in autolyzed *W. anomalus* (Table 5). The quantities of β -glucans, mannans and chitin are higher in in all the inactivated yeast than the autolyzed yeast (Table 5). Although not statistically tested, numerically, β-glucans are slightly higher in the inactivated CJ than in the inactivated *W. anomalus* and vice versa for mannan and chitin. Similarly, the intake of β-glucans is

higher in the inactivated yeast than the autolyzed yeast for both species while both forms of *W. anomalus* with higher daily intake of mannans.

3.4: Changes in Immune response parameters

The negative control had no visible immunohistochemical labeling with a weak diffused background staining in the stratum compactum in some instances. However, some of the samples had no visible labels in both CD3ε and CD8α stains hence were excluded from all further analysis. Both CD3ε- and CD8α-positive labelled cells were observed in all dietary groups with their expression more pronounced in the epithelium than the lamina propria. For T-cells in the epithelium, they were more localized closer to the base of the epithelium among the enterocytes. Both T-cells were more abundant in the epithelium of SBM fed group than the FM and the other diets specifically CD8α. For T-cells in the lamina propria, they were located closer to the base of the stratum compactum (Fig. 8). Most of the T-cells are located individually but some are clustered together especially in the FM fed group with rare occurrence in the lamina propria and the stratum compactum. Simple folds in the SBM, ICJ, ACJ, IWA and AWA fed groups were significantly shorter than the FM fed group with visibly enlarged lamina propria. For the simple fold, epithelium and lamina propria, there was no observed significant differences between the diets in their percentage area occupied by $CD3\varepsilon^+$ cells. However, FM fed group had significantly less percentage coverage of $CD8\alpha^+$ cells in the entire simple fold and epithelium than SBM. In terms of the $CD8\alpha^+$ cells in the lamina propria, the SBM fed group showed a significantly high occurrence than all the other diets. A High within group variation was observed in most dietary groups for both the fold length and area.

Table 5: Composition of autolyzed and Inactivated yeast post drying and the estimated daily beta-glucans and manna intake

		Cell Wall Polysaccharides (% of dry matter)			Estimated β-glucan & Mannan intake (mg/day)	
DIETS	DM (g/kg)	β -Glucans	Mannans	Chitin	β-glucans	mannans
ICJ	890	15.76 ± 3.1	7.66 ± 2.1	0.28 ± 0.1	26.22 ±0.3	12.72 ±0.1
ACJ	889	10.33 ± 0.8	5.55 ± 0.6	0.23 ± 0.0	16.13 ±0.6	8.66 ±0.3
IWA	925	14.41 ± 1.4	10.89 ± 0.9	0.47 ± 0.0	23.78 ±0.9	17.97 ±0.7
AWA	914	11.32 ± 0.7	9.97 ± 0.6	0.39 ± 0.0	17.99 ±1.3	15.84 ±1.2

The percentage of b-glucans, Mannans and Chitin in the cell wall of the yeast and their estimated daily intake is the Mean ± SD from triplicates analysis.

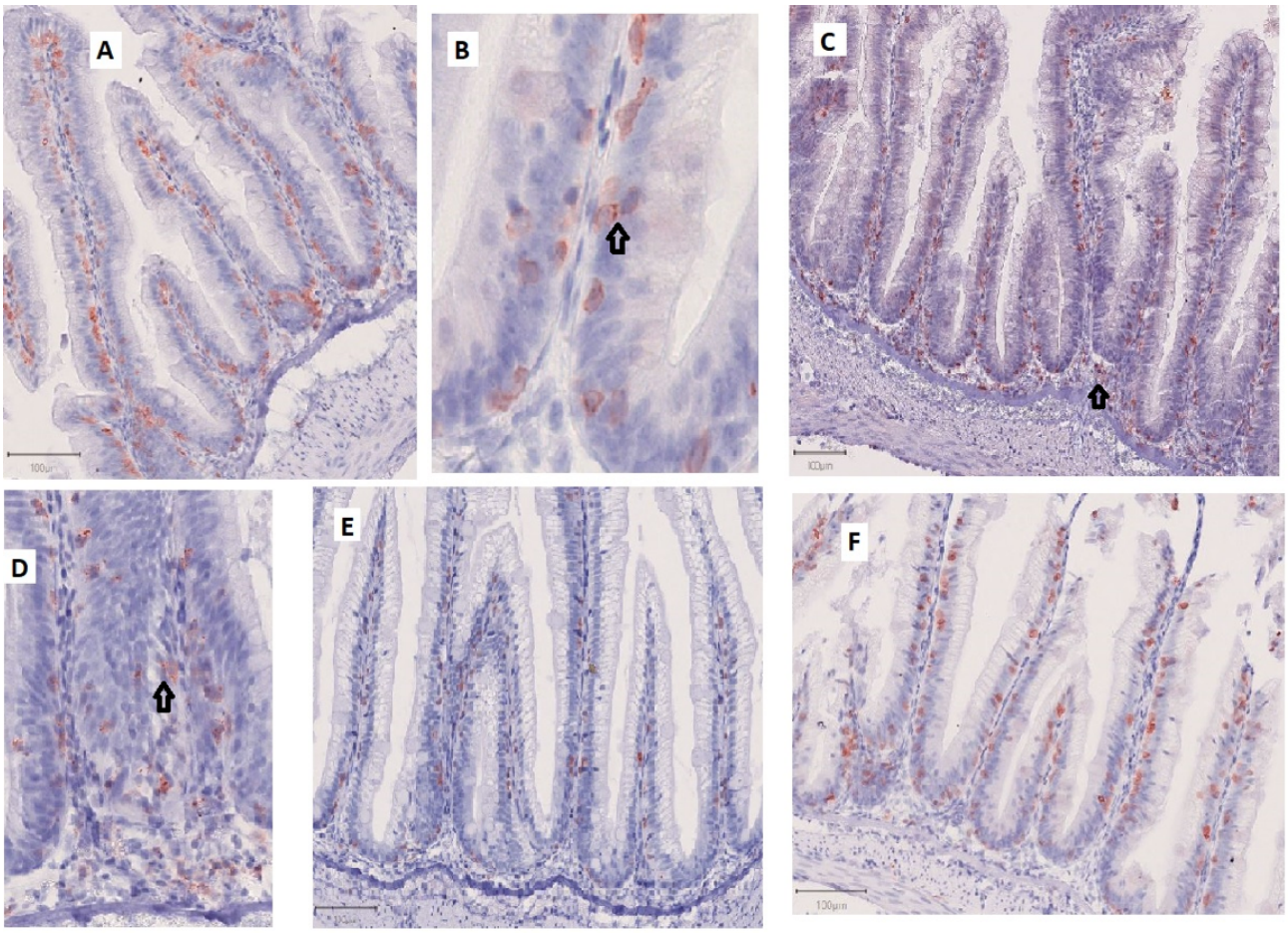
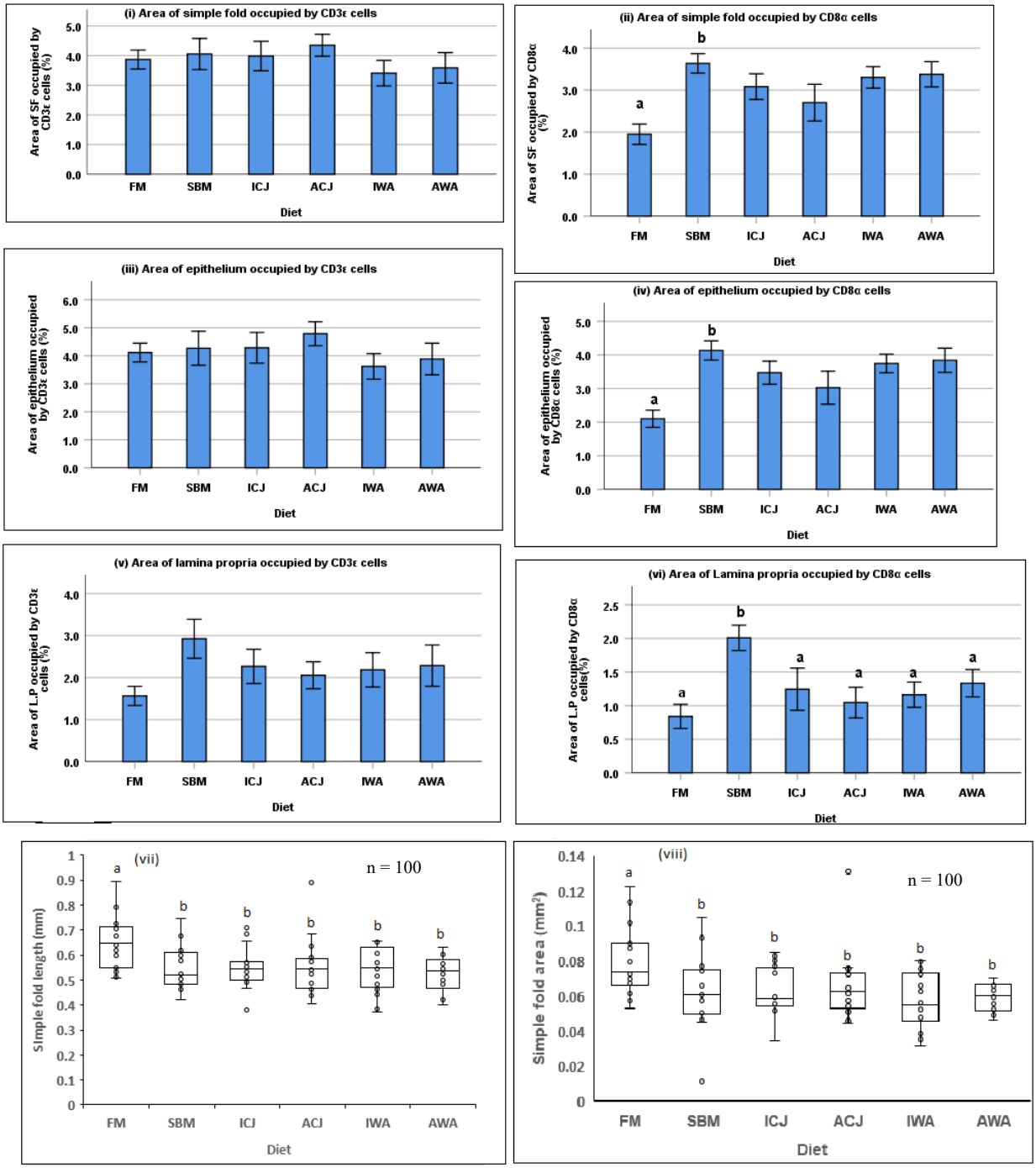


Image A, C, E, F: Magnification = x20. Image B & D: Magnification = x40, scale = 100µm

Figure 8: Immunohistochemical staining of CD3ε⁺ and CD8α⁺ positive cells in the intestines of Atlantic salmon fed six different. CD3ε⁺ immunohistochemical labeling (Black arrowhead) were more abundant (A) in the simple folds than CD8α⁺ and more localized closer to the base of the epithelium (B) across all diets. CD8α⁺ cells were more expressed in SBM fed group (C) than all the other groups (F: ICJ, ACJ, IWA, AWA) and far

less in FM fed group (E). A closer magnification shows that cells were localized in the lamina propria and the submucosa as well adjacent the stratum compactum (C and D) especially in the SBM fed group



SF = simple fold, LP = Lamina propria

Fig. 9: Percentage of simple fold, epithelium and lamina propria covered by T-cells (i-vi) and the morphometry of selected folds (vii, viii) from the distal intestines of Atlantic salmon fed six diets for six weeks. Both fold length and area in the FM fed group are significantly different from all the other diets ($p = 0.05$). Data is expressed as their means ± standard error (S.E) in all cases. For the Dunnet's posthoc test, SBM was set as the control diet and all the other diets were compared with it.

4.0: DISCUSSION

Most research on yeast as a feed ingredient has focused on their nutritional value and health benefits in experiments conducted largely in freshwater rather than on the effect ofdownstream processing and seawater transfer on growth and health. Therefore, this research was designed to acquire further understanding on how Atlantic salmon transferred from fresh- to seawater fed SBM diets supplemented with 10% of either autolyzed or inactivated *C. jadinii* or *W. anomalus* influence the gastro-intestinal health of the fish. Growth parameters, digestibility, yeast cell wall polysaccharides, changes in the morphology of the DI, changes in the immune cell profile in the simple folds, epithelium and lamina propria of the DI of the fish are the focus of this discussion. Since there was no difference in feed intake, changes in intestinal health can be attributed to the composition of the dietary treatments.

The absence of any observed differences in the SGR and FCR is consistent with the findings of Agboola et al., (2021) and Øverland et al., (2013) for *C. jadinii*. However, the experimental duration was relatively short to cause any observable differences in the growth parameters. The appetite and growth of Atlantic salmon can be suppressed for approximately thirty days post seawater transfer with osmoregulatory adaptation usually within ten days of transfer (Usher et al., 1991). Feed intake was depressed for the first week suggesting that fish were not fully acclimatized to the feed and the saltwater environment over this period. For this reason, the dietary effect might be limited to the few remaining weeks of the experiment and not the entirety of the experimental duration. Furthermore, an increase or decrease in salinity has a profound effect on fish growth since it is strongly correlated with energy expenditure for maintenance. Most teleost fishes regulate the osmotic pressure of their plasma such that their internal salinity is in the range of 10-15‰ (Imsland et al., 2001). Flushing out Na^+ and Cl^- across the gills of Atlantic salmon using Na^+- Cl^- ATPase molecules required energy, that could have been used alternatively the first few weeks of the experiment. In general, Atlantic salmon reared at low salinities obtain higher growth, better feed conversion and feed intake compare to full salinity seawater (Imsland et al., 2001; Ytrestøyl et al., 2020). This could be due to lower energy expenditure on osmoregulation. In the present experiment, full salinity (32‰) was reached four weeks into the experiment which may have been too early, hence, affecting both the feed intake and growth rate.

Besides, studies have shown that salinity affects nutrient transport in the intestines of salmonids (Collie, 1985).

Cell membranes of the intestinal mucosa are selectively permeable and regulate the movement of materials between the intestinal lumen and the internal environment. Some plants contain saponins that are used to maintain homeostasis in biological systems to ensure immunity against fungal diseases, kill protozoans and mollusks, as well as acting as antivirals in a hostile/antagonistic interaction with these pathogens (Anisimov, 1987; Bowyer et al., 1995; Francis et al., 2002). Perhaps their amphipathic property allows them to interact with the phospholipids with similar property in cell membranes which leads to the opening of the membranes. Their mode of action with bio-membranes is best explained by their hemolytic properties when administered intravenously. Injecting saponin extracts results in the release of hemoglobin from erythrocytes resulting from the interaction between the saponins and the membrane-bound sterols which increase the permeability of the cell membrane and subsequent death of the cell since osmoregulation and water balance are compromised in the process.

According to Nordrum et al. (2000), compromised mucosal barrier of the DI by the enteritis could increase mucosal permeability. As a result, a significant proportion of absorption could be via carrier independent component leading to a maintained near to or even higher than normal absorption, despite reductions in transporter densities or functions. With an alternate means of nutrient absorption, the effect of the SBMIE, as observed in the present study, on growth for instance may not be observed due to the relatively short duration of the experiment. This is a possible reason for the lack of difference in SGR and FCR between the FM and the other diets even though clear signs of enteritis were observed in the soy containing diets. Furthermore, the higher concentration of Na^+ in the lumen of the intestines of marine fish that must drink seawater is related to the high dependence of carrier systems during seawater adaptations (Collie, 1985). This relationship between permeability and salinity can also affect the transport of neutral amino acids such as methionine (Nordrum et al., 2000). The synergistic effects of all these factors might have played a significant role in the short present study by subverting the effects of the diets leading to limited observed differences in growth and FCR.

The digestibility results were not supported by the FCR and growth rate as FM and SBM clearly showed higher digestibility in terms of protein compared to the yeast containing diets. However, ADC of SBM in turn was not different from the test diets coherent with the results of the histomorphology. A higher digestibility may not imply an improve absorption or growth. Depending on the viscosity and other rheological properties of the digesta, the retention time and the transit time down the gut plays an important role in ADC. Even for a well digested diet, a lower retention time and fluidity may result in lower ADC. Similarly, if the transit time for the digesta through the gut is long, absorption would be enhanced and for that matter higher ADC. This property might have caused the observed disparity between the FM and the test diets in terms of their ADCs.

Reduced total mucosal weight which coincided with the shortening of the mucosal folds in fish fed the SBM and yeast supplemented diets in this study may considerably reduce the absorptive capacity of the DI in salmonids (Nordrum et al., 2000). The decline in the activities of brush border enzymes in the DI of a SBMIE inflicted Atlantic salmon (Krogdahl et al., 1994, Chikwati et al., 2013) may partly explain the reduced protein digestibility and lack of difference between the SBM and the test diets. A decrease in the ADC of lipids have been observed in rainbow trout fed SBM explained by the low levels of bile acid in the chyme (Romarheim et al., 2006) due to the interaction between some ANFs and essential lipid digestion components (Mosberian-Tanha et al., 2018). The findings of this study is in contrast of these previous reports considering that the ADC of lipids in the FM, SBM and the two forms of *C. jadinii* were not different. Yeast products in diets of different fish species have different protein digestibility comparable to conventional protein sources in diets for different fish species (Øverland & Skrede, 2017). The ADC of lipids in fish fed either inactivated or autolyzed *C. jadinii* was similar in FM control diet and are in line with reports by (Øverland et al., 2013). Similarly, rainbow trout fed yeast (*C. lypolytica*) exhibited a better growth and digestibility (Matty & Smith, 1978). This present study affirms that *C. Jadinii* has a better lipid digestibility comparable with the FM control diet. Reports on the digestibility of *W. anomalus* in fish are limited. However, Vidakovic et al., (2020) reported a higher crude protein ADC comparable to FM when 20% FM was replaced by a mix of 30% *W. anomalus* + 70% *S. cerevisiae* for rainbow trout contrasting the results of this study. The fact that it was added in a mixture makes it complex to determine the actual ADC of WA even though the author concluded that the mixture had no negative effect on growth and gut health and can

potentially replace up to 40% of FM. More so, a second possible reason for the difference may be the method of production and feed processing. Molasses was the carbon substrate for the WA fermentation and the feed processed using extrusion at 120°C-130°C was a great difference compared to the current study. Studies have shown that *W. anomalus* has thicker cell walls than *C. jadinii* and can obtain a 20% reduction in their cell wall thickness when autolyzed (Agboola et al., 2021). This property can play a role in the availability of the intracellular nutrients, and thereby reducing their ADC.

Results from the DI histomorphology observations in the current study shows that this enteropathy occurred in varying degrees in the dietary groups with some similar effects between ICJ and the FM diet in terms of reducing the width of the lamina propria and submucosal cellularity suggesting that inactivated *C. jadinii* have the potential to relieve fish of SBMIE consistent with previous studies (Grammes et al., 2013; Agboola et al., 2021). The presence of fishes in all the experimental dietary groups including the SBM with normal histomorphology in the simple folds DI also suggests that perhaps the enteropathy was not fully induced in the test diets and the SBM or the effect of the SBM was rather at the individual level even though signs of SBMIE can occur as early as two days from the start of feeding and fully in one week (Baeverfjord and Krogdahl., 1996).

A study showed that the severity of SBMIE in salmon fed SBM supplemented with 200 g/kg or 100 g/kg of *C. jadinii* was comparable to observations in the diet containing only SBM (Reveco-Urzua et al., 2019). In the same study, supplementation with lower concentration of the yeast (25 or 50g/kg) resulted in in a large variation within group with morphology ranging from normal to moderate SBMIE (Reveco-Urzua et al., 2019) comparable to the results of this current study. Øverland & Skrede (2017) speculated that such inconsistent effects of yeast on host immune response could be attributed to yeast strain, fermentation conditions, downstream processing during the manufacturing process. Notwithstanding, the severity in SBMIE in Atlantic salmon and the variation in the distal intestine histology can be influenced by variation in commercial source of SBM used in studies. A study by Uran et al., (2009) concluded that differences in commercial sources of SBM can influence the severity of SBMIE especially in the loss of supranuclear vacuolization with less variability in the amount of goblet cells. Therefore, comparing studies of such kind can lead to overstating or downplaying the effect of the yeast supplement in SBM based diets. As stated earlier,

genetics and adaptability to plant source proteins have been observed in rainbow trout over time (Abernathy et al., 2017; Callet et al., 2017). Depending on dietary component of the brood stock and the generation, the post-smolts used in this experiment might have developed some resistance to ANFs in plant nutrients partially explaining the variability in the degree of SBMIE particularly in the SBM fed group.

Temperature could be another contributing factor to this observation. Changes in water temperature can delay SBMIE in Atlantic, however, not the type and severity (Uran et al., 2008). With average water temperature throughout the study was about 11°C may be enough to delay the formation of this enteropathy as observed in some of the fish individuals. On the other hand, factors such as the supplementation strategy, the correct dose and duration, fish species, size and conditions determine the improvements in health and growth performance of fish upon supplementation of these immunostimulants (Torrecillas et al., 2014).

Data from the cell wall analysis indicate that inactivation of the yeast resulted in high b-glucans and mannans contrary to the findings of Hansen et al., (2021), while in autolysis, more soluble chitin was produced in both forms of *W. anomalus* than *C. jadinii* which may not be nutritionally available for Atlantic salmon due to their lack of chitinases. The high chitin in both forms of *W. anomalus* may partially explain the poor ADC for lipids and proteins observed in both IWA and AWA. Karlsen et al., (2015) observed that high inclusion of chitin in the diet of Atlantic salmon reduced growth rate and condition with a negative correlation between chitin level and ADC of lipids and proteins. Also, the chitin content of IWA and AWA are consistent with the findings of (Agboola et al., 2021) suggesting that *W. anomalus* might contain more chitin in their cell walls than *C. jadinii*. These results also show that downstream processing influenced the solubility of cell wall polysaccharides with the appreciably high content β -glucans in ICJ being a probable reason for their potential in the assuage of SBMIE in this present study. All the research reports on yeast as an immunostimulant and their potential in alleviating SBM enteropathy involve inclusion of the processed yeast biomass to the test diets. However, the exact amount of mannan and b-glucans or their ratios needed to counteract SBMIE in each of those experiments was not explored. For example, a combination of 20% CJ and 20% SBM showed that CJ could counteract SBMIE (Grammes et al., 2013). Similarly, 5% inclusion of WA in a 40% SBM diet showed that WA could counteract SBMIE (Agboola et al., 2021) with CJ showing a potential to cause similar effect in Atlantic salmon. Lower inclusion of CJ reduces the

severity of SBMIE in Atlantic salmon and no effect with increasing supplementation (Reveco-Urzua et al., 2019). On the contrary, CJ supplement did not counteract SBMIE in Atlantic salmon (Hansen et al., 2019). The estimated daily β -glucans and mannan intake follow the same pattern as observed in the percentage composition of these polysaccharides with more β -glucans consumed in the inactivated variants of each yeast strain but more so in the ICJ than the IWA. Similarly, more mannans were consumed in IWA than ICJ and generally in the *W. anomalus* than *C. jadinii*. Speculatively, the 2:1 ratio of β-glucans to mannan in the ICJ diet potentiated it to counteract SBMIE with respect to this experiment.

Generally, the lack of SBMIE mitigating effects particularly in *W. anomalus* in this study contrary to literature reports might be attributed to the fish age or size at the time of diet administration. For example, Agboola et al., (2021) showed that both *W. anomalus* and *C. jadinii* could potentially alleviate SBMIE in Atlantic salmon through an experiment conducted on fish of average initial weight of 5.71 g. Although the strain of *W. anomalus* used in this study was the same in the in study of Agboola et al., (2021), the lack of counteracting effects of *W. amomalus* in this study could also be attributed to differences in the batch-to-batch fermentation.

Similarly, in the experiment of Grammes et al., (2013), CJ showed the potential to counteract SBMIE on fish of average weight 107 g. Suggestions from Gu et al., (2015) describes that mild SBMI-inflammation in the intestines of Atlantic salmon juveniles could be attributed to immature intestinal functions compared with post-smolts, (136g) used in this current study. Possibly, the high cell division in the intestines is sufficient to subvert this enteropathy in salmon parr. Corroborating this report is Sahlmann et al., (2015), who indicated that SBM can be fed to fry of Atlantic salmon until they reach 4-5g (approx.144 days post hatching) without any adverse effects on the intestinal morphology and function.

SBMIE is not limited to seawater, however, the severity of the enteropathy is less in freshwater with similar characteristics (Sahlmann et al., 2015; Gu et al., 2016). T-cell-like response or mediation is central to SBMIE in Atlantic salmon (Bakke-McKellep et al., 2007). Their role during SBMIE in the DI of Atlantic salmon have also been documented by other authors (Lilleeng et al., 2009; Marjara et al., 2012; Sahlmann et al., 2013; Reveco-Urzua et al., 2019). T-cells are involved in different immune responses that occur in infections, cancer,

allergies and autoimmune diseases making them functionally polarized to offer disease-specific response. Gut intraepithelial lymphocytes (IELs) consist of CD3ε$^+$ T lymphocytes. This population is predominated by CD8$^+$ CD4$^-$ lymphocytes but also significant numbers of CD8$^-$ CD4$^+$ as well as CD8$^+$ CD4$^+$, therefore making their expression different from other peripheral T cell populations (Bernard et al., 2006). CD4 and CD8α are involved in T-receptor signaling and T-cell activation. CD3ε$^+$ are parental T-cells consisting of cytotoxic CD8α$^+$, and T-helper CD4$^+$ cells and may include other lymphocytes. From this study, the percentage of the simple fold, the epithelium and the lamina propria occupied by CD3ε$^+$ was not different across diets suggests that a large percentage of the lymphocyte population was CD8α$^+$ cells especially in the lamina propria. However, CD8α$^+$ cell coverage in the entire simple fold and the epithelium in the SBM fed group was significantly different from the FM and significantly different from all other diets in the lamina propria. An increase in CD3ε$^+$ cell population in the DI of Atlantic showing signs of SBMIE was reported by Lilleng et al., (2009) contrasting the results of this study. However, the higher percentage coverage of the lamina propria by the CD8α$^+$ lymphocytes in the SBM dietary group indicate a much higher activation or migration of lymphocytes in response to the enteropathy compared with the other diets similar to the findings of Lilleeng et al., (2009) who further argued that development of immune response occurs at different stages in time. The local production of immune cells may be an early response towards the SBMIE (Marjara et al., 2012) which involves the activation of resident T-cells in the mucosa with the release of cytokine mediators. This underscores the results of the histological observations that the SBMIE might be in the initial phase and for that matter the observed high within group variability.

Increase in severity may also result in the recruitment of more lymphocytes and/or migration to the site where inflammation is more severe. In higher vertebrates, cell-mediated cytotoxicity is by NK cells and T lymphocytes for innate and adaptive response respectively. While NK cells kill infected cells with peptides that are invariant, T lymphocytes recognize and kill cells with peptides from intracellular antigen synthesis with MHC I molecules (Picchietti et al., 2011) such as those presented during SBMIE.

Gut lymphocytes have been described in fish even though their involvement in immune response is largely unknown. However, Romarheim et al., (2013) argued that it was conceivable that the surveyor role of CD8α$^+$ lymphocytes in epithelium of mammals was

similar to Atlantic salmon and that stress induced by SBMIE presents a challenge to CD8α+ lymphocytes to eliminate affected epithelial cells whether it is appropriate or not. This lack of functional specificity other than the fact that they are cytotoxic makes it difficult to explain the lack of difference in the percentage population of CD8α+ in the epithelium between the SBM and the yeast supplemented diets. The main cause of the uncontrolled CD8α+ T cell-mediated gut inflammation and tissue destruction according to Westendorf et al., (2006) is linked to the recognition of intestinal enterocyte-specific antigens by CD8α+ T cells. Increase in the lamina propria CD8α+ lymphocytes is a sign of cytotoxicity in the SBM fed group. The lower percentage The CD8α+ cell population in the lamina propria of the yeast supplemented diets and FM also indicate that the yeasts had some immunostimulatory effect locally in the DI.

The confinement of T lymphocytes at the base of the mucosal fold largely in the SBM fed diet have also been documented (Lilleng et al., 2009; Reveco-Urzua et al., 2019) and in the lamina propria by (Romarheim et al., 2013) during a SBMIE in Atlantic salmon. Lilleng et al., (2009) also suggested that a limited movement of T cells into the mucosa at the initial phase of enteritis is likely and that this may be a specific response to SBM through proliferation. Consistent with the current study, the result of the immune response underscores the results of the changes in the histomorphology that the SBMIE might be in its early stages. Morphometric measurements of the selected simple folds were also consistent with the result of the histomorphology analysis. Both the fold length and area were higher in the FM fed group than the SBM which was comparable to the yeast supplemented test diets. This further enforces the fact that shortening of the villi is characteristic in a fish presenting SBMIE (van den Ingh et al., 1996; Baeverfjord & Krogdahl, 1996). The reduction in the fold area and weight is likely to reduce the absorptive capacity of the fold.

5.0 CONCLUSION

This study demonstrated a similar growth and feed conversion ratio between fish fed diets supplemented with 10% yeast and those fed the FM control. The protein digestibility was as higher for the control fed fish compared to fish fed SBM containing diets. Further, digestibility of crude lipids was higher in fish fed both variants of *C. jadinii* compared to those fed *W. anomalus* and comparable to the FM control diet. This study also demonstrated that inactivated *C. jadinii* showed the most potent effect on gut health of Atlantic salmon in terms histological changes in the cellularity of the lamina propria and submucosa. This latter proves that downstream processing plays an important role in the functionality of yeast in alleviating SBMIE. The lower $CD8\alpha^{+}$ population in the lamina propria of fish fed the yeast supplemented diets and FM control diets indicate that both versions of the yeast had some immunostimulatory effects.

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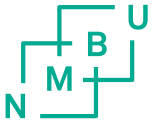

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