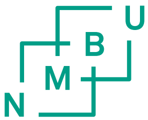

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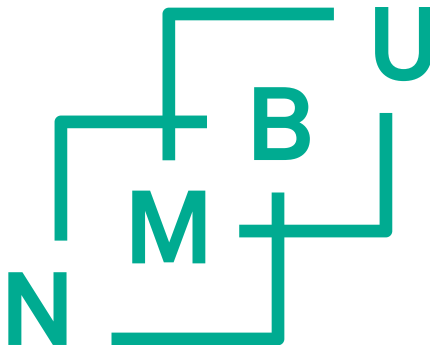

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This dissertation is dedicated to my Parents

Mrs. Agboola Silifat (late)

May Almighty Allah forgive her sins and elevate
her station among those who are guided.
Enlarge for her, her grave and shed light upon her in it.

and

Alhaji Agboola Biliaminu

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1 Abbreviations and definitions

ADCs	Apparent digestibility coefficients
AFM	Atomic force microscopy
ANFs	Anti-nutritional factors
ASVs	Amplicon sequence variants
ICJ	Inactivated *Cyberlindnera jadinii*
ACJ	Autolyzed *Cyberlindnera jadinii*
IWA	Inactivated *Wickerhamomyces anomalus*
AWA	Autolyzed *Wickerhamomyces anomalus*
IBA	Inactivated *Blastobotrys adeninivorans*
ABA	Autolyzed *Blastobotrys adeninivorans*
ICU	a reference inactivated *Cyberlindnera jadinii*
CD3	Cluster of differentiation 3
CD3ε	Cluster of differentiation 3 epsilon
CD4	Cluster of differentiation 4
CD83	Cluster of differentiation 83
CD8α	Cluster of differentiation 8 alpha
ConA	Concanavalin A
CR3	Complement receptor 3
DEGs	Differentially expressed genes
DI	Distal intestine
DSP	Down-stream processing
ELISA	Enzyme-linked immunosorbent assay
FM	Fishmeal
FCR	Feed conversion ratio

GSMMs	Genome-scale metabolic models
IFNγ	Interferon gamma
IgD	Immunoglobulin D
IgM	Immunoglobulin M
IL-10	Interleukin-10
KEGG	Kyoto encyclopedia of genes and genomes
MHCII	Major histocompatibility complex
NIRS	Near infrared spectroscopy
PERMANOVA	Permutation multivariate analysis of variance
qPCR	Quantitative polymerase chain reaction
RTgutF	Rainbow trout intestinal fibroblastic cell line
RTgutGC	Rainbow trout epithelial cell line
SBM	Soybean meal
SBMIE	Soybean meal-induced enteritis
SEM	Scanning electron microscope
SHK-1	Salmon head kidney cell line
SPC	Soy protein concentrate
SGR	Specific growth rate
TEER	Transepithelial electrical resistance
TEM	Transmission electron microscope
Three Rs	Replacement, Reduction and Refinement
TNFα	Tumor necrosis factor alpha
ZBTB46	Zinc finger and BTB domain-containing protein 46

2 List of papers

Paper I
Agboola JO, Øverland M, Skrede A, Hansen JØ. Yeast as major protein-rich ingredient in aquafeeds: a review of the implications for aquaculture production. Reviews in Aquaculture 2020:1-22. https://doi.org/10.1111/raq.12507

Paper II
Agboola JO, Schiavone M, Øverland M, Morales-Lange B, Lagos L, Arntzen MØ, Lapeña D, Eijsink VG, Horn SJ, Mydland LT, Francois JM, Mercado L, Hansen JØ. Impact of down-stream processing on functional properties of yeasts and the implications on gut health of Atlantic salmon (*Salmo salar*). Scientific Reports 2021, 11:1-14. https://doi.org/10.1038/s41598-021-83764-2

Paper III
Agboola JO, Morales-Lange B, Hansen JØ, Lagos L, Øyås O, Mercado L, Mydland LT, Øverland M. The spleen as a target to characterize immunomodulatory effects of down-stream processed *Cyberlindnera jadinii* yeasts in Atlantic salmon exposed to a dietary soybean meal challenge. Frontiers in Immunology 2021:3345. https://doi.org/10.3389/fimmu.2021.708747. Shared first author with Morales-Lange B.

Paper IV
Agboola JO, Lapeña D, Øverland M, Arntzen MØ, Mydland LT, Hansen JØ. Yeast as a novel protein source-Effect of species and autolysis on protein and amino acid digestibility in Atlantic salmon (*Salmo salar*). Aquaculture 2022, 546:1-12. https://doi.org/10.1016/j.aquaculture.2021.737312

Paper V
Agboola JO, Mensah DD, Hansen JØ, Lapeña D, Mydland LT, Arntzen MØ, Horn SJ, Øyås Ø, Press CM, Øverland M. Effects of yeast species and processing on intestinal health and transcriptomic profiles of Atlantic salmon (*Salmo salar*) fed soybean meal-based diets in seawater. International Journal of Molecular Sciences 2022, 23: 1-18. https://doi.org/10.3390/ijms23031675

Paper VI

Agboola JO, Mensah DD, Hansen JØ, Rocha SDC, Øyås Ø, Lapeña D, Mydland LT, Arntzen MØ, Horn SJ, Øverland M. Effect of yeast species and processing on intestinal microbiota of Atlantic salmon (*Salmo salar*) fed soybean meal-based diets in seawater. Submitted to Animal Microbiome

Paper VII

Agboola JO, Chikwati EM, Hansen JØ, Kortner TM, Mydland LT, Krogdahl Å, Djordjevic B, Schrama JW, Øverland M. A meta-analysis to determine factors associated with the severity of enteritis in Atlantic salmon (*Salmo salar*) fed soybean meal-based diets. Submitted to Aquaculture

3 Summary

Yeasts are gaining attention as alternative ingredients in fish feeds. The nutritional and health potentials of non-saccharomyces yeasts in fish are scarce in literature. Three non-saccharomyces yeasts; *Cyberlindnera jadinii* (CJ), *Blastobotrys adeninivorans* (BA) and *Wickerhamomyces anomalus* (WA) are the focus of this thesis. The objective of the current thesis was to investigate the nutritional values and health effects of the three selected yeasts in the diets of Atlantic salmon (*Salmo salar*) with focus on growth performance, intestinal health, gut microbiota, and immune responses of fish. The three yeasts were produced in-house using a growth medium containing a blend of enzymatic hydrolysates of pre-treated spruce wood (*Picea abies*) and chicken by-products. After harvesting, the selected yeasts were processed by direct heat-inactivation with spray-drying (ICJ, IBA and IWA) or autolyzed at 50 ºC for 16 h, followed by spray-drying (ACJ, ABA and AWA). The present thesis comprises of seven papers.

Paper I used a desk study approach to review the state-of-the art on the use of yeasts in fish feeds and identified gaps in literature regarding the use of yeasts as aquafeed ingredients. Yeasts are efficient converter of low-value non-food biomass into high-value resources. Yeasts showed comparatively similar amino acids with fishmeal (FM) and soybean meal (SBM), except for methionine, lysine, arginine, and phenylalanine which need to be supplemented when used in fish feeds. Genetic modification and/or nutrient digestibility through exogenous enzyme supplementation and the use of cost-effective down-stream processing (DSP) are possible strategies to increase the nutritive values of yeasts in fish. Additional investment in large-scale production at competitive price is needed for yeasts to be considered as feasible replacement for FM and SBM in fish feeds.

Paper II investigated the impacts of yeast species and processing on performance, immune response and gut health of Atlantic salmon fry fed SBM-based diet in freshwater. In a 37-day feeding experiment, the fish were fed one of the nine experimental diets: a FM-based diet, a challenging diet with 40% SBM and six other diets containing 40% SBM and 5% each of ICJ, ACJ, IBA, ABA, IWA and AWA yeast products. An additional control containing 40% SBM and 5% of a reference inactivated *C. jadinii* (ICU), known for its ability to counteract SBM-induced enteritis (SBMIE) was used in this experiment. *C. jadinii* and *W. anomalus* yeasts showed the most promising effects on gut health based on widening of lamina propria and

immune response parameters. The AWA was effective in ameliorating SBMIE in fish, while only limited effects were observed for other yeasts products. The ability of yeasts to counteract SBMIE is linked to the activation of immune responses in fish. The results also revealed that the amounts, length, adhesion, and accessibility of cell wall components could be important for the ameliorating effects of yeasts on SBMIE in fish.

Paper III assessed the effects of yeasts species and processing on systemic immune response of Atlantic salmon fry fed SBM-based diet in freshwater and demonstrated whether spleen can be used as a target organ to characterize immunomodulatory effects of functional ingredients in fish. The production of yeasts, experiment diets and fish experimental protocol were fully described in **Paper II**. Four experimental diets (FM, SBM, ICJ, and ACJ) were used in **Paper III.** The immunomodulatory effects of the diets were analyzed in the spleen of fish after 37 days of feeding, using a transcriptomic evaluation by RNA sequencing and protein expression of specific immunological markers through indirect ELISA. The results showed that SBM induced a down-regulation of pathways associated with ion binding and transport, along with an increase at the protein levels of pro-inflammatory cytokines TNFα and IFNγ. The inclusion of ACJ in the diet was able to control the inflammatory profile caused by SBM through activation of biological pathways related to endocytosis, along with increased protein expression of IL-10 and decreased level of TNFα. The functionality of yeasts in improving gut health of fish is dependent on the yeast species and DSP used after harvesting the yeasts. The results also showed that spleen was a good target organ to characterize the immunomodulatory effects of functional ingredients in Atlantic salmon.

Paper IV investigated the effects of yeast species and processing on nutrient digestibility of yeasts in Atlantic salmon. The production and processing of yeasts used in **Paper IV** were as described in **Paper II**. Seven experimental diets were used in paper. The control feeds consisted of 100% reference diet (REF) and six other diets comprising of 70% REF diet and 30% each of the yeast products (ICJ, ACJ, IBA, ABA, IWA and AWA). The protein and amino acids of the three yeast species were moderately digested in Atlantic salmon. Autolysis slightly increased protein digestibility of *C. jadinii* and *W. anomalus* in Atlantic salmon, but not *B. adeninivorans*. The results revealed that cell wall porosity as demonstrated by nitrogen solubility had larger impact on nutrient digestibility of yeasts than cell wall thickness. The nutrient digestibility of yeasts in Atlantic salmon is dependent on the yeast species and DSP used after harvesting the yeasts.

Based on the results of **Papers II, III and IV**, a second batch of *C. jadinii* and *W. anomalus* yeast were produced to understand the response of Atlantic salmon reared in seawater to dietary yeasts. Therefore, **Paper V** evaluated the effects of yeasts species and processing on intestinal health and transcriptomic profile from DI and spleen tissue of Atlantic salmon fed SBM-based diet in seawater. The yeasts were produced and processed following the procedure described in **Paper II**. The ICJ, ACJ, IWA and AWA yeasts products were used in this paper. Six diets were formulated, one based on FM, a challenging diet containing 30% SBM and four other diets containing 30% SBM and 10% each of the yeast products (ICJ, ACJ, IWA and AWA). The inclusion of ICJ and ACJ yeasts reduced the loss of enterocyte supranuclear vacuolization and reduced the population of CD8α positive cells in the lamina propria of fish fed SBM diets. The ICJ and ACJ yeasts controlled the inflammatory profile through up-regulation of pathways connected to wound healing and taurine metabolism. The IWA and AWA yeasts controlled the inflammatory profile in fish fed SBM through down-regulation of pathways associated with toll-like receptor signaling, C-lectin receptor and signal transduction. This paper strengthened our earlier observations (**Papers II** and **III)** that *C. jadinii* and *W. anomalus* are promising novel ingredients with health beneficial effects in terms of controlling distal intestine inflammation associated by feeding plant based diets to Atlantic salmon.

Paper VI investigated the effects of yeast species and processing on gut microbiota of fish. The yeast production and processing, experimental diets and fish experimental protocol were as described in **Paper V**. After 42 days of feeding, six fish from each tank were randomly selected to collect digesta samples from the DI for 16S rRNA sequencing. Water samples (from the source and rearing tanks) and feed samples were also collected for the sequencing analysis. The microbiota of fish fed SBM diet differed from those fed FM diet. The microbiota composition, richness and diversity were similar in fish fed ICJ, IWA and SBM diets. Fish fed ACJ increased relative abundance of *Pediococcus,* and mucin O-glycan degradation pathway, while fish fed AWA diet increased relative abundance of *Bacillaceae* compared with fish fed the other diets. Despite the significant modulation of intestinal microbiota of fish fed the autolyzed yeasts (ACJ and AWA), the histological and transcriptomic results revealed that the autolyzed yeasts did not improve gut health of fish beyond the level observed for the inactivated yeasts (ICJ and IWA) (**Paper V**). These results suggest that the ameliorating effects of yeasts on SBMIE is connected to their ability to stimulate immune responses in Atlantic salmon (**Papers II, III and V**), rather than through modulation of intestinal microbiota (**Paper VI**).

Paper VII used meta-analytic approach to determine various factors associated with severity of SBMIE in Atlantic salmon fed SBM-based diets in seawater. Dataset from 26 articles were extracted, standardized, and analyzed with ordinal logistic model by comparing the SBM treatment(s) with the neutral-reference treatment in each study. The log-odds ratio of the proportional odds model and its standard error were extracted and analyzed using the random effects model to estimate the effect size of dietary SBM on SBMIE in fish. Univariate and multivariate meta-regression, as well as subgroup analysis were used to identify study factors associated with the severity of SBMIE in fish. The results showed that the severity of enteritis in Atlantic salmon fed SBM-based diets was associated with fish production phase, feed type, SBM inclusion level, year of study and water temperature, but not the exposure time. The result of the meta-analysis agreed with our observations in **Papers II and V** that the severity of SBMIE has declined over the years in fish and questioned the validity/sensitivity of using SBMIE as a dietary challenge to investigate the effects of functional ingredients in fish. Furthermore, the regression analysis showed that increased severity of enteritis reduced growth performance of fish fed SBM-based diets.

4 Norsk sammendrag

Gjær får oppmerksomhet som en alternativ ingrediens i fiskefôr. I litteraturen finnes det lite informasjon om de ernærings- og helsemessige egenskapene til ikke-saccharomyces gjær i fôr til fisk. Denne avhandlingen fokuserer på tre ikke-saccharomyces gjærsorter; *Cyberlindnera jadinii* (CJ), *Blastobotrys adeninivorans* (BA) og *Wickerhamomyces anomalus* (WA). De tre gjærsortene ble produsert ved NMBU ved hjelp av et vekstmedium basert på en blanding av enzymatiske hydrolysater av sukker fra grantre (*Picea abies*) og biprodukter fra kylling. Etter høsting, ble de tre gjærsortene direkte varmeinaktivert ved spraytørking (ICJ, IBA og IWA) eller ved autolyse ved 50 ºC i 16 timer, etterfulgt av spraytørking (ACJ, ABA og AWA). Formålet med denne avhandlingen var å undersøke næringsverdiene og helseeffektene av de tre utvalgte gjærsortene i fôr til atlantisk laks med fokus på vekst, tarmhelse, tarmmikrobiota og immunrespons hos fisk. Resultatene fra dette arbeidet er beskrevet i syv artikler.

Artikkel I er et litteraturstudie som gjennomgår det siste innen bruk av gjær i fiskefôr og identifiserte hull i litteraturen ved bruk av gjær som ingrediens i fiskefôr. Gjær kan på en effektiv måte omdanne lav-verdig biomasse som ikke er egnet til menneskemat til høyverdige ressurser. Gjærsortene hadde relativt lik aminosyresammensetning som fiskemel (FM) og soyamel (SBM), bortsett fra metionin, lysin, arginin og fenylalanin som må suppleres ved bruk av gjær i fiskefôr. Genmodifisering og/eller bruk av eksogene enzymer og kostnadseffektiv nedstrømsbehandling (DSP) er mulige strategier for å øke fordøyelighet av næringsstoffene i gjær i fôr til fisk. Ytterligere investeringer i storskala produksjon til konkurransedyktig pris er nødvendig for at gjær skal betraktes som en alternativ fôringrediens til FM og SBM i fiskefôr.

Artikkel II undersøkte effekten av gjærsort og prosessering på tilvekst, immunrespons og tarmhelse hos atlantisk lakseyngel fôret med SBM-basert dietter i ferskvann. I et 37-dagers fôringsforsøk ble atlantisk lakseyngel fôret med et av de ni forsøksfôrene: et FM-basert fôr, et utfordrende fôr med 40% SBM og seks andre fôrblandinger som inneholdt 40% SBM og 5% av hver av gjærproduktene ICJ, ACJ, IBA, ABA, IWA og AWA. En ekstra kontroll med 40% SBM og 5% av en inaktivert *C. jadinii* (ICU) referanse, kjent for sin evne til å motvirke SBM-indusert enteritt (SBMIE) ble også brukt i dette forsøket. Gjærsortene *C. jadinii* og *W. anomalus* ga den mest lovende effekt på tarmhelsen basert på utvidelse av lamina propria og

immunresponsparametere. AWA var effektiv i å motvirke SBMIE hos fisk, mens de andre gjærproduktene hadde begrensede effekter. Gjærens evne til å motvirke SBMIE er knyttet til aktivering av immunresponser hos fisk. Resultatene viste også at mengden, lengden, adhesjonen og tilgjengeligheten av celleveggskomponenter kunne være viktig for de positive effektene av gjær på SBMIE hos fisk.

Artikkel III undersøkte effekten av gjærsort (beskrevet i **Artikkel II**) og bearbeiding på systemisk immunrespons i atlantisk lakseyngel fôret med SBM-basert dietter i ferskvann. Fire av de eksperimentelle diettene fra **Artikkel II** ble brukt (FM, SBM, ICJ og ACJ). Resultatene viser at milten kan brukes som et målorgan for å karakterisere immunmodulerende effekter av funksjonelle ingredienser hos fisk. De immunmodulerende effektene av diettene ble analysert i milten hos fisk etter 37 dagers fôring, ved å evaluere gen- og og proteinuttrykk av spesifikke immunologiske markører gjennom h.h.v. RNA sekvensering og indirekte ELISA. Resultatene viste at SBM førte til nedregulering av reaksjonsveier forbundet med ionebinding og transport, og en økning på proteinnivå av de proinflammatoriske cytokinene TNFα og IFNγ. Tilskudd av ACJ i fôret førte til kontroll av den inflammatoriske profilen forårsaket av SBM gjennom aktivering av biologiske reaksjonsveier relatert til endosytose, sammen med økt proteinuttrykk av IL-10 og redusert nivå av TNFα. Funksjonaliteten til gjær med hensyn til forbedring av tarmhelsen til fisk er avhengig av gjærsort og DSP som brukes etter høsting av gjæren.

Artikkel IV undersøkte effekten av gjærsort (beskrevet i Artikkel II) og bearbeiding på fordøyelighet av næringstoffene i gjær hos atlantisk laks. Syv forsøksfôr ble brukt i artikkelen, hvor kontrollfôret besto av en 100% referansediett (REF) og de seks andre diettene besto av 70% REF-diett og 30% av hver gjærsort (ICJ, ACJ, IBA, ABA, IWA og AWA). Protein- og aminosyrene til de tre gjærsortene hadde en moderat fordøyelighet hos atlantisk laks. Autolyse økte proteinfordøyeligheten av *C. jadinii* og *W. anomalus* i atlantisk laks, men ikke *B. adeninivorans*. Resultatene viste at porøsiteten av celleveggen, målt som nitrogenløselighet, hadde større innvirkning på fordøyelighet av næringsstoffer fra gjær enn celleveggtykkelsen. Fordøyeligheten av næringsstoffer fra gjær i atlantisk laks er avhengig av gjærsort og DSP som brukes etter høsting av gjær.

Basert på resultatene fra **Artikkel II, III** og **IV**, ble et annet parti med *C. jadinii* og *W. anomalus* gjær produsert for å forstå responsen av å fôre gjær på tarmhelse hos atlantisk laks i sjøvann. **Artikkel V** evaluerer effekten av gjærsort og prosessering på tarmhelse og transkripsjonsprofil i baktarm (DI) og miltvev fra atlantisk laks fôret med SBM-baserte dietter i sjøvann. Gjærsortene ble produsert og behandlet etter protokollen beskrevet i **Artikkel II**. Seks fôrblandinger ble formulert, en basert på

FM, en utfordrende diett som inneholdt 30% SBM og fire andre dietter som inneholdt 30% SBM og 10% hver av gjærsortene (ICJ, ACJ, IWA og AWA). Inkluderingen av ICJ og ACJ gjær reduserte tapet av enterocytt supranukleær vakuolisering og reduserte mengden av CD8α positive celler i lamina propria hos fisk fôret med SBM-baserte dietter. ICJ- og ACJ-gjær regulerte den inflammatoriske profilen gjennom oppregulering av reaksjonsveier knyttet til sårheling og taurin metabolisme. IWA- og AWA-gjær regulerte den inflammatoriske profilen i fisk fôret med SBM gjennom nedregulering av reaksjonsveier forbundet med toll-lignende reseptorsignalering, C-lektinreseptor og signaltransduksjon. Disse resultatene styrket tidligere observasjoner (**Artikkel II og III**) om at *C. jadinii* og *W. anomalus* er lovende nye ingredienser med helsemessige gunstige effekter når det gjelder å kontrollere betennelse forbundet med fôring av plantebaserte dietter til atlantisk laks.

Artikkel VI undersøkte effekten av gjærsort (beskrevet i **Artikkel V**) og bearbeiding på tarmmikrobiota hos fisk. Etter 42 dagers fôring ble gjødselprøver fra DI tatt fra seks tilfeldige fisker per tank for 16S rRNA-sekvensering, samt vannprøver (fra ras-vann og oppdrettstanker) og fôrprøver. Mikrobiotasammensetningen hos fisk fôret med SBM dietten var forskjellig fra de som ble fôret med FM dietten. Mikrobiotasammensetningen, antall og mangfoldet var lignende mellom fisk gitt ICJ, IWA og SBM. Fisk fôret med ACJ hadde økte relativ mengde av *Pediococcus*, og mucin O-glykan nedbrytningsveien, mens fisk fôret med AWA dietten hadde økt relativ mengde av *Bacillaceae* sammenlignet med fisk fôret med de andre diettene. Til tross for den signifikante moduleringen av tarmmikrobiota av fisk fôret med de autolyserte gjærsortene (ACJ og AWA), viste de histologiske og transkripsjonsresultatene at de autolyserte gjærsortene ikke forbedret tarmhelsen til fisk utover nivået som ble observert for de inaktiverte gjærsortene (ICJ og IWA) (**Artikkel V**). Disse resultatene tyder på at de positive effektene av gjær på SBMIE er knyttet til deres evne til å stimulere immunresponser hos atlantisk laks (**Artikkel II, III og V**), i stedet for gjennom modulering av tarmmikrobiota (**Artikkel VI**).

Artikkel VII brukte meta-analysetilnærming for å kartlegge ulike faktorer knyttet til alvorlighetsgraden av SBMIE hos atlantisk laks fôret med SBM-baserte dietter i sjøvann. Et datasett fra 26 fiskeforsøk ble hentet ut, standardisert og analysert med en ordinal logistisk modell, hvor SBM-behandlingen(e) sammenlignes med en nøytral referansebehandling i hvert studie. Log-odds-forholdet mellom den proporsjonale oddsmodellen og standardfeilen ble hentet ut og analysert ved hjelp av «tilfeldige effekter» modellen for å estimere effektstørrelsen av SBM i fôret på SBMIE hos fisk. Univariat og multivariat meta-regresjon, samt undergruppeanalyse ble brukt til å identifisere studerte faktorer knyttet til alvorlighetsgraden av SBMIE hos fisk.

Resultatene viste at alvorlighetsgraden av enteritt hos atlantisk laks fôret med SBM-baserte dietter var forbundet med fiskeproduksjonsfase, fôrtype, inkluderingsnivå av SBM, studieår og vanntemperatur, men ikke eksponeringstiden. Resultatet av meta-analysen var i samsvar med våre observasjoner i **Artikkel II og V** om at alvorlighetsgraden av SBMIE har blitt redusert gjennom årene i fisk og stiller spørsmål ved egnetheten/følsomheten ved bruk av SBMIE som modell for å undersøke effekten av funksjonelle ingredienser på helse i fisk. Videre viste regresjonsanalysen at økt alvorlighetsgrad av enteritt reduserte vekstytelsen til fisk fôret med SBM-baserte dietter.

5 Synopsis

5.1 Introduction

Aquaculture is important for global food supply and is pivotal in addressing malnutrition, hidden hunger, and poverty around the world [1]. Aquaculture is the fastest growing food production sector in the world, with an annual growth rate of 5.3% since 2010 [2]. Fish contributes high-quality proteins, poly-unsaturated fatty acids, and micro-nutrients to dietary intake for people [2]. At present, fish accounts for about 7% of all proteins, and 17% of the total animal protein intake in the world [2]. The continuous growth of aquaculture is necessary to meet the future demand for protein and other essential nutrients of a growing human population. However, the growth in the aquaculture sector is challenged by limited supply of sustainable high protein feed resources. Traditionally, fishmeal and fish oil have been the gold-standard ingredients for commercial farming of carnivorous fish species [3]. However, the stagnation in the forage fish stocks, high market prices and sustainability concerns indicate that high inclusion of fishmeal and fish oil in aquafeeds is no longer sustainable [3].

In recent years, there has been a reduction in the use of marine ingredients as more plant ingredients are used in salmon feeds [4, 5]. Among the available plant resources, soybean meal (SBM) is attractive for feed production due to its high protein content, its availability, and its competitive market prices [6]. The use of SBM is, however, limited in salmon feeds due to the presence of various anti-nutritional factors (ANFs) [7], food-feed competition [4] and environmental concerns [8]. A multitude of studies have demonstrated that SBM inclusion in the diets can induce enteritis in the distal intestine (DI) of Atlantic salmon, a condition widely known as SBM-induced enteritis (SBMIE) [9-15]. Alcohol soluble fractions of SBM (especially saponin) have been implicated as the etiological agents of SBMIE in fish, but there are indications that the presence of other ANFs (such as protease inhibitors, trypsin inhibitors and lectins) amplified the severity of the enteritis [15-19]. To overcome these challenges, a more refined soy-product known as soy protein concentrate (SPC) with low level of ANFs is currently being used in commercial salmon farming. However, the use of SPC also raises serious ethical and sustainability concerns such as increased pressure on land, water, and energy use, as well as competition for human food [8, 20]. In addition, a recent study has revealed that DI inflammation is

still frequently observed in commercial salmon production in Norway [21]. In addition, the Norwegian government has stated that all fish feed resources shall come from sustainable sources by 2030 due to sustainability concerns and is driven by consumer perception [22]. Therefore, there is emerging needs for sustainable alternative ingredients in fish feeds.

Microbial ingredients such as yeasts, bacteria and microalgae are gaining increasing attention as promising ingredients for aquaculture [23, 24]. Yeasts are considered potential ingredients because of their nutritional contents [23], low environmental footprint [25], ability to convert low-value resources into high-value nutrients [26, 27] and their functional properties in fish [28, 29]. Yeasts have high protein content (about 40-50%) and contain other bioactive components beneficial to fish health and robustness [23, 24]. The cell wall represents about 26-32% of the yeast dry weight and contains mannan-oligosaccharides, β-glucan, and chitin [30, 31]. Over the years, extensive research [32-35] and reviews [28, 29] have elucidated the nutritional and functional values of cell wall components derived from *Saccharomyces cerevisiae* in various fish species. However, there are limited information on the inclusion of yeasts as major protein ingredients in fish feeds. Furthermore, research on the use of yeast as fish feed ingredients have focused on *S. cerevisiae*, and limited information exists on the potential of other yeast species in aquaculture.

Three different non-saccharomyces yeast species; *Cyberlindnera jadinii*, *Blastobotrys adeninivorans* and *Wickerhamomyces anomalus* have been the focus in this thesis. These yeasts were selected based on their ability to utilize media containing blend of hydrolysates from spruce trees and chicken by-products, their high growth rate, and their high protein content, as well as their low production of side products, such as alcohol [26, 27]. In addition, inactivated forms of *C. jadinii* and *W. anomalus* as approved by the European Commission can be incorporated in animal feed (Regulation No 68/2013). Despite their authorization, the production volumes of yeasts are still too small to guarantee consistent use in fish feeds. As a result, yeasts are currently not price competitive compared with conventional aquafeed ingredients (such as fishmeal (FM) and SBM).

One approach to increase the premium on yeasts is to document their functional values, beyond their nutritional values in fish. To achieve this, it is imperative to standardize the down-stream processing (DSP) used after harvesting the yeasts, in order to optimize their use and guarantee reproducible effects in fish. Øverland and Skrede [24] stated that the choice of DSP is crucial to preserve valuable nutrients and bioactive components present in the yeasts. Hitherto, different DSPs such as chemical,

enzymatic, physical, and mechanical have been applied to increase the nutritional and functional values of yeasts for various industrial application [36-38]. A recent study showed that different DSP methods have varying impacts on nutrient digestibility of yeasts [36]. Autolysis and mechanical homogenization increased the protein digestibility of *S. cerevisiae* in Atlantic salmon by 60% and 45%, respectively [36]. While DSP methods may increase the digestibility of nutrients in the yeasts, it is imperative that the methods are benign and do not negatively alter the bioactive components present in the yeasts. Additionally, cost-effectiveness, scalability and ease of commercialization are factors to be considered when selecting DSP methods for yeast production. Considering these factors, autolysis was selected as the DSP method in the present thesis. Although the impact of autolysis on nutrient digestibility of *S. cerevisiae* in fish is documented in literature [36], it is uncertain how it modifies the nutritional and functional values of other yeast species in fish. Understanding how autolysis influences the immune response, gut health and microbiota composition of fish deserves attention and may be an important step towards the commercialization of the three selected yeast species as fish feed ingredients.

In the current thesis, it was hypothesized that the selected yeasts can be included in the diets to improve the nutritional and health of Atlantic salmon and that the degree of success depends on the types of yeast species and DSP method used after harvesting the yeasts. The present work aims to reveal possible nutritional and health effects of including yeasts in the diets of Atlantic salmon with focus on performance, intestinal health, gut microbiota, and immune responses of fish. To achieve these, the present work consists of seven papers. In **Paper I**, we used a desk study approach to review the state-of-the-art on the use of yeasts in fish feeds and identified gaps in literature regarding the use of yeasts as aquafeed ingredients. In **Paper II**, we investigated the impacts of yeast species and processing on performance, immune response and gut health of Atlantic salmon fed SBM-based diets in freshwater, while **Paper III** focused on the effects of yeast species and processing on systemic immune response of Atlantic salmon fed SBM-based diets in freshwater and demonstrated whether spleen can be used as a target organ to characterize the immunomodulatory effects of functional ingredients in Atlantic salmon. In **Paper IV**, we investigated the effects of yeast species and processing on nutrient digestibility of yeasts in Atlantic salmon and also understood whether protein digestibility of yeasts in Atlantic salmon were influenced by nitrogen solubility and cell wall thickness of yeasts, as well as digesta viscosity and dry matter.

Based on the results of **Papers II, III and IV**, *C. jadinii* and *W. anomalus* were selected to understand the response of Atlantic salmon reared in seawater to dietary yeasts. Therefore, in **Paper V**, we studied the effects of yeast species and processing on intestinal health and transcriptomic profile from DI and spleen tissues of Atlantic salmon fed SBM-based diets in seawater, while **Paper VI** evaluated the effects of yeast species and processing on gut microbiota of fish. A number of recent studies revealed that fish fed SBM developed mild to moderate enteritis compared to those fed SBM in early years, suggesting that fish are developing tolerance to dietary SBM. To investigate this hypothesis, a meta-analysis was conducted in **Paper VII** to determine various factors associated with the severity of enteritis in Atlantic salmon fed SBM-based diets.

5.2 Background

5.2.1 Production of yeast as fish feeds: from low-value to high-value resources

Yeasts are efficient bio-converter of low-value non-food biomass into high value protein resources. Yeasts are capable of building up all the amino acids necessary for protein synthesis using the carbon chain derived from metabolites from intermediate metabolic pathways and the amino groups from aminization or transamination process [39]. Molasses is used as the primary sugar source in the production of yeasts. Previously, molasses derived from industrial production of sugar cane [40], sugar beet [41], fruits [42] and whey [43] have been used in the production of yeasts. However, the increased price and the use of molasses in other industrial processes [44] imply the needs for new substrate sources for yeast production. In terms of environmental and social sustainability, the non-food resources such as lignocellulosic biomass are gaining increasing interest as new substrate sources for yeast production [26, 27]. Figure 1 shows the schematic for yeast production using sugars from lignocellulosic biomass and nitrogen substrate from animal or fish-by-products. Lignocellulosic biomass contains highly complex polysaccharides and thus needs to be broken down into fermentable sugars before being used in yeast fermentation.

The breaking down of lignocellulosic biomass into fermentable sugars involves two processing steps: pre-treatment and enzymatic hydrolysis (Figure 1). The pre-treatment of lignocellulosic biomass is necessary to achieve optimal enzymatic hydrolysis of the different polysaccharides to fermentable sugars, which can then be

used for yeast production [45]. The pre-treatment methods commonly used for delignifying the lignocellulosic biomass are; physical (e.g. chipping), physico-chemical (e.g. steam explosion), chemical (e.g. acid or base) or biological (e.g. enzymes or fungi) [45]. For in-depth understanding of the different pre-treatment methods and their suitability for the lignocellulosic biomass under consideration, see the reviews of Mosier et al. [46], Chandra et al. [47] and Van Dyk and Pletschke [48]. To obtain enriched nitrogen substrate for yeast fermentation, animal by-products undergo five major processing steps: grinding, enzymatic hydrolysis, heat treatment, separation, and filtration (Figure 1). The process generates side-streams such as oil, solids, retentate and permeate, which can be used in the production of biodiesel, poly-unsaturated fatty acids, biogas, and protein concentrate (Figure 1).

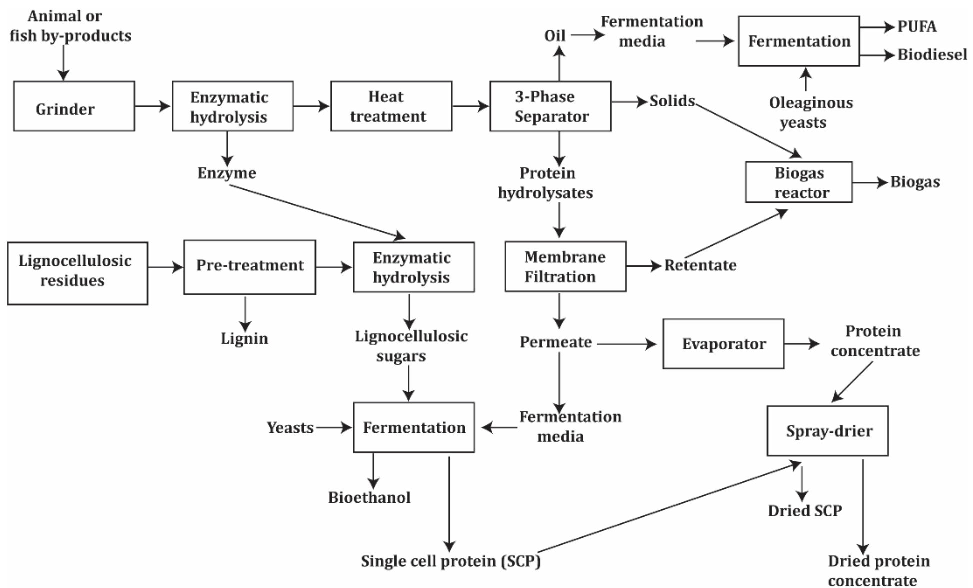

Figure 1. The schematic for yeast production using sugars from lignocellulosic biomass and nitrogen substrate from animal by-products. Illustration adapted from Lapena [45].

After the treatment of lignocellulosic biomass and the animal by-products, the sugars and the enriched nitrogen substrate are combined in a blend and used for yeast fermentation. The fermentation is carried out in a fermenter, a controlled, aseptic environment that can be used for yeast production for a prolonged period of time. A fermenter should provide a system for dissolved oxygen, temperature, and pH control, as these are crucial for efficient yeast production [45]. The efficiency of a

fermenter depends on the fermentation strategies used during yeast production. The fermentation strategies can be categorized into: batch/fed batch, continuous, and repeated fed-batch fermentations [45]. In-depth description of the three different strategies and their applicability to small or large scale yeast production can be found in Lapena [45]. Briefly, in batch, yeast inoculum is cultured under a fixed starting substrate concentration and the fermentation will come to an end after depletion of one of the important components of the medium. In fed-batch, the fermentation is initially started in batch mode, after which the fermenter is continuously or sequentially, fed with fresh medium without removal of the culture. The continuous fermentation is a process in which the media is constantly fed into the fermenter and the culture containing the microbial biomass are continuously removed. The continuous fermentation enables stable process conditions and high biomass yield compared with batch and fed-batch fermentation. Repeated fed-batch combines features of batch/fed batch fermentation with continuous fermentation. Regardless of the fermentation strategy used, the yeast culture obtained at the end of the fermentation process should be washed, centrifuged, and separated to obtain yeast cream which can then undergo further DSP (such as spray drying) before being incorporated in fish feeds.

5.2.2 Down-stream processing of yeasts: Why is it important?

The choice of DSP methods is important to optimize the use of yeasts in future fish feeds. Øverland and Skrede [24] reported that DSP is crucial to preserve the nutritional and functional values of yeasts as aquafeed ingredients. After harvesting, separated yeast cream can be dried to obtain a meal that can be used in fish feeds. Methods such as spray-drying, drum-drying, oven-drying, and freeze-drying can be used to dry the yeast cream. Each drying method has its advantages and disadvantages, and selection of an optimal drying method should be based on the requirements of the final products. An in-depth discussion of the different drying methods is beyond the scope of this thesis, and further information on this topic can be found in this review [49]. Scalability, cost-effectiveness, and the effects on nutritional quality of yeasts are important factors to consider when selecting the drying process. The drying method and temperature can influence the nutritional quality of yeasts. In a recent study, Hansen et al. [36] observed that spray-drying with inlet temperature of 250 ºC reduced the protein digestibility of *S. cerevisiae* compared with spray-drying at 180 ºC. The thermal process can have both beneficial and detrimental effects on the nutritional quality of meal produced after the drying

process. The drying of yeasts causes denaturation [39], which changes the structure of proteins and increases their digestibility and utilization in animals [50]. Studies have demonstrated that thermal treatment can increase the susceptibility of protein to proteolytic enzymes [51, 52], and inactivates the ANFs present in the ingredients [50]. Thermal treatment can also reduce the pathogenic burden present in feed ingredients [50, 53].

Conversely, heat treatment especially at high temperature promotes protein aggregation [51, 54, 55] and cross-linking [54, 56] between two amino acids, which could decrease protein digestibility of an ingredient. In addition, during heat treatment, the amino group of protein present in an ingredient can react with reducing sugars to produce Maillard reaction products, with negative consequence on digestibility and utilization in animals [50, 52, 57]. The formation of Maillard reaction products can be divided into early, advanced, and final stages (Figure 2). The wide range of products formed due to Maillard reaction and their effects on nutritional quality of ingredients has been extensively reviewed elsewhere [52, 53, 57]. Although, the impacts of thermal treatment on formation of Maillard reaction products in yeast is not reported in literature, however, previous study has demonstrated that drum-drying leads to formation of different Maillard reaction products such as furosine, carboxymethyllyisne and carboxethyllysine in *Nannochloropsis gaditana* microalgae biomass, with negative consequences on nutrient digestibility of the microalgae biomass in fish [58].

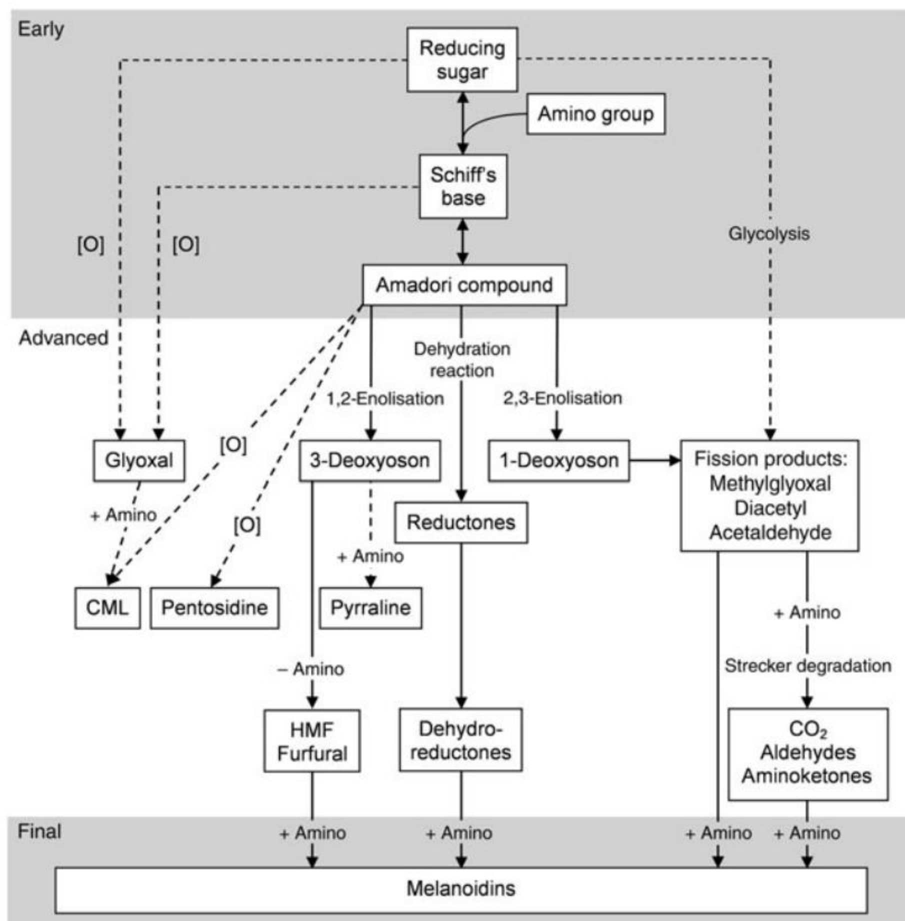

Figure 2. Schematic showing the steps involved in Maillard reaction. [O], oxidation; CML, N^{ε}-(carboxymethyl)lysine; HMF, hydroxymethylfurfural. Figure taken from van Rooijen et al. [57] with the authors' permission.

High nucleic acid content can limit the use of yeasts as feed ingredients [59]. The nucleic acid contains purine compounds, which can be metabolized into uric acid whose high concentration can lead to gout or renal stones in human [59]. The level of nucleic acid in the yeasts is influenced by the fermentation condition, growth rate and carbon/nitrogen ratio of the medium [39]. Methods such as chemical treatment using alkaline extraction at high temperature or enzymatic treatments can be used to reduce the amount of nucleic acid present in the yeasts [60]. This method can increase protein yield but may cause the formation of secondary compounds such as lysinoalanine, which may reduce the quality of protein in yeasts [60]. Alternatively,

the removal of nucleic acid in yeasts can be carried out using endogenous or exogenous RNA degrading enzymes (ribonucleases) [61].

Another limitation to the use of yeasts in fish feed is their rigid cell wall, which prevents accessibility of intracellular nutrients of yeasts to digestive enzymes produced by fish [38, 39, 62, 63]. Effective cell wall disruption techniques are technical strategies to improve digestibility and utilization of nutrients from yeasts in fish. Previous studies have demonstrated that partial or complete disruption of yeast cell wall increases protein digestibility and nutritional values of yeasts in rainbow trout [38], Atlantic salmon [36], shrimp [64] and Arctic charr [65]. Chemical, enzymatic, physical, and mechanical methods can be used to rupture the cell wall of yeasts [59, 66-68]. Chemical disruption entails the use of acidic or alkaline treatments or a combination of both methods [31]. Mechanical cell wall disruption can be achieved through the use of mechanical forces such as solid-shear forces (e.g., bead milling, high speed homogenization), liquid-shear forces (e.g., high pressure homogenization, microfluidization), transfer of energy through waves (e.g., ultrasonication, microwave), currents (e.g., pulse electric field) or heat (e.g., thermolysis, autoclaving) [66-68]. Enzymatic disruption of yeast cell wall can be performed either by the use of endogenous enzymes (autolysis) [27, 36] or exogenous enzymes [27, 31]. Cost-effectiveness, the thickness of the cell wall and intended use of the final products are important factors to consider when selecting the cell wall disruption methods. Among these methods, autolysis is the focus of this thesis and is further addressed below.

Autolysis is a slow process in which endogenous enzymes break down the intracellular components of yeast cell [39, 59, 69, 70]. It is a phenomenon in which the endogenous enzymes such as proteases, β-glucanases and chitinases are activated at low or high temperature to break the yeast components [69, 70]. The autolytic process can influence the ultrastructure and biophysical properties of yeasts, and consequently, determines the nutritional and functional values of yeasts in fish. Electron microscopic images of yeast after the autolysis process revealed a decrease in the cell wall thickness and disorganization of the intracellular components of yeasts [70]. The reduction in cell wall thickness agrees with the results of previous work [36], which showed that protein digestibility of yeast in Atlantic salmon increases with the autolytic process. Autolysis also modifies the adhesive properties of mannoprotein present on the yeast cell wall, and can have implication on the health stimulating properties of this cell wall constituent when used in fish diets [31]. Autolysis is also used in commercial production of yeast autolysates and yeast extracts for various industrial purposes [39]. The production of autolysates and yeast

extracts involves separation of the cell wall from cell contents and can increase the quality of the final products [39]. Previous studies have revealed that protein digestibility was higher in extracted yeast compared with intact yeasts in fish [38, 65]. The autolysis process is cost-effective, less energy demanding and can be used for large scale production of yeasts compared with mechanical disruption methods.

5.2.3 Nutritional and bioactive components of yeast

The nutritional values of yeasts

Yeasts can be used as major protein ingredients in fish feeds. The protein content of yeasts ranged from 40-50% [23, 24, 71]. The amino acid composition of yeast depends on yeast species, yeast strain, growth media, fermentation conditions and DSP used after harvesting the yeast biomass [71]. The amino acid compositions of yeasts are comparable with conventional SBM, except for sulphur-containing amino acids such as methionine and cysteine which are limiting in yeasts [24, 71]. However, methionine-rich yeasts can be produced through genetic engineering of mutants producing higher levels of methionine [39]. Yeast protein contains high amounts of non-protein nitrogen in the form of nucleic acids [39, 72]. The nucleic acid can account for about 10-30% of yeast protein depending on yeast species, growth media and the growth rate of yeasts [39]. Contrary to other animals, salmonids can metabolize high levels of nucleic acids due to their ability to synthetize considerable level of urate oxidase [72-74]. The nucleic acids are semi-essential especially during early life stages of fish and can contribute to added benefits of yeasts. Thus, additional DSP method to remove the nucleic acid may not be necessary when using yeasts in the salmonid diets. In addition, nucleic acid may have protein sparing effects [24], with the possibility of partitioning protein away from non-protein metabolic process (e.g., gluconeogenesis).

Yeasts contains low level of lipids, except for oleaginous yeasts which are able to accumulate high amounts of lipids in their cells [75-77]. *Yarrowia lipolytica* yeast contain up to 20% lipid and has been used to replace fish and plant oils in fish diets without compromising growth performance of fish [76, 77]. The main value of lipid in oleaginous yeasts lies in their specific fatty acid profile rather than their total lipid content [23]. Oleaginous yeasts are able to synthesize long-chain omega-3 poly-unsaturated fatty acids [78, 79], which are essential for fish growth and fillet quality. Aside from protein and lipids, vitamins and minerals are moderately present in yeasts [39]. The mineral content of the yeasts is determined by the amount of corresponding mineral in the fermentation media [39]. When grown on selenium-rich media, yeasts

are able to incorporate high level of selenium into their cells and is the mechanism behind production of selenium-rich yeasts, a specialty yeasts used to increase antioxidant status of animals [80-82]. Additional information on the nutritional values of yeasts and their potential to meet the nutrient requirements of Atlantic salmon and rainbow is further discussed in **Paper I** of this thesis.

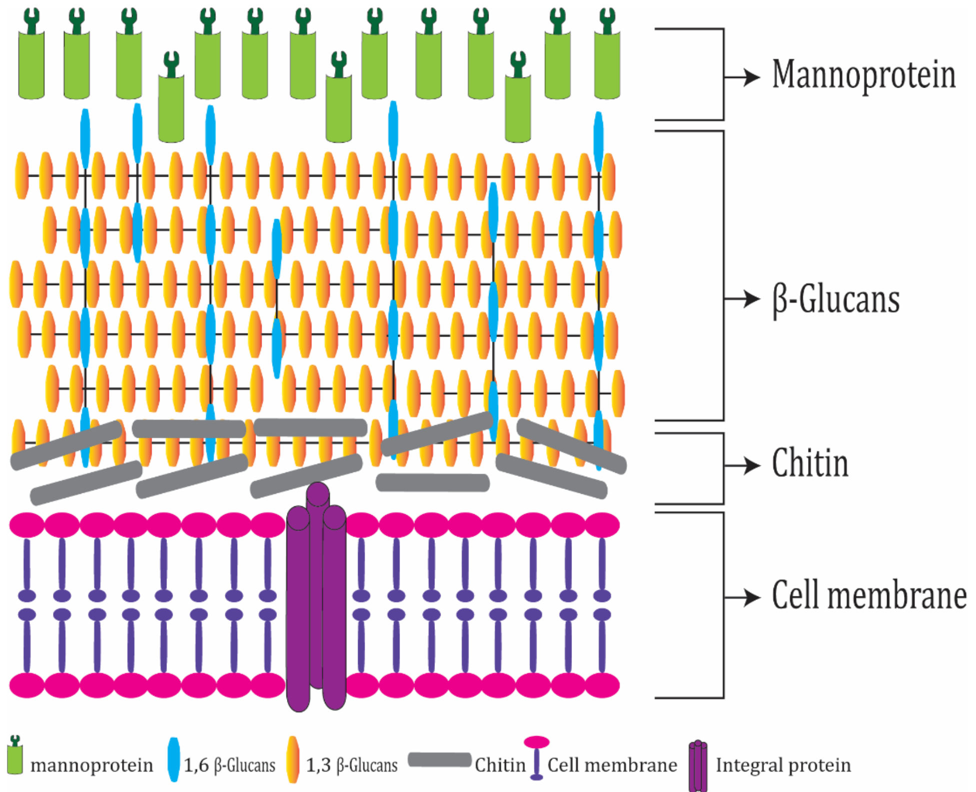

Figure 3. Bioactive components of yeast cell wall. Figure inspired by Anwar et al. [83].

The cell wall components: functional values of yeasts

The cell wall is made up about 26-32% dry weight of the yeast [30]. The yeast cell wall contains about 85-90% polysaccharides and 10-15% protein [31]. Glucans, mannans and chitin are the main polysaccharides present in the yeast cell wall (Figure 3). The cell wall polysaccharides of yeasts principally comprised of 30-60% glucans, 25-50% mannans and 5-10% chitin [31]. The compositions of yeast cell wall can vary depending on growth conditions, time of harvesting, as well as species and strains of yeasts [30, 84]. The glucan which is responsible for the shape and mechanical strength of the cell wall contains highly branched polysaccharides with β-

1,3 and β-1,6 linked glucose residues [30, 39]. The *S. cerevisiae* yeast is used for producing high quality β-glucans for many therapeutic and industrial applications [85]. The β-glucan is widely used as immunostimulant in aquaculture and plays an important role in activation of both innate and immune functions [29]. The β-glucans have been used in a multitude of studies to stimulate health functions in fish and protect them against multi-stressor conditions (see review of Meena et al. [29]). Glucans are believed to elicit their immune functions by binding to surface receptor (e.g., complement receptor (CR3) and dectin-1 receptor) present on the surface of several immune cells such as macrophages, neutrophils, natural killer cells, and dendritic cells [86, 87]. For instance, β-glucans binds to the dectin-1 receptor to activate the macrophages and subsequently leads to the production of inflammatory cytokines and stimulation of phagocytosis for microbial killing (Figure 4).

The mannans are highly branched polymer of mannose with α-1,6 main chain, as well as α-1,4 and α-1,3 linked chains [39]. The mannan polysaccharides are in complex with proteins and are generally known as mannoprotein [30]. The *S. cerevisiae* yeasts have been used in commercial production of mannan-oligosaccharides for different industrial applications [88]. Mannan-oligosaccharides from yeasts elicit relevant health effects, such as inhibition of pathogen adherence, modulation of gut microbiota and improvement of immune response in various fish species (see review of Torrecillas et al. [28]). The mannan-oligosaccharides bind to the lectin-type receptor to prevent adhesion of enteropathogenic bacteria to the intestinal villi [89]. Chitin is a polymer of β-1,4 linked N-acetyl glucosamine [39], and is predominately present around the bud scars [30]. Chitin is present in relatively small amount (5-10% of the cell wall) in yeast [30]. The role of yeast chitin on performance and health of fish is less studied in literature, however, there are suggestions that chitin can have both detrimental and beneficial effects in fish. High inclusion of chitin may interfere with digestibility and utilization of nutrients in the diets. Conversely, chitin can serve as substrate for microbial growth, which may confer beneficial effects in fish. Therefore, the role of yeast chitin in fish needs to be investigated in future studies.

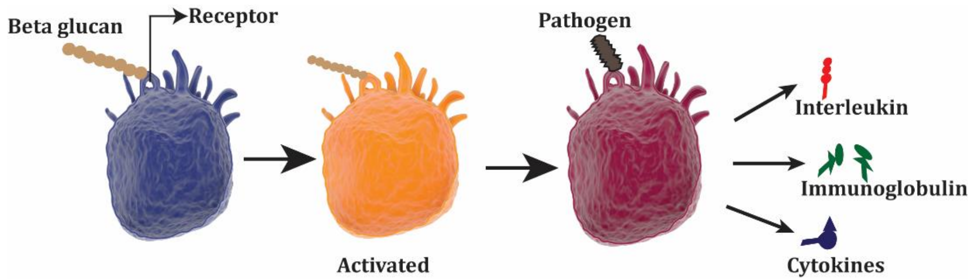

Figure 4. β-glucan activation of the immune system. β-glucan activates the macrophage by binding to the dectin-1 surface receptor. Figure inspired by Bell et al. [90].

The biophysical properties of yeast cell wall components

The biophysical properties of yeast cell wall components can be determined using Atomic Force Microscope (AFM). The AFM technique is based on the measure of interaction force between the AFM tip and the sample [91]. The AFM can be operated in two different modes i.e., single force spectroscopy and single molecule force spectroscopy modes (Figure 5). The single force spectroscopy mode is done by scanning the tip over the sample and recording the force versus distance curves created as a result of this interaction (mode 1 in Figure 5). The force versus distance curves consist of a non-contact and a deformation component, with an indentation which represents the nanomechanical properties of the yeast cell wall [91]. The elasticity (i.e., the Young modulus), stiffness and roughness of the yeast cell wall can be determined using the single force spectroscopy mode [92-94]. Schiavone et al. [93] has demonstrated that the nanomechanical properties of yeast cell walls are influenced by yeast strain and the autolytic treatment. Understanding the nanomechanical properties of yeast cell walls and how they are influenced by the different cell disruption treatments, may give insights to strategies that can be used to optimize the digestibility and utilization of nutrients from yeasts in fish.

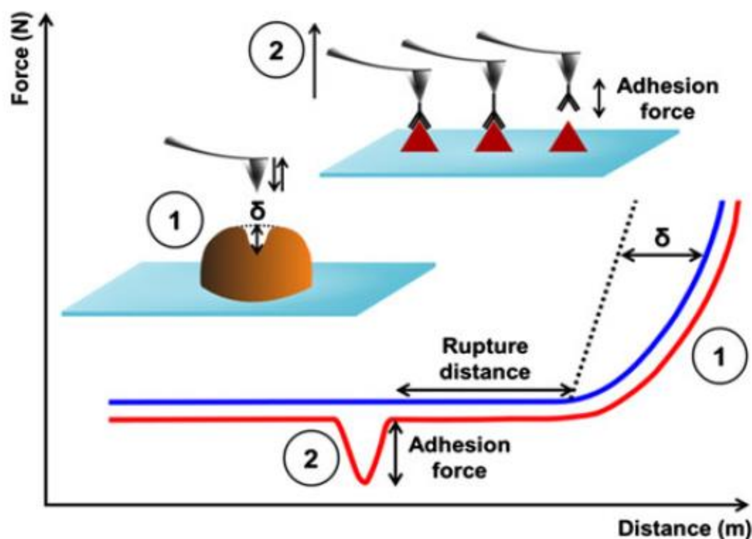

Figure 5. schematic of atomic force microscopy used to determine the biophysical properties of yeast cell wall components. Figure taken from Francois et al. [91] with the authors permission.

The single-molecule force (mode 2 in Figure 5) entails probing the yeast cell with an AFM tip that is functionalized with a specific ligand that could interact with molecules on the yeast cell wall [95]. Previous studies have showed that probing the yeast with an AFM tip functionalized with concanavalin A (ConA tip), a lectin protein with specificity with D-mannose can provide quantitative information on frequency of adhesion events, distribution, and flexibility of mannoprotein at the surface of different yeast strains [92-96]. Likewise, probing the yeast with an AFM tip functionalized with wheat germ agglutinin and anti-β-1,3/anti-β-1,6 antibodies have been used respectively to determine the structure of chitin and β-1,3/β-1,6-glucans present on the yeast cell wall [94, 97]. The interaction between the functionalized AFM tips and the molecule on the yeast surface is given by the adhesion forces on the retracted force curve (red curve in Figure 5). For further reading on the use of AFM to determine the biophysical properties of yeast cell wall components, see review of Francois et al. [91]. Investigating the biophysical properties of yeast cell wall components may give an understanding of the mechanisms in which yeasts are able to exert their functional effects in fish.

5.2.4 Soybean meal-induced enteritis (SBMIE) as a challenge model: strategies to alleviate this in fish

Plant protein sources such as SBM contain a wide range of ANFs such as protease inhibitors, lectins, saponins, phytic acids, and trypsin inhibitor which can compromise performance and gut health of fish [6, 7]. Some ANFs can be eliminated/reduced by different processing methods. Methods such as heat treatment, enzyme supplementation and alcohol extraction can be used to reduce the levels of trypsin inhibitors, protein inhibitors, lectin, phytic acids and saponin present in plant ingredients [7]. An extensive discussion of the individual ANFs and their implications in fish growth and health is beyond the scope of this thesis, however, previous literature [6, 7] provide comprehensive reviews for further reading. The SBM, produced from the residue after oil extraction from soybeans, is an important plant protein ingredient for aquaculture, especially for Carp and Tilapia. Studies have revealed that SBM can induced inflammation in the DI of Atlantic salmon [9-15]. This condition is known as SBMIE, and it is characterized by loss of enterocyte vacuolization, reduction in mucosal fold height, and infiltration of inflammatory cells in the lamina propria and epithelial submucosa [9].

The precise etiological agents of SBMIE in fish are yet to be fully understood, but several studies have implicated the alcohol soluble ANFs (especially saponin) as the likely candidate [15-18]. The saponin content of SBM ranged from 2 to 4 g/kg depending on the cultivation conditions, strain, maturity, and processing [98, 99]. Soyasaponin can be categorized into two main groups i.e., group A and group B depending on the position of glycosylation to the sapogenin. The glycosylation of group A soyasaponin occurs at the C-3 and C-22 position of soyasapogenol A, while group B soyasaponins are glycosylated at the C-3 position of soyasapogenol B [100]. Saponins are amphipathic molecules, which affect functions of intestinal epithelial by increasing the permeability of intestinal mucosal cells, inhibiting active mucosal transport and facilitates uptake of substances, such as antigens and toxins, that are normally not absorbed by the enterocytes [7, 96]. A number of studies have documented that dietary inclusion of soyasaponin induced enteritis in DI of Atlantic salmon [15-19]. Krogdahl et al. [19] and Knudsen et al. [16] showed dose-dependent increased in severity of enteritis when Atlantic salmon were fed FM or lupin meal-based diets with increasing levels of soyasaponin. Previous studies have demonstrated that the inclusion of SPC, a highly-refined soy product in which several ANFs including saponin are removed or inactivated, did not induced enteritis in Atlantic salmon [14, 101]. In contrast, a recent study has documented that the

removal of three proteinaceous ANFs (Kunitz trypsin inhibitor, lectin, and allergen P34/Gly m Bd 30 k) did not mitigate SBMIE in Atlantic salmon. These observations strengthened the speculations that soyasaponins are the potential causes of SBMIE.

Several studies have demonstrated that SBMIE increased gut permeability in fish [16, 102-105]. Nordrum et al. [102] observed increased permeability and reduced carrier-mediated transport in the DI epithelial of Atlantic salmon and rainbow trout fed SBM diets. Similarly, studies have revealed that fish fed SBM responded to inflammatory changes through down-regulation of genes related to tight junction protein (such as aquaporin and claudin) at both local and systemic levels [104-107], which is an indication of increased gut permeability in fish. Increased gut permeability may lead to translocation of bacteria, antigenic peptide, and other toxic constituents to the underlying mucosa (Figure 6).

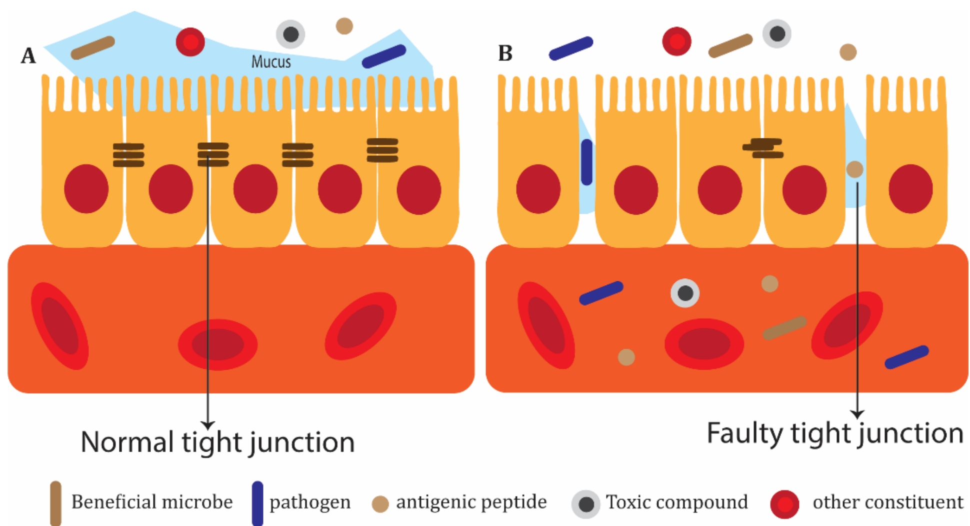

Figure 6. A schematic showing normal (A) and increased gut permeability in fish fed soybean meal-based diets. The schematic was made based on the papers of Krogdahl et al. [7], Nordrum et al. [102] and Merrifield et al. [108].

Fish respond to gut permeability through alteration of genes needed to manage the damage to the epithelial layer or to compensate for the immune response during the healing process [109-111]. Sahlmann et al. [109] through transcriptomic profiling of the DI of Atlantic salmon fed SBM observed an initial (after 1-3 days of feeding) up-regulation of functional genes relating to lipid metabolism, proteolysis, transport, and detoxification possibly as an attempt for the tissue to compensate for initiating the

immune response. However, after 3 days of feeding, gene associated with tissue repair and remodeling were up-regulated and the initial genes were down-regulated, indicating a dysfunction of the metabolic and digestive functions as a result of the inflammation [109]. Studies have shown that T-cell mediated hypersensitivity could be central to the development of SBMIE in fish [110, 112, 113]. Using immunohistochemistry, previous studies have demonstrated increased population of CD3ε (Cluster of differentiation 3 epsilon) and CD8α (Cluster of differentiation 8 alpha) positive lymphocytes in the lamina propria of fish fed SBM diets compared with fish fed reference FM diet [110, 114]. Bakke-McKellep et al. [110] also observed an increased reactivity of IgM (immunoglobulin M) in the lamina propria possibly due to leakage of IgM through the weakened epithelial barrier. These results showed that increased gut permeability due to SBMIE alters both local and systemic immune responses and consequently influences the growth performance and health of fish fed SBM-based diets.

Considering body of evidence on the consequence of SBMIE on nutrient absorption, nutrient utilization, performance, and health of fish, it is an ideal model to investigate the effects of functional diets in fish. Thus, a multitude of studies have investigated potential effects of functional ingredients (especially microbial ingredients) in counteracting SBMIE in fish [106, 114-118]. Yeast such as *S. cerevisiae* and *C. jadinii* can be used to alleviate SBMIE in fish [106, 114, 115]. Similarly, meal produced from *Methylococcus capsulatus* bacteria grown on natural gas have been used to mitigate adverse effects of SBM in Atlantic salmon. Also, a previous study has revealed that microbial feed additive (Bactocell®) can be used to abate intestinal inflammation in Atlantic salmon [119]. In the latter study, fish were chemically induced by oxazolone compared with the use of SBM in former studies. It is important to note that the effect of microbial ingredients in alleviating SBMIE is inconsistent in literature. This has been attributed to difference in species, strains, and their levels of bioactive components, as well as the DSP used after harvesting the microbial ingredients.

5.2.5 Modulation of intestinal microbiota in Atlantic salmon fed soybean meal-based diets

Studies have demonstrated that microbiota plays a fundamental role in digestive functions, nutrient utilization, barrier functions, immune responses, welfare, and health of fish [120-122]. The diets and their compositions are important modulators of intestinal microbiota of fish [123, 124]. Different components of the diets can

selectively promote specific microbial taxa, which can positively or negatively alter the intestinal milieu of fish. As such, a number of studies have documented the effects of SBM on intestinal microbiota of Atlantic salmon [125-128]. Studies have reported that the richness and diversity of gut microbiota of fish fed SBM-based diets are lower than fish fed FM-based diets [125, 126]. The gut microbiota of fish fed SBM are consistently dominated by members of the lactic acid bacteria [125-127], known for their ability to improve growth performance and health of fish [129-131]. The high abundance of lactic acid bacteria in the gut of fish fed SBM-based diets was attributed to the presence of oligosaccharides (such as stachyose and raffinose), which can be metabolized by the microbes [125]. The abundance of these lactic acid bacteria is contradictory to the development of SBMIE and challenged the general understanding that microbiota plays a critical role in the development of SBMIE in Atlantic salmon [125, 126, 128]. Thus, it remains unclear whether the dominance of lactic acid bacteria is a cause or a consequence of fish response to the development of SBMIE.

5.2.6 Methods to evaluate functional effects of yeasts in fish

Achieving three Rs in fish research

Animal welfare is an important concern regarding the use of animal for experimentation. This is why research on animals in different countries are governed by legal regulations that control the minimal acceptable standards that must be followed in conducting animal experiments. These regulatory frameworks are governed and emphasized by the Three Rs (Replacement, Reduction and Refinement) principles of animal research [132]. The authors recommended that the Three Rs principles should applied whenever possible in animal research [132]. Replacement implies the application of methods which avoid or replace the use of animals, Reduction means reduction in the number of animals used to obtain information of a given amount and precision, while Refinement entails optimizing the animal welfare by avoiding pain and distress to those animals which still have to be used [132].

In vitro methods can be used to achieve the Three Rs principles of animal research. *In vitro* methods are advantageous in the sense that they reduced the number of animal use for research and can be replicated in large-scale with minimal cost compared to animal research. The main disadvantage of *in vitro* methods is that they cannot entirely replicate the conditions that occur inside an animal. Different *in vitro* methods can be used to evaluate the effects of functional diets in fish. This thesis focuses on the use of cell culture and *in vitro* gut model.

Cell culture

Cell culture refers to laboratory methods that allow the proliferation of eukaryotic or prokaryotic cells in physiological conditions. Cell culture can be categorized into primary cultures and cell lines. Primary culture is a culture in which the cells are isolated from freshly collected tissues (e.g., spleen) and proliferated under the appropriate conditions until they used up all the available substrates (i.e., reach confluence) [133]. Cell lines arise from primary cultures and cells can be propagated repeatedly and sometimes indefinitely [133]. Rainbow trout intestinal (RTgutGC) [134] and salmon head kidney (SHK-1) [135] are two cell lines that can be used to evaluate the effects of functional ingredients in fish. The approach entails culturing and maintaining the cells under appropriate conditions, followed by a period of induction with functional ingredients. For overview of how *in vitro* method can be used to evaluate the effects of functional ingredients in fish, see the paper of Wang et al. [134]. Contamination represents a major problem for the maintenance of cells *in vitro* and can alter the phenotype and genotype of the cultured cell line. Therefore, the cell culture should be checked regularly to recognize early signs of contaminations to prevent the spread of contaminants to other cells or cell culture products [136].

In vitro gut model

Fish-gut-on-chip [137] and SalmoSim [138] are two models that can be used to simulate the fish gut. Fish-gut-on-chip model relies on microfluidic technology and is based on reconstruction of the intestinal barrier by culturing two rainbow trout intestinal cell lines, namely epithelial RTgutGC and fibroblastic RTgut F, in an artificial environment [137]. Critical barrier functions such as tight junction integrity and permeability can be studied using a fish-gut-on-chip system under a controllable platform in the presence of relevant physiological cues, including fluid flow and coexistence of supporting fibroblasts [137]. SalmoSim is a continuous gut fermentation system which simulates the three compartments (stomach, pyloric caeca and midguts) of intestinal tract of fish and can be used to mimic the microbial communities present in the intestine of marine phase Atlantic salmon [138]. For instance, a recent study employed SalmoSim to document the impact of mannan-oligosaccharides on the gut microbiome of Atlantic salmon [139].

Methods to obtain relevant indices on fish health

Regardless of whether the experiment is conducted with fish and/or with *in vitro* models, different methods can used to obtain indices on impacts of functional ingredients in fish. Tissues such as spleen, gut, liver, and skin isolated from fish can be fixed, dehydrated, embedded, stained (e.g., eosin and hematoxylin) and viewed blindly under a light microscopy for histological assessment of the tissue samples. Such analysis is considered a good indicator of how dietary compounds alter morphology of different tissues and consequently provide information on the effect of diets on fish health [140]. The histological analysis can be complemented with immunohistochemistry approaches to evaluate the distribution of a specific antigen in fish tissues [141]. Immunohistochemistry technique is based on the use of monoclonal and polyclonal antibodies for the detection of specific antigens in tissue sections [141], and the fluorescent-labeled antibody is detected using a fluorescence microscope or confocal laser scanning microscope.

Fish respond to external stimuli such as pathogens through innate and adaptive immune defense system. In fish, the mucus layer of the skin and intestine contains proteins such as complements, lysozyme and cytokines which can give indications of health status of the fish tissues after a dietary or a pathogenic challenge. Techniques such as ELISA (which denotes enzyme-linked immunosorbent assay) and western blot can be used to identify the presence of specific antigen in a tissue of interest. The ELISA and western blot rely on the use of antibodies to detect a target antigen using highly specific antibody-antigen interactions. In ELISA, the antigen is mobilized to a solid surface either directly or via the use of a capture antibody [142]. Western blot is used to identify specific protein from a complex mixture of protein, and the protein is separated based on molecular weight and type through gel electrophoresis [143]. Membrane producing band for each protein is then label with antibody specific for each protein of interest [143].

Flow cytometry is another technique that can be used to document the impact of functional ingredients in fish. Population of immune cells in the fish tissues (head kidney and spleen) after a feeding or induction (in the case of cell culture) period with functional ingredients can be detected using a flow cytometry. Flow cytometry uses lasers to detect and measure the physical and chemical characteristics of a cell population [144]. Epithelial barrier measurement is a good indicator of nutritional and health status of fish. Methods such as Transepithelial Electrical Resistance (TEER) and translocation assays can be used to determine cell barrier integrity and permeability [145]. The TEER measurements are based on measuring how much of electrical signals is blocked by the cellular layer, thereby quantifying barrier integrity

[145]. Translocation assay is based on measuring the permeation of a fluorescent-labeled molecule through the cell [134]. Wound healing assay mimics cell migration during wound healing *in vivo* [146]. Would healing assay involves creating a “wound” in a cell monolayer and capturing the images at the beginning and at regular intervals after an induction with functional ingredients to quantify the effects of these ingredients on cell migration, tissue remodeling and wound resealing process.

Multi-omics techniques can provide better insights to how dietary compounds affect fish health. Methods such as transcriptomics, quantitative polymerase chain reaction (qPCR), metabolomics and proteomics can be used to evaluate how different genes, metabolites and proteins are altered after a fish feeding or a cell induction with functional ingredients. Transcriptomic analysis provide a global overview on the complete set of RNA transcripts that are produced by the genome, under specific circumstances or in a specific cell, using high-throughput methods [147]. This is currently done using high-throughput RNA sequencing, which can detect all transcripts in a sample [147]. The qPCR method can be used to validate specific genes of interest after a transcriptomic analysis. Changes in fish metabolites or protein after a feeding period with functional ingredients can be detected using a metabolomics or a proteomics analysis, respectively. Changes in microbiota (gut, skin, and mucus) of fish can provide useful information on the nutritional and function effects of dietary compounds. This can be measured using a 16S amplicon rRNA sequencing which is the sequencing of the 16S rRNA gene that code for the small subunit of the ribosome (the hypervariable region) found in prokaryotes such as bacteria and archaea. Advanced meta-omics techniques such as metagenomics, metatranscriptomics and metaproteomics can also be used to document the effects of functional ingredients on metabolic capacity of microbiota of fish.

5.2.7 Role of yeast in achieving circular bioeconomy in aquaculture

Circular bioeconomy is an important concept to achieve food security and increase the sustainability of food supply chain around the world. Food sectors are shifting from traditional methods of food production, which is linear to a more circular bioeconomy approach. Linear food production system is based on the concept of take-make-dispose, which implies that the raw materials are processed into a product and then disposed after use [148]. On the contrary, circular bioeconomy is based on the concept of reduce, reuse, and recycle [148]. In this concept, resources used are minimized (reduce), waste or co-products generated in the value-chain are

maximized (reuse) and raw materials are reused to a high standard (recycle) [148]. Aquaculture can play a significant role in achieving a circular bioeconomy. The concept is not new to aquaculture as there are numerous examples of such approach being used to produce fish in different parts of the world, especially in Asia. Traditional integrated-aquaculture system being practiced for many years such as the mulberry dike-fish pond system in China, East Kolkata Wetlands Ramsar site in India and Rice-fish-livestock system in Bangladesh take advantage of synergies among farm components to efficiently recycle and reuse nutrients [149].

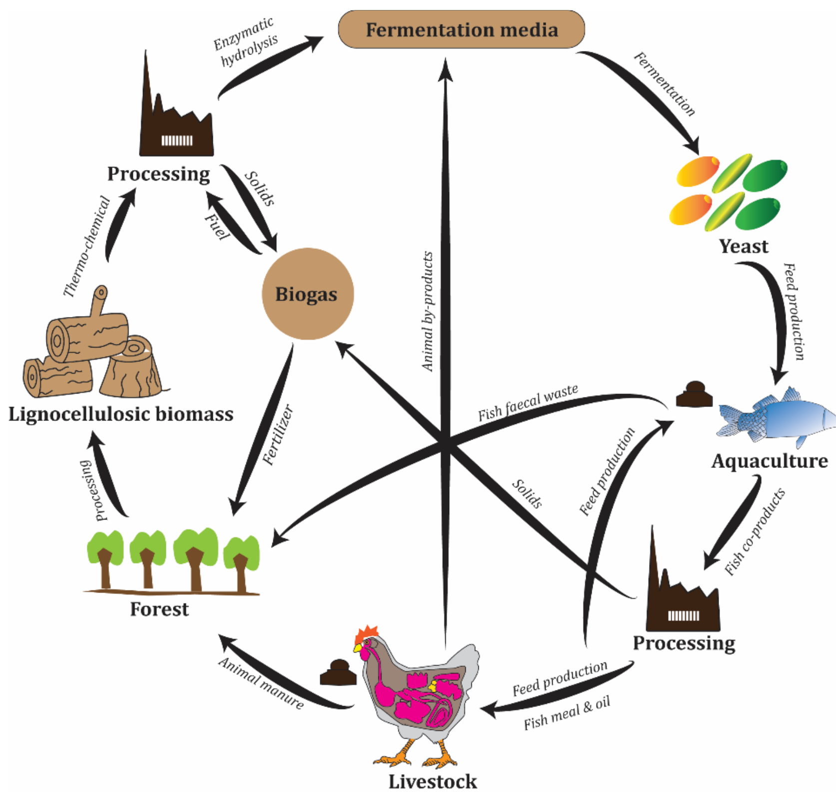

Figure 7. A conceptual framework showing how yeasts can contribute towards achievement of circular aquaculture.

Circular bioeconomy can be implemented in different facet of aquaculture value-chain, but the focus of the present thesis is on how it can be implemented in fish feed production. One-way to achieve this is through the use of co-products across different sectors in production of sustainable novel feed ingredients. Yeast produced from lignocellulosic biomass can contribute to circular bioeconomy as presented in

Figure 7. Lignocellulosic biomass such as wood waste from forestry industry can be saccharified and enzymatically hydrolyzed into sugars that can be used as substrate for yeast production. Animal co-products (such as chicken offal) from the agricultural sector can be hydrolyzed into nitrogen-rich substrate and used for yeast fermentation. The yeast can be directly dried or processed into bioactive products and used as feed ingredients in the aquacultural sector. In turn, the co-products of fish from the aquacultural sector can be processed into fishmeal and fish oil that can be used for both livestock and fish feeds. The faecal waste from fish and livestock can be used as fertilizer to support sustainable forestry productivity. Also, the side stream (solids) from processing of lignocellulosic biomass into sugar substrate can be used in biogas plant where the gas produced can be used to process the lignocellulosic biomass. In addition, solids from biogas production can be used as fertilizer for the sustainable forestry productivity, thereby closing the loop.

5.2.8 Economic feasibility of using yeast as novel ingredient for fish feeds

Price competitiveness is an important factor contributing to the sustainability of an ingredient in fish feeds. To be considered as feasible replacement for conventional ingredients, a novel ingredient most not only meet the technical feed quality, and nutritional and health requirements of fish, but it should also be available at competitive market price for end users (e.g., feed producer). Information on the unit price of yeast protein produced from lignocellulosic biomass is scarce. Economic cost analysis of replacing yeast with conventional ingredients was, therefore, calculated in this thesis (Table 1). Based on previous study, the amount (kg) of glucose, nitrogen-rich substrate and urea needed to produce 1 kg of yeast were estimated as 1.53, 1.02 and 0.08, respectively [27]. Five different scenarios based on price of BALI (Borregaard Advanced Lignin) substrates and potential improvement of protein content and apparent digestibility of protein in yeast were used in the current calculation. The estimated cost of producing 1 kg of dried yeast ranged from 3.68 – 3.74 USD. Adjusting the price of yeast based on their total and digestible protein contents would be the best possible approach to make comparison with conventional protein ingredients. For this reason, the price of yeast, FM and SBM were calculated based on their total and digestible protein contents. By increasing the price of BALI substrate from 0.69 to 0.73 USD, the price per kg of digestible yeast protein increased by 0.17 USD (Scenarios 1 *vs.* 3 and Scenarios 2 *vs.* 4). Also, increasing the protein content (from 50 to 55%) and digestibility coefficient of protein (73 to 82%)

decreased the price per kg of digestible yeast protein by 1.92 USD (Scenarios 1 *vs.* 2 and Scenarios 3 *vs.* 4). Considering scenarios 1-4, the cost of yeast (per kg of digestible protein) was at least 4 and 8 times as expensive as FM and SBM, respectively, implying that yeast is currently not price competitive as replacement for conventional ingredients in fish feeds.

The major driver of yeast price is the cost of substrates for fermentation. This is influenced by the competition between food, fuel, and feed for biomass, as the substrates for yeast fermentation can also be used for other industrial applications, particularly bioethanol production. The food-fuel-feed competition contributes to the significant increase in price of sugar and nitrogen-rich substrates in recent years. The high sugar price is influenced by the increasing demand for bioenergy as a result of subsidy imposed on clean/renewable energy by the European Union (Norwegian) government. The high substrate cost limits the ability to commercialize the innovation to produce yeast (from lignocellulosic biomass) as fish feed ingredients. Based on scenario 5, it became clear that a 50% reduction in the price of BALI and nitrogen-rich substrates would decrease (to about half) the price of digestible yeast protein, but not to a level that is still competitive with both FM and SBM. Thus, the use of cheaper substrates (such as ammonia, urea) can help to improve the price competitiveness of yeasts and increase the possibility to commercialize them for fish feeds. For this reason, ongoing research are focusing on the production of filamentous fungi from cheaper, low-value sugar and non-sugar side streams from forestry by-products. The filamentous fungi contain about 65% crude protein content and is highly digestible (protein digestibility of 90%) in fish (Foods of Norway, personal communication).

Additional approach to further improve the price competitiveness of yeasts as viable replacement for conventional ingredients is by optimizing their bioactive components (such as β-glucan and mannan) and documenting their functional effects in fish. Production of high-value fractions such as β-glucan and mannan for fish, livestock and human consumption would further improve the economic values of yeast. It is important to note that the current calculation did not account for the cost of machinery (such as fermenter, separator, and spray drier), overhead costs and other running costs which may be incurred during the yeast production. The estimated price of yeast was based on production cost rather than market price, and the profit margin of the yeast producer was not accounted for in the current calculation. Likewise, other valuable side streams (such as solids and retentate) generated during yeast production, which can bring additional revenue were not accounted for in this calculation. Thus, the estimated price of yeast in the current

analysis may change based on the different variables used and those not accounted for in this calculation.

Table 1. Estimated economic feasibility of replacing yeast with conventional ingredients in fish feeds.

Parameters	Units	Estimated cost of Yeast					Fishmeal	Soybean meal
		Scenerio 1	Scenerio 2	Scenerio 3	Scenerio 4	Scenerio 5		
Amount of glucose needed to produce 1 kg of yeast[a]	kg	1.53	1.53	1.53	1.53	1.53		
Amount of nitrogen-rich substrate to produce 1 kg of yeast[a]	kg	1.02	1.02	1.02	1.02	1.02		
Amount of urea needed to produce 1 kg of yeast[a]	kg	0.08	0.08	0.08	0.08	0.08		
Price per kg of BALI substrate	USD	0.69[b]	0.69[b]	0.73[c]	0.73[c]	0.37[h]		
Price per kg of nitrogen-rich substrate[d]	USD	2.5	2.5	2.5	2.5	1.25[h]		
Price per kg of urea[e]	USD	0.90	0.90	0.90	0.90	0.90		
Price of glucose needed to produce 1 kg of yeast	USD	1.06	1.06	1.12	1.12	0.56		
Price of nitrogen-rich substrate needed to produce 1 kg of yeast	USD	2.55	2.55	2.55	2.55	1.27		
Price of urea needed to produce 1 kg of yeast	USD	0.07	0.07	0.07	0.07	0.07		
Total cost of 1 kg dried yeast	USD	**3.68**	**3.68**	**3.74**	**3.74**	**1.90**		
Protein content of the dried yeast (%)	%	50[f]	55[g]	50[f]	55[g]	50[f]		
Price per kg of protein in dried yeast	USD	**7.35**	**6.69**	**7.48**	**6.80**	**3.81**		
Apparent digestibility coefficient of protein[5]	%	73[f]	82[g]	73[f]	82[g]	73[f]		
Price per kg digestible protein	USD	**10.07**	**8.15**	**10.24**	**8.29**	**5.22**		
Price per kg conventional ingredients[4]	USD						1.50	0.50
Protein content of conventional ingredients[4]	%						65	50
Price per kg of protein in conventional ingredients	USD						**2.31**	**1.00**
Apparent digestibility coefficient of protein[5]	%						90	85
Price per kg digestible protein	USD						**2.56**	**1.25**
Price per kg of protein	**USD**	**7.35**	**6.69**	**7.48**	**6.80**	**3.81**	**2.31**	**1.00**
Price per kg of digestible protein	**USD**	**10.07**	**8.15**	**10.24**	**8.29**	**5.22**	**2.56**	**1.25**

[a]Value obtained from Lapeña et al. [27]; [b]Price per kg of BALI substrate as at year 2020. Value obtained from Borregaard; [c]Price per kg of BALI substrate as at year 2022. Value obtained from Borregaard; [d]Price per kg nitrogen-rich substrate as at year 2022. Value obtained from Norilia; [e]Price per kg urea as at year 2021. Value obtained from IndexMundi [150]; [f]Value obtained from this thesis; [g]Future target protein content and digestibility coefficient of protein in yeast; [h]Sensitivity cost analysis when the price of BALI and nitrogen-rich substrates are 50% the current price due to in-house production of these substrates.

5.3 Status of knowledge

Narrative reviews on the role of cell wall components of yeast in fish diets is common in literature [28, 29, 151], but there are fewer reviews on the use of yeast as major ingredient in fish feeds. Majority of studies in literature have documented the effects of β-glucan and mannan derived from *S. cerevisiae* on nutrient utilization, growth performance and health of various fish species [28, 29]. There is limited data on the potential of non-saccharomyces as aquafeed ingredients. A number of studies have reported the nutritional and health values of *C. jadinii* yeast in fish [36, 71, 106, 115], but there is no available information on the inclusion of *B. adeninivorans* in fish feeds. Previous studies have reported the nutritional potential of a microbial biomass (containing 70:30 mix of *W. anomalus* and *S. cerevisiae*) in fish [152, 153], but there is no information on the feeding potential of only *W. anomalus* in fish diets. Data on protein digestibility (and other nutrients) are lacking in literature which is necessary for yeast to be used as major protein ingredients in fish feeds. Intact cell walls can impede the nutrient digestibility of yeasts in fish. There is scarcity of information on the cell disruption treatments that can be used to optimize the digestibility and utilization of yeasts in fish. Previous studies have demonstrated that cell wall disruption treatments improved the nutrients digestibility of *S. cerevisiae* in fish [36, 38], but little is known on how the disruption treatments can influence the digestibility and utilization of the three yeast species under consideration in this thesis. Protein digestibility of *S. cerevisiae* improved by 60% after the autolytic treatment [36]. Also, previous study has demonstrated that the composition, ultrastructure, and biophysical properties of *S. cerevisiae* yeast were modified after the autolytic process [93]. Similar studies on *C. jadinii, B. adeninivorans* and *W. anomalus* are scarce in literature. There is limited information on how the changes on the yeast cell wall as a result of the autolysis process influences the functionalities of yeast in fish feeds.

A number of studies have documented inconsistent effect of yeast in counteracting SBMIE in Atlantic salmon [106, 114, 115]. These studies are mostly conducted with *S. cerevisiae* and *C. jadinii*, but no data on the potential of *B. adeninivorans* and *W. anomalus* in alleviating SBMIE in fish. The ability of *S. cerevisiae* to stimulate gut health of fish is linked to their cell wall components. However, similar studies with *C. jadinii, B. adeninivorans* and *W. anomalus* are scarce in literature. Also, our understanding of the impact of autolysis process on immunomodulatory functionality of yeasts in fish is scarcely available in literature. While the local

immune effects of yeast in counteracting SBMIE in fish have been extensively studied in literature, there is less information on the systemic immune response in the spleen and head-kidney of Atlantic salmon. Also, no data exists on how the DSP of yeasts would affect their ability to counteract SBMIE and improve gut health of fish.

Previous studies have reported alteration to the intestinal microbiota of fish fed SBM-based diets [125-128], but there is little information in literature on the effects of yeast inclusion on intestinal microbiota of fish fed SBM-based diets. The bioactive components of yeast can be used as substrate for microbial growth and, thus, modulate the intestinal microbiota of fish. However, the understanding of how the autolysis of yeasts alters the intestinal microbiota of fish fed yeast-based diets is lacking in literature. Majority of studies on gut microbiota of fish fed yeast-based diets are limited to taxonomic profiling of the digesta or mucus samples, but the role of yeast and its cell wall components on metabolic functions of gut microbiota in Atlantic salmon are less reported in literature. There is substantial evidence that dietary inclusion of SBM causes SBMIE in Atlantic salmon, and the severity of SBMIE was attributed to various factors in literature. The reported effects of SBM in the diets of Atlantic salmon have not been quantitatively summarized using a meta-analytic approach. Analyzing these data using meta-analysis approach can help to identify the various factors associated with the severity of SBMIE in Atlantic salmon fed SBM-based diets.

5.4 Hypotheses, objectives and aims

Overall hypothesis: The selected yeasts (i.e., *C. jadinii, B. adeninivorans* and *W. anomalus*) produced from wood sugar and nitrogen-rich substrate can be used as feed ingredients for Atlantic salmon without compromising the growth performance and health of fish, and that the degree of success is dependent on the type of yeast species and DSP method used after harvesting the yeasts.

General objective: To investigate the nutritional value and health effects of yeasts in the diets of Atlantic salmon with focus on growth performance, intestinal health, gut microbiota, and immune responses of fish.

The thesis comprises of seven papers, the hypotheses and objectives of each paper are stated as follows:

Paper I

Hypothesis: Yeasts can be used as major protein ingredients in fish feeds.

Objective: To review the state-of-the-art on the use of yeasts in fish feeds and identify gaps in literature regarding the use of yeasts as aquafeed ingredients using a desk study approach.

Paper II

Hypothesis: The selected yeasts contain bioactive components that can improve growth performance, immune response and gut health of Atlantic salmon fed SBM-based diet in freshwater.

Objective: To examine the impacts of yeasts species and processing on performance, immune response and gut health of Atlantic salmon fed SBM-based diets in freshwater.

Paper III

Hypothesis: The selected yeasts contain bioactive components that can improve systemic immune response of Atlantic salmon fed SBM-based diet freshwater.

Objective: To determine the effects of yeast species and processing on systemic immune response of Atlantic salmon fed SBM-based diets in freshwater and demonstrate whether spleen can be used as a target organ to characterize the immunomodulatory effects of functional ingredients in Atlantic salmon.

Paper IV

Hypothesis: The nutrient digestibility of yeast in fish is affected by the type of yeast species and the DSP used after harvesting the yeast.

Objective: To investigate the effects of yeast species and processing on nutrient digestibility of yeasts in Atlantic salmon. Also, to understand whether protein digestibility of yeasts in Atlantic salmon are influenced by nitrogen solubility and cell wall thickness of yeasts, as well as digesta viscosity and dry matter.

Paper V

Hypothesis: Yeast contains bioactive components that can alter local and systemic immune response and improve the gut health of Atlantic salmon fed SBM-based diets in seawater.

Objective: To study the effects of yeast species and processing on intestinal health and transcriptomic profile from DI and spleen tissues of Atlantic salmon fed SBM-based diets in seawater.

Paper VI

Hypothesis: Inclusion of yeasts alters the intestinal microbiota of Atlantic salmon fed SBM-based diets in seawater and that DSP of yeast influences the modulation of gut microbiota of fish.

Objective: To evaluate the effects of yeast species and processing on composition, diversity and predicted metabolic capacity of gut microbiota in Atlantic salmon fed SBM-based diet in seawater.

Paper VII

Hypothesis: The severity of SBMIE in Atlantic salmon are associated with fish production phase, SBM inclusion level, water temperature, and adaptation to SBM over time.

Objective: To determine various factors associated with the severity of enteritis in Atlantic salmon fed SBM-based diets.

5.5 Materials and Methods

This thesis consists of seven papers (five papers from three fish experiments and two literature reviews) (Figure 8). Detailed descriptions of the methodology used are presented in each paper. A summary of the applied methodologies in each paper is described in this section.

5.5.1 Paper I

A desk study approach was used to collate, synthesize, and discuss the prospects of yeasts as major protein ingredients in fish feeds. A literature search was performed using search engines Google Scholar, Web of Science and Scopus to gather relevant data on the nutritional, amino acids, and cell wall composition of five major yeast species which have potential to be used as fish feed ingredients. Based on available information in the literature, the five yeast species; *S. cerevisiae, C. jadinii, Kluyveromyces marxianus, B. adeninivorans and W. anomalus* were considered in this paper. The paper discussed the potential of yeast as an efficient bio-converter of non-food biomass and addressed the multi-functional values of yeast cell wall components

in fish. The nutritional composition of yeast was discussed in the paper and the nutritional adequacy of yeast as potential fish feed ingredients for both Atlantic salmon and rainbow trout was estimated using chemical score, essential amino acid index and ideal protein concept based on limiting methionine. Studies in which *S. cerevisiae* and non-saccharomyces yeasts have been used as macro-ingredient were collated and summarized in this paper. Information on strategies to improve utilization of yeast in fish; environmental footprint of yeast as feed ingredients; and legislation for use of yeast in animal feed were collated and discussed in the paper.

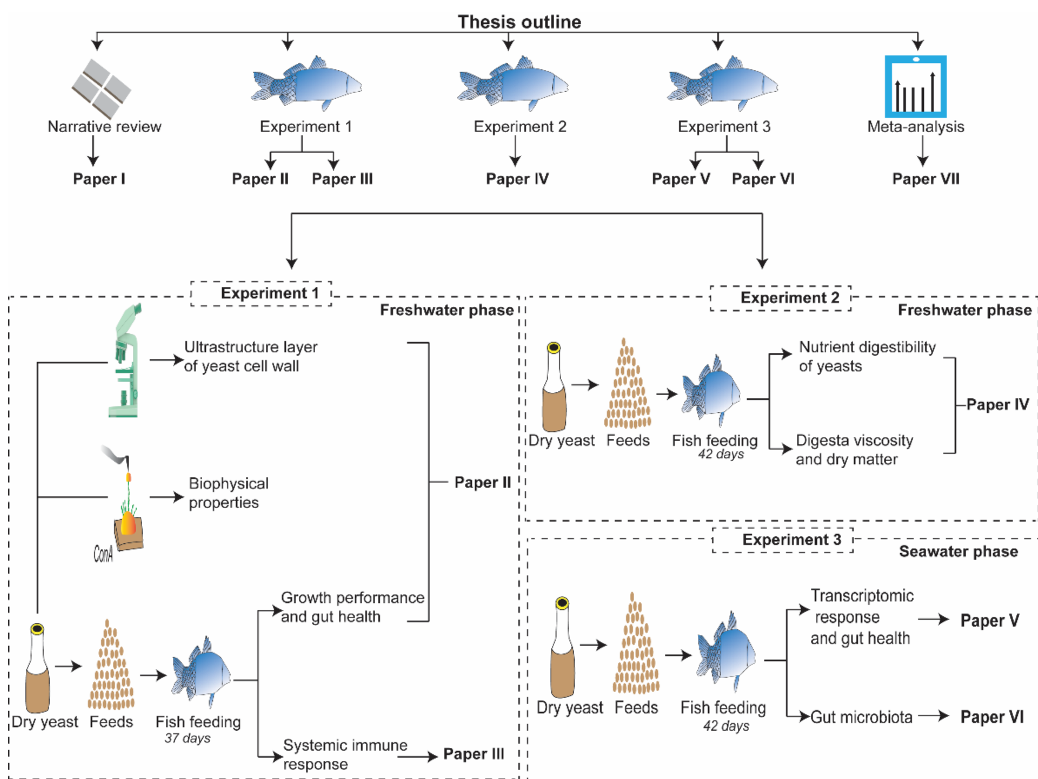

Figure 8. Overview of papers and fish experiments in the present thesis.

5.5.2 Paper II

Detailed description of the yeast production and processing, the experimental diets, the fish experimental protocol and sampling procedure can be found in **Paper II** of this thesis. Briefly, after 37 days of feeding the experimental diets, six fish from each tank were randomly sampled to collect DI and pyloric caeca tissues for further

analysis. The DI and pyloric caeca tissues were used for histological analysis. The histological sections of the DI were assessed for loss of supranuclear vacuolization, widening of lamina propria in the mucosal folds, increase of connective tissue between the basal folds and stratum compactum, and length of villi. For pyloric caeca, enterocytes height as well as number and average size of mucous cells in the mucosal area were evaluated. The protein expression of pro-inflammatory cytokines (Tumor necrosis factor alpha (TNFα) and Interferon gamma (IFNγ)), anti-inflammatory marker (Annexin 1), IgM and antigen presenting marker (Cluster of differentiation 83 (CD83)) were evaluated using indirect ELISA in the DI tissues of fish. The tissue samples and the analysis used to evaluate gut health in this paper is presented in Figure 9. The nutritional and cell wall compositions of yeasts and diets were analyzed using the standard protocol.

In addition to the fish experiment, the morphology and ultrastructure of the yeast pastes with and without autolysis were evaluated using a scanning electron microscope (SEM) and a transmission electron microscope (TEM). The cell surface properties of yeasts (i.e., Young modulus, length of mannoprotein unfolded, rupture distance, adhesion force and adhesion frequency) were evaluated using the AFM with and without functionalized tips with ConA. The specificity of mannan for ConA was assessed using an immunofluorescence approach. The fish performance, morphometric and immune response data were analyzed using a one-way ANOVA. For fish performance and morphometric data, significant differences among dietary groups were detected using the Tukey HSD test, whereas the significance differences for the immune response parameters were detected using Dunnett's multiple comparison test. Histology data were analyzed using a non-parametric Kruskal-Wallis test followed by Dunn's multiple comparison test.

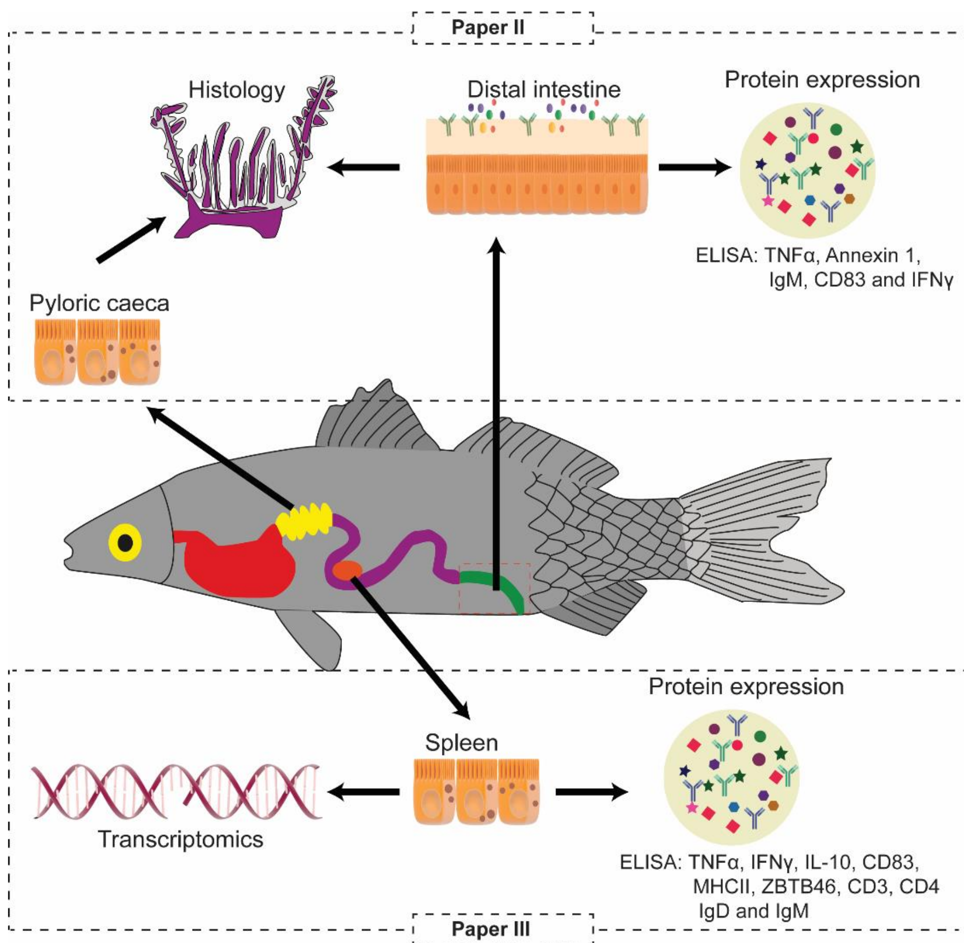

Figure 9. The analyses used in **Paper II and III** to evaluate the immune response (local and systemic) and gut health of Atlantic salmon fed the experimental diets.

5.5.3 Paper III

The full description of the yeast production and processing, production of experimental diets and fish experimental protocol for **Paper III** are documented in **Paper II**. Fish were fed four experimental diets (FM, SBM, ICJ and ACJ) in this paper. The tissue samples and the analysis used to evaluate gut health in this paper is presented in Figure 9. For each dietary group, six spleen samples were analyzed using indirect ELISA to determine protein expression of lymphocytes markers (Cluster of differentiation 3 (CD3), Cluster of differentiation 4 (CD4), immunoglobulin D (IgD) and IgM), cytokines (TNFα, IFNγ and Interleukin-10 (IL-10)) and antigen presenting

markers (CD83, Major histocompatibility complex (MHCII) and Zinc finger and BTB domain-containing protein 46 (ZBTB46)). Additionally, total RNA from four spleen tissues per dietary group was used to assess the transcriptomic profile of fish fed the experimental diets using RNA sequencing technology. Data from indirect ELISA were analyzed using one-way ANOVA and significant differences among the dietary groups were detected based on Tukey's test for multiple comparison. The raw reads from the RNA sequencing data were cleaned and aligned to *Salmo salar* genome ICSASG_v2 using HISAT (v2.1.0). FeatureCount was used for mapping and differentially expressed genes (DEGs) were estimated between diets using SARTools R package. Functional classification was performed using g:profiler and Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Enrichment Analysis was used to determine gene biological functions.

5.5.4 Paper IV

The production and processing of yeasts used in **Paper IV** are reported in **Paper II** of this thesis. Seven experimental diets were used in this paper. The control feeds consisted of 100% reference diet (REF). Six test diets (ICJ, ACJ, IBA, ABA, IWA, AWA) comprising of 70% REF diet and 30% each of the yeast products (**as in Paper II**). The diets were cold-pelleted using a P35A pasta extruder (Italgi, Carasco, Italy) and thereafter dried at 60 ºC in small experimental dryers before being used in fish trial.

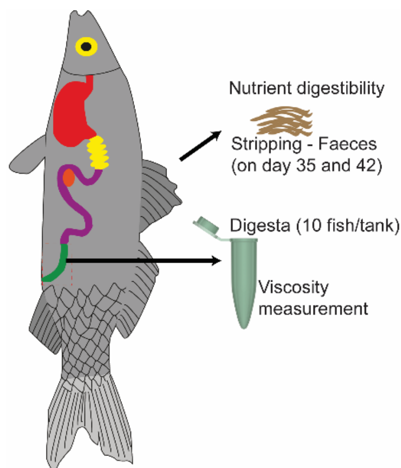

Figure 10. The samples collected in **Paper IV** to analyze the nutrient digestibility of the experimental diets and yeasts.

The analyses used in **Paper IV** are presented in Figure 10. On day 35 and 42, fish were anesthetized and stripped for faeces. The faecal samples collected on both days were pooled by tank and freeze-dried for proximate analysis and used for calculation of nutrient digestibility. Digesta samples were collected (ten fish per tank) and used for determination of digesta viscosity and dry matter. Growth performance and apparent digestibility coefficients (ADCs) of nutrients in the diets and the yeasts were calculated according to the established equations in literature. Viscosity of yeasts, diets and digesta were determined according to standard protocol. Additionally, the size distribution of yeasts with and without autolysis were examined using flow cytometry. Nitrogen solubility, *in vitro* protein digestibility and free amino acids in the yeast samples were evaluated following standard protocol with some modifications.

5.5.5 Paper V

Based on the results of **Papers II, III and IV**, a new batch of *C. jadinii* and *W. anomalus* were used in **Paper V**. Detailed description of the yeast production and processing, the experimental diets, the fish experimental protocol and sampling procedure can be found in **Paper V** of this thesis. After 42 days of feeding, six fish were randomly selected from each tank, anesthetized, and dissected for DI and spleen samples. The tissue samples and the analysis used to evaluate gut health in this paper is presented in Figure 11.

The DI tissues were used for histological analysis and immunohistochemistry analysis. The histological sections of the DI were assessed for shortening of mucosal fold height, loss of supranuclear vacuolization, and infiltration of lamina propria and submucosal with inflammatory cells. The population of T-lymphocytes (CD3ε and CD8α positive cells) were evaluated by immunolabeling of DI samples with CD3ε and CD8α monoclonal primary and secondary antibodies. Total RNA extracted from the DI and spleen tissues were used to evaluate the transcriptomic profile of fish using the RNA sequencing technology. RNA sequence data analysis was performed using the publicly available nf-core/RNA-seq pipeline (v3.3) implemented in Nextflow (v21.04.0). ShinyGO (v0.741) was used to determine the functional profile of DEGs between a pair of diet comparison and EnrichmentMap (v3.3.3) in Cytoscape (v3.8.1) was used to visualize enriched Gene Ontology terms for all diet comparisons in a network. The nutritional and cell wall compositions of yeast was determined following standard protocol.

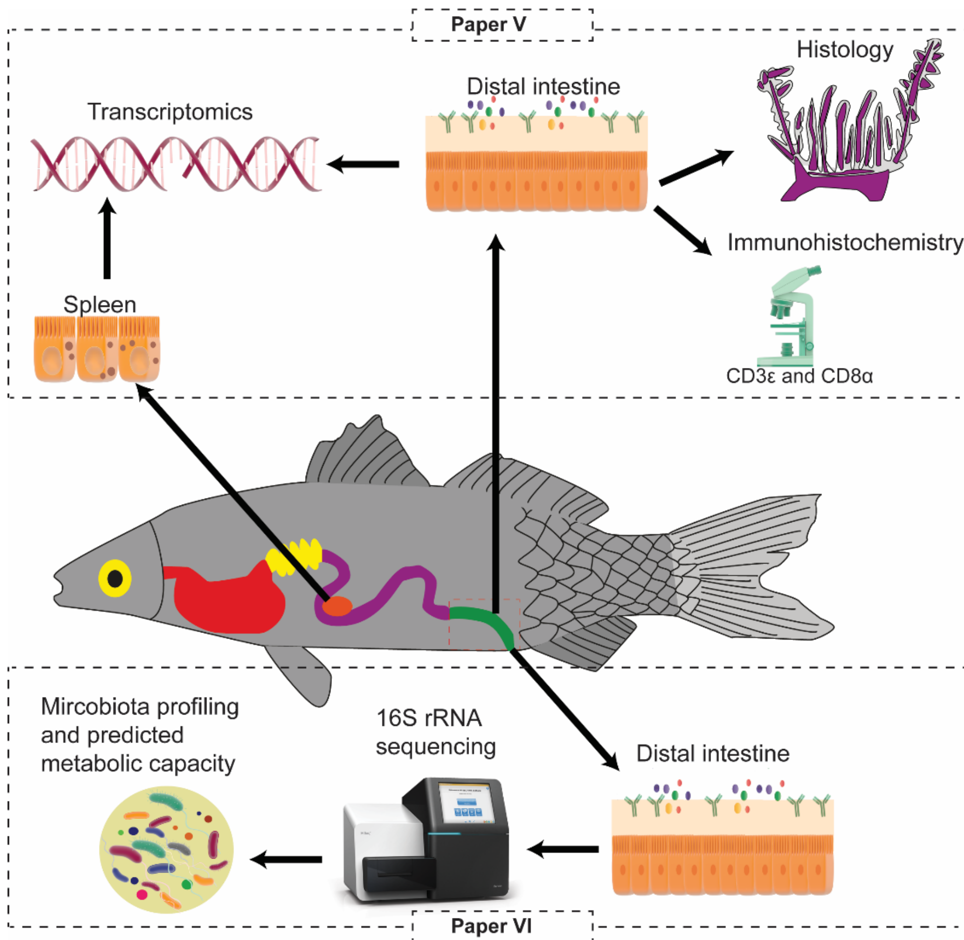

Figure 11. The analyses used in **Paper V and VI** to evaluate the immune response (local and systemic) and gut health of Atlantic salmon fed the experimental diets

5.5.6 Paper VI

The yeast production and processing, experimental diets and fish experiment in **Paper VI** were as presented in **Paper V.** The tissue samples and the analysis used for microbiota profiling of fish fed the experimental diets is presented in Figure 11. At the end of the feeding trial, six fish from each tank were randomly selected to collect digesta sample from the DI for 16S rRNA sequencing. Water samples (from the source and fish rearing tanks) and feed samples were also collected for the analysis. Total DNA was extracted from the digesta, water and feed samples following standard protocol with some modifications. Then, library preparation was conducted

according to the Illumina 16S Metagenomic Sequencing Library Preparation protocol. The V3-V4 hypervariable regions of the 16S rRNA gene were amplified, cleaned and the cleaned PCR products were examined using 1% agarose gel electrophoresis. The library was sequenced using the Miseq Reagent kit v3 (600 cycle) (Illumina; catalog no., MS-102-3003) on the Illumina Miseq system (Illumina, San Diego, California, USA). The retrieved raw sequence from the Miseq system were processed using DADA2 (v1.18.0) in R (v4.0.4) to infer amplicon sequence variants (ASVs) and taxonomic assignment was conducted using the SILVA (v138.1) reference database.

The observed ASVs, Pielou's evenness, Shannon's index, and Faith's phylogenetic diversity were used to compute alpha diversity indices. Similarly, Jaccard, unweighted Unifrac, Aitchison and PhILR transformed Euclidean distances were computed for beta-diversity. The statistical differences among the dietary groups for the microbial compositions at genus or lowest taxonomic ranks (top 15 most abundant taxa) and alpha diversity were evaluated using Kruskal-Wallis test followed by Wilcox pair-wise comparisons test. For beta-diversity, the statistical differences among the dietary groups were computed using permutation multivariate analysis of variance (PERMANOVA) with 999 permutations using the R package vegan (v2.5.7) followed by a pair-wise comparison test. The ASVs were mapped to an available genome-scale metabolic models (GSMMs) of gut microbes to predict the metabolic capacity of intestinal microbiota of fish fed the experimental diets. Enriched subsystem pathways were obtained from the GSMMs model and analyzed using Fisher's exact test and significantly enriched pathways between pair of diets were detected using Benjamini-Hochberg procedure.

5.5.7 Paper VII

A systematic literature search was conducted in Oria, Web of Science, Scopus and Google scholar using the terms; "soybean meal", "induced", "enteritis", "enteropathy", "intestinal/gut health", "Atlantic salmon" and "*Salmo salar*" to identify studies that meet the selection criteria. After duplicate removal, a total of 46 articles were assessed for their eligibility for the meta-analysis. A total of 26 articles (16 published and 10 unpublished in peer-review literature were included in the meta-analysis after the eligibility assessment. Semi-quantitative scores for the four histological variables reported to be associated with inflammatory changes in fish were extracted from each article. The four histological variables were reduction in mucosal fold height; disappearance of supranuclear vacuolization; increases in cellularity of lamina propria and submucosal due to infiltration by inflammatory cells. Data on

inclusion level (%) of SBM, SBM types, feed type (i.e., whether the feed contained SBM with or without microbial ingredients), and fish production variables (initial and final body weight, water temperature, water salinity, specific growth rate, thermal growth coefficients and the fish exposure time to diets) were either extracted or calculated using the available information. The semi-quantitative scores were extracted based on number of fish recorded for each scoring category – normal, mild, moderate, marked, and severe. Then, the data were transformed into trichotomous outcomes and analyzed using ordinal logistic regression by comparing the SBM treatment(s) with neutral-reference treatment in each study. The neutral-reference treatment was considered as the dietary group without SBM – the diets contain ingredients such as fishmeal, SPC and wheat gluten meal which are known for their inability to induce enteritis in fish. The log-odds ratio and its standard error from the ordinal logistic regression was used to perform the meta-analysis using the Comprehensive Meta-analysis software.

A meta-regression analysis was performed to identify factors associated with the severity of enteritis using both categorical (production phase and feed type) and continuous (SBM inclusion level, year of study, water temperature and exposure time) variables. Both univariate (using individual variable) and multi-variate meta-regression analyses were performed on the dataset. The meta-regression was conducted using the random-effects model and heterogeneity across studies were assessed using the chi-squared (Q) test and the I^2 statistics. To control for variation across studies, sub-group analyses were conducted by stratifying the studies into different production phase (seawater *vs.* freshwater), feed type (SBM with *vs.* SBM without microbial ingredients), SBM inclusion level (< 20%, 20%, and > 20%), year of study (< 2014 *vs.* ≥ 2014) and rearing water temperature (≤ 10 ºC *vs*. > 10 ºC). The effect size for the analyzed variables was determined as log-odds ratio for the meta-regression and odds ratio for the sub-group meta-analysis at 95% level of confidence intervals. Linear and quadratic regression analyses were performed between log-odds ratio and fish production parameters to determine the effects of enteritis on fish growth performance.

5.6 Results

5.6.1 A review of the implications for using yeast as major protein ingredients in aquafeeds (Paper I)

Review of existing information in the literature showed that yeasts were efficient bio-converter of low-value non-food biomass into high-value nutrients. The cell wall polysaccharides can be used to support growth performance, immune responses, and health status of fish. Yeasts showed comparatively similar composition of amino acids with FM and SBM, except for a lower content of the sulphur-containing amino acids i.e., methionine, and cysteine. The results of the chemical score, essential amino acids index and ideal protein concept based on limiting methionine indicated that methionine, lysine, arginine, and phenylalanine were potential limiting amino acids when yeasts are used in the diets of Atlantic salmon and rainbow trout. These limiting amino acids may require further exogenous supplementation when yeasts are used as major ingredients in fish feeds. **Paper I** also documented that genetic manipulation and/or improvement in nutrient digestibility through exogenous enzyme supplementation and use of cost-effective DSP are possible strategies to increase the utilization of yeasts in fish feeds. Additional investment in large-scale production at competitive price is needed for yeasts to be considered as feasible replacement for FM and SBM in fish diets.

5.6.2 The effects of down-stream processing on functional properties of yeasts and the implications on gut health of Atlantic salmon fry fed soybean meal-based diets in freshwater (Paper II)

Effects of yeast species and processing on nutritional and cell wall compositions of yeasts

The nutritional and cell wall contents of yeasts were dependent on yeast species and DSP used after harvesting the yeasts. The crude protein, crude lipids and ash contents differed among the three yeasts and were unaffected by the autolysis process. The lowest crude protein level (37 – 39%) was recorded for *B. adeninivorans*, compared with *C. jadinii* (45 – 48%) and *W. anomalus* (52 – 53%). Conversely, *B. adeninivorans* and *W. anomalus* had the higher crude lipid content compared with *C. jadinii* (6 – 6.2%). The ash content varied among the three yeast species and ranged from 3 – 8%.

The β-glucan, mannan and chitin contents differed among the three yeasts species and were affected by the autolysis process. Higher β-glucan content (% dry mass) was reported for *C. jadinii* (20.4%) compared with *B. adeninivorans* (12.3%) and *W. anomalus* (11.1%). Autolysis reduced the β-glucan content by 20%, 13% and 18% for *C. jadinii, W. anomalus* and *B. adeninivorans,* respectively. Higher mannan content (18%) was reported for *W. anomalus* compared with *C. jadinii* (11%) and *B. adeninivorans* (10%). The mannan content of *C. jadinii* was unaffected by the autolysis process, whereas the mannan contents of the other yeasts were reduced by 5 – 15%. The chitin content of the three yeasts was very low, ranging from 1 – 3% of the yeast dry mass.

Effects of yeast species and processing on ultrastructure and biophysical properties of yeast cell wall

The SEM micrographs showed that both *C. jadinii* and *W. anomalus* are ovoid-like in shape, whereas the shape of *B. adeninivorans* was rod-like. The SEM images also showed that inactivated yeast cells had smooth surfaces with no wrinkles, whereas the autolyzed yeast cells appeared wrinkled and partly disintegrated. This was further confirmed by the TEM images, which indicated that the inactivated yeast cells contain compact and visible intracellular components, whereas the intracellular components of autolyzed yeast cells were visibly distorted. The cell wall thickness differed among the three yeasts and was influenced by the autolysis process. Higher cell wall thickness was documented for *W. anomalus* (ca. 160 nm) compared with *B. adeninivorans* (ca. 104 nm) and *C. jadinii* (ca. 96 nm). Autolysis reduced the cell wall thickness by 16%, 40% and 28% for *C. jadinii, B. adeninivorans* and *W. anomalus,* respectively.

Higher Young modulus (i.e., cell elasticity) was reported for *W. anomalus* compared with the other yeasts. Autolysis reduced cell elasticity of the three yeast species, but the effect was most pronounced for *B. adeninivorans.* The adhesion frequency, adhesion force, length of mannoprotein and rupture distance varied among the three yeasts and was affected by the autolysis process. The adhesion frequency was 65%, 86% and 52% for *C. jadinii, B. adeninivorans* and *W. anomalus,* respectively. Autolysis decreased the adhesion frequency by 22% in *C. jadinii,* 29% in *B. adeninivorans* and slightly increased in *W. anomalus.* The length of mannoprotein unfolded was 48 nm, 123 nm, and 152 nm for *C. jadinii, B. adeninivorans* and *W. anomalus,* respectively. Autolysis decreased length of mannoprotein unfolded by 23% in *C. jadinii,* 46% in *B. adeninivorans* and remained unchanged for *W. anomalus.* The correlation analysis showed that there were significant positive correlations between

mannan content, cell wall thickness and Young modulus. Likewise, there were positive but insignificant correlations between length of mannoprotein unfolded, adhesion force and mannan contents of the yeasts. Conversely, there was a significant negative correlation between β-glucans contents of the yeasts and AFM-derived parameters.

Effects of yeasts species and processing on growth performance, gut health, and immune responses of fish

Fish were reared from 5 to 25 g during the experimental period. No mortality and abnormal behavior were observed during the experiment. There was no effect of diets on feed intake, biomass gain and specific growth rate of fish. Fish fed FM diet showed lower feed conversion ratio (FCR) compared with the other diets. Based on all the histological measurements, fish fed FM diet had normal intestinal morphology compared with fish SBM with mild signs of SBMIE. Based on widening of lamina propria, fish fed AWA and ICU were effective in counteracting SBMIE, while only limited effects were observed for other yeast products. Considering changes in supranuclear vacuolization and connective tissues in absorptive enterocytes, there was no differences among the dietary treatments. The measurement of protein expression showed that fish fed FM diet had increased level of TNFα compared with fish fed SBM diet. Similarly, fish fed the yeast diets showed higher expression of TNFα compared with those fed SBM diet. On the contrary, fish fed yeast-based diets showed lower expression of Annexin 1 compared with fish fed SBM diet. There was no significant difference among the dietary treatments for protein expression of CD83, IFNγ and IgM. Correlations among all the diets based on five immunological markers showed a positive and significant correlation between fish fed FM diet and those fed ACJ diet. Also, there was a negative and significant correlation between Annexin 1 expression in the DI and glucan intake.

5.6.3 The effects of down-stream processing on transcriptomic profiles and systemic immune responses of Atlantic salmon fry fed soybean meal-based diets in freshwater (Paper III)

Effects of yeasts species and processing on transcriptomic profile of fish

Fish fed FM showed the highest DEGs (313 down-regulated, 448 up-regulated) compared with those fed SBM diet. Fish fed ACJ diet showed a lower number of DEGs (95 down-regulated, 51 up-regulated) compared with fish SBM diet. In addition, a fewer number of DEGs (21 down-regulated, 4 up-regulated) were observed when

comparing fish fed ICJ diet with those fed SBM diet. The Gene Ontology analysis comparing fish fed FM diet with those fed SBM diet showed that the up-regulated terms were associated with ion binding, transporter, and metabolic activity, whereas the down-regulated terms were related to semaphoring activity, biological adhesion, and cell adhesion. When comparing fish fed ACJ diet with those fed SBM diet, the down-regulated terms were associated to intrinsic apoptotic signaling pathway, while the up-regulated terms were related to molecular binding and gas transporter activity. Furthermore, network analysis showed that fish fed FM and ACJ diets (compared with SBM) shared similar pathways related with the up-regulation of tetrapyrrole binding, oxygen transport, oxygen carrier activity, hemoglobin complex, heme binding, gas transport, molecular carrier activity, oxygen binding and cytosol.

Effects of yeasts species and processing on systemic immune responses of fish

The detection of immunological markers in the spleen tissues by indirect ELISA indicated that fish fed FM diet had lower levels of CD83 compared with fish fed SBM diet. Fish fed both ICJ and ACJ diets showed increased expression of MHC II compared with fish fed SBM diet. Lower levels of ZBTB46 were observed in fish fed ICJ and ACJ diets compared with those fed FM and SBM diets. Level of CD4 was significantly higher in fish fed ACJ diet compared with fish fed FM diet. There were no dietary effects on the protein expression of CD3, IgD, and IgM in the spleen tissue of fish. Fish fed ICJ diet showed higher expression of IFNγ and TNFα compared with SBM diet, whereas fish fed ACJ showed opposite trends. Fish fed ACJ diet showed higher levels of IL-10 compared with fish fed SBM diet. Correlation analysis of all the immunological markers showed a significant positive correlation between fish fed FM and ACJ diets, whereas correlations between other diets did not show significant results.

5.6.4 The effects of yeast species and processing on nutrient digestibility of yeast in Atlantic salmon (Paper IV)

Effects of yeasts species and processing on protein and amino acid digestibility of yeast in fish

The ADCs of protein in inactivated yeasts (ICJ, IBA and IWA) ranged from 63 to 72% with the highest values recorded from IBA. The effect of autolysis on ADCs of protein was inconsistent among the three yeast species. Autolysis increased the ADCs of protein in *C. jadinii* and *W. anomalus,* while no effect was observed for *B. adeninivorans.* The ADCs of amino acids were influenced by the yeast species and

processing used after harvesting the yeast. The ADCs of total amino acids were in line with the ADCs of protein. The ADCs of total amino acids in inactivated yeasts were 57%, 73% and 68% for *C. jadinii, B. adeninivorans* and *W. anomalus,* respectively. Autolysis increased the ADCs of total amino acids (by 5 to 16%) for all the three yeasts. The ADCs of lysine in inactivated yeasts ranged from 67 to 79% with the highest value documented for *B. adeninivorans*. Autolysis improved the ADCs of lysine by 15%, 7%, and 13% for *C. jadinii, B. adeninivorans* and *W. anomalus,* respectively. The ADCs of methionine in inactivated yeasts were 47%, 81% and 74% for *C. jadinii, B. adeninivorans* and *W. anomalus,* respectively. The impact of autolysis on ADCs of methionine differed among the three yeast species. Autolysis improved the ADCs of methionine in *C. jadinii* and *B. adeninivorans*, while slightly reduced in *W. anomalus.*

Effects of processing on viscosity and cell wall integrity

There were no definite trends on the impact of processing on viscosity of yeasts. The viscosities of IBA and IWA yeasts were similar and higher than the other yeast products. There were no effects of diets on digesta viscosity and digesta dry matter. As detected by flow cytometry, autolysis reduced size distribution of the three yeast species. The nitrogen solubility ranged from 23 to 48% in activated yeasts. Autolysis increased nitrogen solubility by 49%, 30% and 75% for *C. jadinii, B. adeninivorans* and *W. anomalus,* respectively. *In vitro* protein digestibility in inactivated yeasts were 85%, 83% and 76% for *C. jadinii, B. adeninivorans* and *W. anomalus,* respectively. Autolysis increased the *in vitro* protein digestibility of *C. jadinii* and *B. adeninivorans* but had no effect on *W. anomalus*. The regression analysis showed that the ADCs of protein in yeasts were positively correlated with digesta viscosity and nitrogen solubility of yeasts. The cell wall thickness of yeasts showed low but positive correlation with ADCs of protein in yeasts. Contrarily, the ADCs of protein in yeasts were negatively correlated with digesta dry matter.

5.6.5 The effects of yeast species and processing on intestinal health and transcriptomic profiles of Atlantic salmon fed soybean meal-based diets in seawater (Paper V)

Effects of yeast species and processing on nutritional and cell wall compositions of yeasts

There was batch-to-batch variation in the protein content of ICJ and IWA yeasts presented in **Paper II** and **Paper V**. Protein content ICJ and IWA yeasts (second batch) used in **Paper V** were 47% and 43%, respectively. The protein contents of yeasts

were not influenced by the autolytic process. Crude lipid content (2.8 – 2.9%) was similar for both ICJ and IWA yeasts. Autolysis improved the crude lipid content from 2.9 to 5.7% in *C. jadinii* and from 2.8 to 4.1% for *W. anomalus.* The ash, total phosphorus, and gross energy contents of the two yeasts were similar and unaffected by the autolytic process. The β-glucan contents (15 – 16%) were similar for both yeasts. The mannan content of ICJ (8%) was lower compared with IWA (11%) yeast. Autolysis reduced the content of β-glucan and mannan by 21 – 33% and 8 – 24% for both yeast species. Chitin contents (0.3 – 0.5%) were low for the two yeast species.

Effects of yeast species and processing on performance, nutrient digestibility, and intestinal health of fish

No abnormal fish behavior or mortality was observed during the experimental period. There were no effects of diets on biomass gain, specific growth rate (SGR) and FCR of fish. The ADC of protein was higher in fish fed FM diets compared with those fed the other diets. Fish fed IWA diet had lower ADC of lipid compared with fish fed the remaining diets. For histological data, fish fed FM showed normal and healthy DI morphology compared with the other diets. Based on shortening of mucosal fold height, infiltration of lamina propria and submucosal cellularity, similar inflammatory changes were observed in the DI of fish fed SBM diet compared with fish fed yeast-based diets. On the other hand, fish fed ICJ and ACJ diets were significantly different from fish fed SBM diet with marked inflammatory changes based on loss of supranuclear vacuolization. The population of T-lymphocytes detected using immunohistochemistry analysis showed that the expression of both CD3ε and CD8α positive cells were more evident in the DI epithelium than the lamina propria. Regardless of the diets, there was higher abundance CD3ε positive cells compared with CD8α positive cells in the DI of fish. There was no effect of diets on the area of lamina propria occupied by CD3ε positive cells. The area of lamina propria occupied by CD8α positive cells was significantly higher in fish fed SBM diet compared with fish the other diets.

Effects of yeast species and processing on transcriptomic profiles from the distal intestine and spleen tissues of fish

Higher DEGs were observed between diet comparisons in the DI tissue compared with spleen tissue. Fish fed SBM showed DEGs (173 down-regulated, 143 up-regulated) compared with those fed FM diet. A fewer number of DEGs were observed when fish fed ICJ and ACJ diets were compared with fish fed FM diet. Fish fed AWA and IWA diets showed the highest DEGs when compared with those fed FM diet. The

Gene Ontology analysis comparing fish fed SBM diet with FM diet showed that the up-regulated terms were mainly related to transport-channel activity, lysosome, and tight junction protein, whereas the down-regulated terms were associated to metabolic pathways. The up-regulated Gene Ontology when comparing fish fed ICJ and ACJ diets with those fed FM diet were associated with metabolic process, wound healing, vitamin B6 binding, taurine and hypotaurine metabolism, whereas the down-regulated terms were associated with transport activity and biosynthetic processes. Furthermore, when comparing fish fed IWA and AWA diets with those fed FM diets, the up-regulated terms were associated with energy metabolism, while the down-regulated terms were related to immune response pathway and oxidation-reduction process. There were no differentially significant Gene Ontology terms between diet comparisons for the spleen tissue.

5.6.6 The effects of yeast species and processing on intestinal microbiota of Atlantic salmon fed soybean meal-based diets in seawater (Paper VI)

Effects of yeast species and processing on taxonomic composition of intestinal microbiota of fish

The taxonomic composition of the digesta samples at phylum level were dominated by Firmicutes, Proteobacteria and Actinobacteriota. Fish fed ACJ and AWA diets had higher abundance of Firmicutes (97%) and lower abundance of Proteobacteria (2.2 – 2.5%) compared with fish fed the other diets. Fish fed ACJ and AWA diets had lower relative abundance of Actinobacteriota compared with fish fed the remaining diets. At genus or lowest taxonomic rank level, the digesta of fish fed ACJ and AWA diets were dominated by *Pediococcus* (92%) and *Bacillaceae* (88%), respectively. *Lactobacillus* and *Limosilactobacillus* were higher in the digesta of fish fed FM diet compared with those fed the other diets. Fish fed ICJ, IWA and SBM had higher abundance of *Enterococcus, Streptococcus, Peptostreptococcus, HT002, RsaHf231, Weissella,* and *Photobacterium* compared with fish fed ACJ and AWA diets. Fifteen ASVs (*Peptostreptococcus, Limosilactobacillus, Weissella, Ligilactobacillus, Streptococcus* and *Lachnospiraceae*) classified as core microbiota were identified in all the dietary groups. The composition of the gut microbiota was similar to that of the feed samples but differed from that of water samples. The ASVs overlap between the gut and the feed was higher than between the gut and water.

Effects of yeast species and processing on alpha-diversity, beta-diversity and predicted metabolic capacity of intestinal microbiota of fish

The microbial diversity of fish fed ACJ and AWA diets was lower compared with fish fed the other diets, based on the four alpha-diversity indices. The microbial diversity of fish fed ICJ, IWA and SBM were similar based on the four alpha-diversity indices. Based on observed ASVs and Faith's phylogenetic diversity, fish fed FM had higher microbial diversity compared with those fed the remaining diets. Considering the four beta-diversity indices, the microbiota of fish fed FM diet were clearly distinct from those fed the other diets. Also, the microbiota of fish fed ICJ, IWA and SBM diets were similar and clearly clustered from those fed the other diets. Based on Jaccard distance, unweighted Unifrac distance and PHILR transformed Euclidean distance, the microbiota of fish fed ACJ diet was distinct from those fed AWA diets. In contrast, the microbiota of fish fed ACJ and AWA diets were similar based on Aitchison distance. The PERMANOVA tests showed that the beta-diversity were influenced by the dietary groups.

For metabolic capacity of gut microbiota, ten pathways were enriched in pairwise comparisons between the dietary groups. The gut microbiota of fish fed FM diet showed predicted enrichment of metabolic pathways related to mucin O-glycan degradation, valerate metabolism and O-Glycan degradation, as well as lower enrichment of purine and pyrimidine catabolism pathways compared with fish fed ICJ and SBM diets. The gut microbiota of fish fed ACJ diets showed predicted enrichment of mucin O-glycan degradation pathway compared with fish fed ICJ, IWA, AWA and SBM diets. The predicted enrichment of metabolic pathways was similar for fish fed FM and ACJ diets, except for glycerophospholipid pathway (enriched in fish fed FM) and nucleotide interconversion (enriched in fish fed ACJ).

5.6.7 Meta-analysis: Factors associated with the severity of enteritis in Atlantic salmon fed soybean meal-based diets (Paper VII)

The meta-regression analysis showed that the severity of enteritis was associated with the fish production phase, feed type, SBM inclusion level, year of study and water temperature, but not exposure time. Fish fed SBM-based diets in seawater were more prone to develop enteritis compared with fish reared in freshwater. Also, regardless of the production phase, the results showed that loss of supranuclear vacuolization was the most sensitive variable to evaluate SBMIE in fish. There was inconsistent effect of dietary inclusion of microbial ingredients on severity of enteritis in Atlantic

salmon fed SBM-based diets. The severity of enteritis was higher in fish fed SBM-based diets before 2014 compared with those fed SBM-based diets after 2014. The severity of enteritis in fish fed SBM-based diets decreased with increasing water temperature. There were negative relationships between log-odds ratio and specific growth rate of fish fed SBM-based diets. There were neither linear nor quadratic relationships between log-odds ratio and final weight of fish. There was negative quadratic relationship between log-odds ratio and thermal growth coefficients of fish fed SBM-based diets in freshwater.

5.7 Discussions

The overall objective of this PhD thesis was to investigate the nutritional and health effects of yeasts in the diets of Atlantic salmon with focus on growth performance, intestinal health, gut microbiota, and immune responses of fish. The results are discussed in detail in the seven included papers. In this section, the results of these papers are discussed in a broader context.

5.7.1 The composition of yeast is influenced by yeast species, batch-to-batch variation and processing methods used post harvesting

Characterization is the first crucial step in the evaluation of ingredients as potential aquafeed resources [154, 155]. Chemical composition, bioactive components, presence of ANFs, variability in composition, source and species are all important factors needed to be documented when evaluating whether a novel ingredient is a feasible replacement for conventional ingredients in fish feeds [154, 155]. Therefore, in **Paper I**, we demonstrated that the nutritional composition differed among the different yeast species and are possibly influenced by their genetics, the fermentation condition used, and DSP used after harvesting the yeasts. Also, we showed in **Paper I** that the different yeast species had similar composition of amino acids with FM and SBM, except for a lower level of sulphur-containing amino acids i.e., methionine and cysteine. Thus, supplementation of crystalline amino acids to achieve a balance amino acid profile is recommended when yeasts are used as ingredients for fish feeds, particularly for Atlantic salmon and rainbow. These observations were reinforced by the analyzed compositions of the three different yeasts used for the fish feeding experiments in **Papers II-VI**. The nutritional compositions of *C. jadinii, B. adeninivorans* and *W. anomalus* varied partially due to differences in their ability to

utilize substrate and the fermentation conditions used. The *C. jadinii* and *W. anomalus* yeasts had the highest protein and amino acid contents among the three yeasts and these were similar to reported values in literature [26, 45, 71]. The non-protein nitrogen content varied significantly among the three yeast species, which highlight the importance of amino acid composition as a measure of protein values of yeasts rather than crude protein content. It is also important to highlight that the nutritional composition varied between the first (**Papers II-IV**) and second (**Papers V and VI**) batches of *C. jadinii* and *W. anomalus* yeasts used in the current thesis. Variation in nutritional content between different batches is an important factor to consider when developing novel ingredients for fish feeds. Thus, for yeasts to be used as major ingredients in fish feed, it is important to optimize their production process to achieve limited variability between different batches of the same yeast specie. It is crucial to note that batch-to-batch variation of ingredients seems to be of less concern in feed manufacturing in recent time due to the use of rapid analysis techniques for ingredient composition, such as near infrared spectroscopy (NIR). The NIR can be used for on-spot analysis of the nutritional composition of raw ingredients such as crude protein, moisture, and crude lipid, and can thus help to improve diet formulation when using different batches of ingredients. The use of NIR in feed formulation is limited by the availability of robust digestibility data and competent calibration curves for use as reference [155].

Aside from their nutritional contents, the three yeast species contain variable amount of β-glucan, mannan and chitin. Although, substantial body of literature on the role of β-glucan and mannan derived from *S. cerevisiae* in fish are available in literature [28, 29], no/scarce information exists on the functional effects of the components derived from the three yeast species under consideration in this thesis. Therefore, further research on this is recommended in the future. In **papers II and V**, we demonstrated that autolysis influences the cell wall composition of yeasts, although the effect varied among the three different yeast species. The micrographs of yeasts presented **in Paper II** showed that the yeasts retained their shapes and largely remained unbroken due to the autolytic process, which was in line with previous findings in literature [70, 156, 157]. The reason for this observation may be attributed to the core β-1,3 glucans responsible for wall rigidity, which remain undegraded during the autolysis process; and that the loss of mannoprotein is expected to alter the porosity of the cells, but not cell wall integrity [70, 158]. Furthermore, in **Paper II**, we showed that yeasts appeared wrinkle and losses their intracellular organization due to the autolysis process, which may be linked to the loss of water during plasmolysis reaction by the cell [70, 156, 157]. The loss of

intracellular organization modified the yeast cells and may account for the changes observed on the cell wall components of yeasts after the autolytic process. The reduction in β-glucan and mannan after the autolysis of yeasts in **Paper II** was in discordance with previous study that observed no loss of these components [93]. The variation between these two studies may be explained by the difference in yeast species and conditions employed during the autolysis process. Our findings (**Papers II and V**) that the nutritional composition of yeasts was mainly unaffected after the autolytic process contradicts the results of a previous study [70]. The discrepancy could be attributed to difference in autolysis process or the decision to separate the soluble fractions before yeast inactivation. In this thesis, the soluble nutrients were not separated before drying, and that may account for the unchanged nutrient contents observed for these yeasts. The images of dried yeast presented in **Paper II** indicated that the morphology and structure of yeast remain unchanged after the drying process. This suggests that the yeasts were stable during the thermal treatment and may have significant implications on the use of yeast in aquafeeds, as fish feed production is highly thermal dependent. Further research on the possible impacts of heat treatment on the nutritional and cell wall compositions of yeasts is needed in the future.

5.7.2 Nutrient digestibility differed among yeast species and slightly influenced by the autolytic process

Fish diets are formulated based on digestible nutrients. Nutrient digestibility is an important information required for an ingredient to be used in fish feeds. In line with previous speculations [36, 38], the cell wall components of yeasts possibly reduced their nutrient digestibility as demonstrated by moderate protein digestibility (63 – 72%) of three different yeast species under consideration in the current thesis (**Paper IV**). Previous studies have reported both similar [36, 71, 159, 160] and higher [65, 71, 152] protein digestibility values for intact yeasts in various fish species. The discrepancies in protein digestibility of yeasts across different studies could be attributed to the yeast species, yeast strains, fermentation and drying conditions used during the yeast production and fish-related factors. As demonstrated in **Paper IV,** autolysis had minimal impact (increased by 9-12%) on the protein digestibility of yeasts. This finding was contrary to the results of a recent study which revealed that autolysis increased protein digestibility of *S. cerevisiae* by 60% in Atlantic salmon [36]. Similarly, a recent study reported that the protein digestibility of autolyzed *C. jadinii* yeast was 90% in Atlantic salmon reared in freshwater (Foods of Norway,

unpublished). These implies that the effect of autolysis on protein digestibility could be dependent on the species and batch of yeasts. It is of note to state that the yeasts used in **Paper IV** were stored frozen for 3-8 months prior to the autolytic process, which is different from the fresh yeast paste used in the studies of Hansen et al. [36] and Foods of Norway (unpublished). Thus, it is plausible that the long freezing step reduced the activity of endogenous enzymes in the yeasts used in **Paper IV**, and consequently reduced the efficiency of the autolytic process. The effect of storage conditions (before autolyzing the yeasts) on the efficiency of autolysis process in yeasts could be a subject of further investigation. Additional use of exogenous glucanases, mannanase and chitinase either singly or as cocktail may be warranted to optimize the effect of autolysis on protein/nutrient digestibility of yeasts in fish. The use of these enzymes during the autolytic process is already of common practice with commercial yeast producers.

Based on the microscopy images of yeasts presented in **Paper II**, it was demonstrated the yeasts retained their shapes and largely remained unbroken due to the autolytic process, which was similar to the findings of Hansen et al. [36]. Thus, it is possible that the autolysis process only had minimal impact on nutrient digestibility of yeasts, which is line with our observations in **Paper IV**. Therefore, additional methods such as cell wall extraction and the use mechanical disruption may be needed to optimize nutrient digestibility of yeasts in fish. Protein digestibility of *S. cerevisiae* yeast after cell wall extraction process increased from 71% to 96% in Arctic charr and Eurasian perch [65]. In this study [65], *S. cerevisiae* was autolyzed followed by the removal of the insoluble fractions after centrifugation. This further strengthens the argument for separation of soluble and insoluble fractions of yeasts after the autolysis process, which is what is commonly practiced by the commercial yeast producers. The separation of the insoluble and soluble fractions is normally used for food grade yeast rather than feed grade yeast, as the separation process can impose additional cost on yeast production thereby making it too expensive to be used in fish feeds. A previous study has revealed that protein digestibility of *S. cerevisiae* yeast in Atlantic salmon increased from 56 to 81% after cell crushing with microfluidizer [36]. Mechanical cell disruption methods such as bead-milling, microfluidizer and homogenizer can be used to optimize the nutrient digestibility of three different yeast species used in this thesis. The drawbacks of these methods are their cost ineffectiveness in terms of energy demand and their difficulty to commercialize for large scale yeast production.

In **Paper IV**, we showed that sulphur containing amino acids, methionine, and cysteine in *C. jadinii* had low digestibility values (47%) in Atlantic salmon. The low

ADCs of these amino acids could be attributed to structural changes in protein due to thermal treatment of yeasts during spray drying. The structural changes in protein may lead to formation of disulfide cross-linkage [50, 56], which can reduce the bioavailability and digestibility of protein in fish. Hansen et al. [36] observed that spray-drying with inlet temperature of 250 ºC reduced the protein digestibility of *S. cerevisiae* compared with spray-drying at 180 ºC. Similarly, a previous study has reported lower amino acid digestibility in fish protein hydrolysate spray-dried at 180 ºC compared with those spray-dried at 150 ºC [161]. Information on the impact of heat treatment on nutrient digestibility and protein quality of the three yeasts used in this thesis is lacking in literature and should be of consideration in future research. Also, the effect of extrusion conditions on protein quality of the three yeasts are also scarce in literature. The conditions used during the extrusion processing (heat, shear, and moisture) can improve protein denaturation and starch gelatinization of feed ingredients and consequently increased their digestibility in animals [50]. It is possible that the nutrient digestibility of the three yeasts (**in Paper IV**) in Atlantic salmon may change when the feed is extruded rather than cold-pelleted as done in the current thesis (**Papers II-VI**). This needs to be further investigated in future research.

5.7.3 Yeasts had inconsistent effects in counteracting SBMIE in fish

The present thesis showed that 5-10% inclusion of the three different yeasts species did not compromise growth performance of fish. In **Papers II** and **V**, 5-10% inclusion of different yeast products did not compromise the feed intake of fish. This implies the inclusion of yeasts in the diets did not affect palatability of feeds. Feed intake is the major driver of fish growth performance as depressed feed intake will lead to reduction in growth rate and vice versa. High inclusion level of plant ingredients may reduce feed intake and growth rate of fish [7]. Therefore, dose-dependent experiments with high inclusion level are needed to investigate the palatability of the different yeast species in fish. The results presented in **Papers II** and **V** showed that the FCR and SGR of fish fed the yeast-based diets were similar compared with that of fish fed the FM and SBM diets. In this thesis, fish fed SBM-based diets in freshwater (**Paper II**) and seawater (**Paper V**) developed mild to moderate inflammatory changes, which contradicts earlier findings where fish developed marked to severe inflammation after feeding on SBM-based diets [9-15]. Our observations in **Papers II** and **V** were strengthened by the meta-analysis results (**Paper VII)**, which showed that the severity of enteritis in Atlantic salmon has declined over the years based on

the four histological variables, except for loss of supranuclear vacuolization. The reduction in severity of enteritis over the years could be associated with increased tolerance of fish to plant-based diets, changes in physico-chemical characteristics of SBM and improved formulation and processing in recent years. The tolerance of fish over the years could be as a result a conscious or unconscious selection of fish for improved growth performance and adaptability to plant-based diets. Although, studies on improving the tolerance of Atlantic salmon against plant-based diets are limited in literature, a number of available studies have reported improved nutrient utilization, growth performance and no signs of DI inflammation in strain of rainbow trout selected on plant-based diets compared with non-selected strain [162-165]. Based on the results of the fish experiments (**Papers II** and **V**) and the meta-analysis review (**Paper VII**), the validity/sensitivity of using SBMIE as a dietary challenge to investigate the effects of functional ingredients in fish may need to be re-evaluated in the future. As such, subsequent studies may focus on the use of other challenges e.g., pathogenic, stress, and hypoxia to optimize the health effects of these functional ingredients in fish.

Despite mild symptoms of SBMIE in **Paper II**, our findings based on widening of lamina propria showed that AWA and ICU yeasts were effective in counteracting SBMIE, whereas ACJ and IWA had partial effects. Similarly, based on loss of supranuclear vacuolization in **Paper V**, inclusion of ICJ and ACJ yeasts partially reduced the inflammatory changes caused by SBM in fish. It is of note to state that based on the histological results in **Paper V**, the inclusion of IWA and AWA yeasts did not prevent SBMIE. This implies that the effects of microbial ingredients in ameliorating SBMIE is inconsistent across studies. Both positive [114-118] and no [106, 115] effects of microbial ingredients in counteracting SBMIE have been reported in literature. These observations were supported by the subgroup analysis conducted in **Paper VII**, which showed that there was inconsistency regarding the ameliorating effects of microbial ingredients on severity of enteritis between the two production phases (freshwater *vs.* seawater) and the four histological variables. In **Paper VII**, we proposed that the large variability on the impact of microbial ingredients to reduce severity of SBMIE may be linked to the types, the strain, the bioactive components, the batch-to-batch variation, inclusion level and processing of microbial ingredients.

In **Paper II**, we proposed two mechanisms in which yeasts could alleviate SBMIE in fish. The first speculation is the activation of immune system through binding of the yeast cell wall components (particularly β-glucans) to the dectin-1 receptor expressed on the surface of the several innate immune cells such as dendritic cells,

neutrophils, eosinophils, macrophages, monocytes, and some T-cells. Our second proposal is linked to the ability of yeasts through their cell wall components to prevent adhesion of enteropathogenic bacteria to the surface glycoprotein of the villi. In **Paper II**, we demonstrated using AFM that the ability of yeasts to carry out these two mechanisms is linked to the amount, the flexibility and the adhesive properties of the components present on their cell walls. The ability of yeasts to counteract enteritis through the immune response pathways was demonstrated in Papers **II, III and V**. This will be discussed further in the next section. In **Paper VI**, we showed that microbial composition, diversity, and richness in the digesta of fish fed SBM diets were similar compared with fish fed ICJ and IWA diets, implying that the ameliorating effects of yeast on SBMIE is linked to their capability to stimulate the immune response rather than through modulation of intestinal microbiota, as earlier proposed in **Paper II**.

5.7.4 The ability of yeast to counteract SBMIE in fish is linked to the activation of both local and systemic responses

As stated in previous section, the ability of yeast to counteract SBMIE is linked to immune system activation. In **Paper II**, fish fed ICJ, ACJ, AWA and ICU diets had elevated production of TNFα in the DI compared with fish fed SBM diet. Similarly, using the spleen tissue in **Paper III**, fish fed ICJ diet had increased production of TNFα and IFNγ compared with fish fed SBM diet. As excessive activation of the immune system may cause damage to the host, fish tends to normalize the immune response by counterbalancing elevated level of TNFα with reduced level of Annexin 1 in fish fed ICJ and ICU diets (**Paper II**). Annexin 1 plays a role in glucocorticoid-mediated dampening of inflammatory response and has been found to be up-regulated in fish suffering from SBMIE [105]. Our results in **Papers II** and **III** showed that autolysis dampened the ability of yeasts to trigger immune responses in fish. Fish fed ACJ diet seems to control the inflammatory response elicited at the local level (**Paper II**) by dampening the production of TNFα and IFNγ at the systemic level (**Paper III**) compared with fish fed SBM diet. The results bear to fore the importance of nutritional programming based on the physiological and environmental state of the fish. The use of inactivated yeasts (ICJ and IWA) may be crucial during the vulnerable stage of fish such as during seawater transfer, during pathogen infection or at the inception of cold period (e.g., in Norway). On the other hand, the autolyzed yeasts (ACJ and AWA) which dampened the immune response of fish may be use during the freshwater stage to increase fish robustness in preparation for seawater transfer or

pathogen infection. The difference in immune stimulating ability of inactivated and autolyzed yeasts highlights the importance of DSP when using yeasts as functional ingredients in fish feeds. Furthermore, a recent study has revealed that the use of functional ingredients in diets of Atlantic can impose additional metabolic cost on the fish, thereby partitioning energy away from growth and other productive processes [107]. Therefore, studies on long-term implication of continuous feeding of diets containing functional ingredients are warranted in the future.

The ability of yeasts to activate the immune response may be linked to the activation of MI/M2 macrophages. This is evidence by the increased level of IL-10 in fish fed ACJ diet (**Paper III**), implying that ACJ was able to control the inflammatory profile associated with SBM, by controlling TNFα production. The IL-10 is a suppressor cytokine and involved in the down-regulation of inflammatory responses in fish [166]. Additionally, the regulatory activities of IL-10 may not be associated only with immunosuppression and M2 macrophage but could also be related to the maintenance of memory cells over time (adaptive immunity) [167]. The suppression of TNFα and IFNγ production in the spleen tissue of fish fed ACJ diet (**Paper III**) is supported by the reduced expression of CD83, an antigen presenting marker involved in the activation of T helper cells which in turn can reduce the expression of TNFα and IFNγ in higher vertebrates [166, 168].

Previous studies have reported that T-cell mediated hypersensitivity is crucial to the development of SBMIE in Atlantic salmon [110, 112, 113]. Thus, the ability of yeasts to ameliorate SBMIE in fish may be linked to the regulatory effects of T-lymphocytes. In **Paper III**, fish fed ACJ diets increased CD4 and CD3 levels compared with fish SBM diet. The CD4 and CD3 cells are lymphocyte markers which regulate immune responses through antigen recognition and subsequent secretion of effector and regulatory cytokines [169]. The elevated level of CD4 marker in fish fed ACJ diet is supported by the increased production of MHCII marker in the spleen of fish fed this diet compared with fish fed SBM diet. MHCII is involved in the antigen-presentation of peptides derived from exogenous proteins to $CD4^{+}$ T-cells [170]. Furthermore, population of CD8α T cells in the lamina propria of fish fed ICJ, ACJ, IWA and AWA diets was lower compared with fish fed SBM diets (**Paper V**). This finding was similar to the result of previous study which showed that inclusion of 2.5% *C. jadinii* reduced the population CD8α in the DI of fish fed SBM-based diet [114]. Understanding how the yeasts activate the T-lymphocytes to regulate the inflammatory profile caused by SBM in fish could be the key to the development of functional additives with reproducible effects in ameliorating this dietary challenge in fish.

5.7.5 Inclusion of yeast in the diets changes the transcriptomic profile of fish

Dietary inclusion of SBM can induce transcriptomic changes in the distal intestine of fish [109, 171]. In **Paper V**, SBM decreased gut barrier functions through up-regulation of genes associated with increased gut permeability such as solute carriers and channel proteins. The increased gut permeability in **Paper V** is supported by up-regulation of tight-junction proteins such as aquaporin, nucleoporin and claudin, which plays a crucial role in intestinal fluid permeability in fish. The transcriptional changes were also detected at the systemic level based on the results of **Paper III**. In addition to inducing inflammatory responses in fish (**Paper III)**, SBM decreased barrier function through down-regulation of genes related to iron-binding proteins, detoxification, transport, and metabolic process. Aside from changes in barrier functions, changing diet from FM to SBM induced rapid changes associated with immune responses in fish [109]. Sahlmann et al. [109] reported that prominent gene expression changes observed during the first 5 days of feeding SBM to fish were related to immune response and the genes linked to gut function start dominating from day 5 onwards. In the current thesis, lysosomal pathway was up-regulated in fish fed SBM compared with those fed FM (**Paper V**).

Global transcriptomic profiling of tissues can provide important insights to mechanisms in which yeasts are able to counteract SBMIE in fish. In **Paper III**, the inclusion of ACJ yeast in fish diets activated pathways such as phagosome and amino acid metabolism which are linked to immune responses through processes involved in the ability of cells to engulf solid particles to form internal vesicles and maintenance of antioxidant activity in fish. In addition, ACJ yeast was able to activate pathways connected to endocytosis and signaling pathways of pattern recognition receptors (**Paper III**). In **Paper V**, fish fed both ICJ and ACJ diets activated pathways relating to wound healing processes, as well as taurine and hypotaurine metabolism, suggesting that the ability of yeasts to counteract SBMIE may be linked to tissue repair and remodeling processes, as well as maintenance of homeostasis through regulation of antioxidant activity in fish. Fish fed IWA and AWA diets (**Paper V**) revealed alteration of genes associated with immune responses such as cytokines (TNFα, Interleukin-12, IFNγ) and pattern recognition receptor (Toll-like receptor-7), suggesting that the ameliorating effect of yeasts on SBMIE is connected to the activation of M1/M2 macrophages. This strengthened the results of protein expression of different immune markers earlier discussed in this thesis. It is noteworthy to state that, contrary to our observations in **Paper III**, the autolyzed

yeasts did not further confer additional beneficial effects beyond the level observed with inactivated yeasts in **Paper V**. This finding highlights the importance of batch-to-batch variation on the effects of DSP on functional properties of yeasts. Also, contradicting our findings in **Paper III**, the transcriptomic analysis of the spleen tissue showed that the experimental diets did not induce systemic effects in fish. The reason for this discrepancy was not possible to explain in the current thesis and should be a subject of further investigation in future studies.

5.7.6 Is microbiota modulation a cause or a consequence of SBMIE in fish?

In line with previous studies [125-127], in **Paper VI,** we demonstrated that the digesta of fish fed SBM were dominated by lactic acid bacteria such as *Lactobacillus, Limosilactobacillus, Ligilactobacillus, Weissella, Enterococcus* and *Streptococcus*. These are beneficial taxa known to promote nutrient utilization, growth performance and health of fish [129-131]. The high abundance of lactic acid bacteria in the digesta of fish fed SBM has been associated with the presence of soluble and insoluble oligosaccharides in SBM, which can be used as substrate for growth and metabolism by the microbiota [125]. The abundance of lactic acid bacteria in the digesta of fish suffering from SBMIE contradicts the general understanding that microbiota plays a critical role in the development of SBMIE in Atlantic salmon [125, 126]. The current thesis was unable to answer whether the dominance of lactic acid bacteria is a cause or a consequence of fish response to the development of SBMIE. It has been earlier speculated that the increased relative abundance of lactic acid bacteria could be related to their production of antimicrobial peptides against some certain bacteria in fish presenting SBMIE [126]. Nevertheless, this calls for further investigation in future studies. Techniques such as metagenomics, metaproteomics or metabolomics with the ability to provide functional profile of the modulated microbiota can be used to clarify the role of microbiota in the development of SBMIE in fish.

5.7.7 The ability of yeasts to counteract SBMIE is linked to immune activation rather than modulation of intestinal microbiota of fish

Paper VI showed that the microbial composition, richness, and diversity were similar in the digesta of fish fed SBM, ICJ and IWA diets. Also, the gut microbiota of fish fed ACJ and AWA were dominated by a single taxon, *Pediococcus* and *Bacillaceae,*

respectively (**Paper VI**). The increased dominance of a single bacteria in fish fed the autolyzed yeasts can be connected to the autolysis process, feed-borne microbiota and/or the composition of the feed. It should be recalled that the inactivated yeasts were able to dampen the inflammatory profile caused by SBM in fish (**Paper V**). Also, the autolyzed yeasts did not improve the gut health of fish beyond the level observed with inactivated yeasts (**Paper V**). Based on these results, we could hypothesize that the microbial modulation did not contribute to the ability of yeasts to counteract SBMIE in fish. This strengthened our earlier assertion that the ameliorating effects of yeasts on SBMIE is connected to their ability to stimulate immune response (**Papers II, III and V**) rather than through modulation of intestinal microbiota of fish (**Paper VI**).

5.8 Identified gaps for future study

The present thesis showed that the three yeast species are potential aquafeed resources. Nonetheless, there are some research questions that may need to be answered before they can be feasible replacement for the conventional ingredients in fish feeds. In this thesis, we demonstrated that the yeasts are currently not economical compared with the price of FM and SBM. The expensive price per unit of yeasts is largely influenced by the price of substrate used during the fermentation process. Thus, research into the use and optimization of cheap substrate sources is warranted in the future. In **Paper II**, we demonstrated using AFM (functionalized with ConA) that the biophysical properties of yeasts changed with the autolyzed and that these changes could be linked to their functional effects in fish. Further investigation could focus on biophysical changes related with β-glucan component of the yeast cell wall using the AFM functionalized with ligands that have specificity for glucan. This investigation could provide further insights on how autolysis/DSP modifies the glucan component of yeasts and possibly explain its functional effects in fish. In **Paper IV**, the moderate nutrient digestibility of yeasts was slightly increased by the autolytic process. Concerted efforts should be directed on improving the nutrient digestibility of the three different yeasts beyond the levels reported in this thesis.

The current thesis revealed that yeasts could be used to ameliorate SBMIE in fish (**Papers II, III and V**). However, **Paper VII** showed that the severity of SBMIE in Atlantic salmon has declined over the years, which question the validity of using SBM as a challenging model in future experiments. Future works may consider the functional effects of three yeasts under clinical conditions. Disease of economic

importance such as *Piscirickettsia salmonis* and *Moritella viscosa* may be considered as challenging models in future studies. Furthermore, the current thesis considered the nutritional values of the three yeasts in terms of their nutrient digestibility (**Paper IV**) and their ability to support growth performance of fish during a SBM challenge model (**Papers II and V**). Thus, proper growth trials in which the yeasts are replacing equal (on digestible nutrient basis) amounts of FM, SBM or other protein sources in the diets would be of worthy interest in the subsequent experiments. In such trial, a dose-response approach could be used to determine the optimal inclusion level of the three yeasts in fish diets.

The fish feeds used in the current thesis (**Papers II-VI**) were cold-pelleted, which does not reflect the extrusion process used by the commercial feed companies. Future research should consider the effects of yeasts on extrusion processing, technical quality of the pellets, nutrient digestibility, and health of fish. In the current thesis (**Paper VI**), we speculated that the effects of yeasts on modulation of intestinal microbiota of fish could be linked to the microbial transfer from the yeasts to the diets. Thus, similar research in the future should consider not only sequencing the microbiota in the feeds and digesta, but also the microbiota in the yeast samples. Also, the metabolic capacities of the different microbiota may be key to understand how yeasts are able to elicit their functional effects in fish. Thus, techniques such as metagenomics, metatranscriptomics and metaproteomics can be used to gain further insights to the effects of yeasts on intestinal microbiota and consequently on the nutritional and health of fish. These techniques can also be used to understand whether microbial modulation is a consequence or a cause of SBMIE in fish fed SBM-based diets. The present thesis did not take into consideration the effects of yeasts on fillet quality of fish, which is an important area of research that needs to be covered in subsequent experiments. Information on the impact of heat treatment on nutrient digestibility and protein quality of the three yeasts used in this thesis is lacking in literature and should be of consideration in future research.

5.9 Conclusions

The present thesis showed that *C. jadinii, B. adeninivorans* and *W. anomalus* can be used as feed ingredients for Atlantic salmon without compromising growth performance and health of fish and the degree of success is dependent on the type of yeast species and down-stream processing used after harvesting the yeasts. In **Paper I**, the review of available information revealed that yeasts can be used as major protein ingredients in aquafeeds. The amino acid compositions of yeasts are

comparable with FM and SBM, except for methionine, arginine, lysine, and phenylalanine, which can be supplemented when yeasts are used in fish feeds (**Paper I)**. In **Paper II**, *C. jadinii* and *W. anomalus* yeasts showed the most promising effects on gut health based on histological changes in the distal intestine and immune response parameters. The AWA was effective in ameliorating soybean-meal induced enteritis in fish, while only limited effects were observed for other yeasts products (**Paper II).** The ability of yeasts to counteract soybean-meal induced enteritis is linked to the activation of both local (**Paper II**) and systemic (**Paper III**) immune responses in fish. The results of **Paper II** also revealed that the amounts, length, adhesion, and accessibility of cell wall components could be important for the ameliorating effects of yeasts on soybean-meal induced enteritis in fish. The functionality of yeasts in improving gut health of fish is dependent on the yeast species and down-stream processing used after harvesting the yeasts (**Papers II and III**).

The **Paper IV** showed that nutrient digestibility differed among the three yeast species. The protein and amino acids of the three yeast species were moderately digested in Atlantic salmon. Autolysis slightly increased protein digestibility of *C. jadinii* and *W. anomalus* in Atlantic salmon, but not *B. adeninivorans* (**Paper IV**). **Paper IV** revealed that cell wall porosity as demonstrated by nitrogen solubility had larger impact on nutrient digestibility of yeasts than cell wall thickness. The nutrient digestibility of yeasts in Atlantic salmon is dependent on the yeast species and down-stream processing used after harvesting the yeasts (**Paper IV**). In **Paper V**, ICJ and ACJ improved gut health by reducing loss of supranuclear vacuolization and population of CD8α cells in the DI of Atlantic salmon fed SBM-based diet in seawater. The *C. jadinii* and *W. anomalus* also controlled the inflammatory profile caused by SBM by inducing transcriptomic changes associated with wound healing and immune response pathway in the DI of fish. **Paper V** strengthened our observations in **Papers II** and **III** that *C. jadinii* and *W. anomalus* are promising novel ingredients with health beneficial effects in terms of controlling inflammation associated by feeding plant based diets to Atlantic salmon.

In **Paper VI**, we demonstrated that the microbiota of fish fed SBM diet differed from those fed FM diet. The microbiota composition, richness and diversity were similar in fish fed ICJ, IWA and SBM diets (**Paper VI**). Fish fed ACJ increased relative abundance of *Pediococcus,* and mucin O-glycan degradation pathway, while fish fed AWA diet increased relative abundance of *Bacillaceae* compared with fish fed the other diets (**Paper VI**). Despite the significant modulation of intestinal microbiota of fish fed the autolyzed yeasts (ACJ and AWA) (**Paper VI**), the histological and

transcriptomic results revealed that the autolyzed yeasts did not improve gut health of fish beyond the level observed for the inactivated yeasts (ICJ and IWA) (**Paper V**). These results suggest that the ameliorating effects of yeasts on soybean-meal induced enteritis is connected to their ability to stimulate immune responses in Atlantic salmon (**Papers II, III and V**), rather than through modulation of intestinal microbiota (**Paper VI**). The result of the meta-analysis (**Paper VII**) agreed with our observations in **Papers II and V** that the severity of soybean-meal induced enteritis has declined over the years in fish and questioned the validity/sensitivity of using SBMIE as a dietary challenge to investigate the effects of functional ingredients in fish. **Paper VII** showed that the severity of enteritis in Atlantic salmon fed SBM-based diets was associated with fish production phase, feed type, SBM inclusion level, year of study and water temperature, but not the exposure time. Furthermore, the regression analysis showed that increased severity of enteritis reduced growth performance of fish fed SBM-based diets (**Paper VII)**.

6 References

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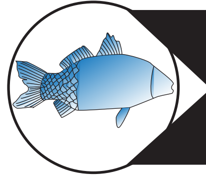
7 Appendices

7.1 Appendix: Papers I-VII



Paper I

REVIEWS IN Aquaculture

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Yeast as major protein-rich ingredient in aquafeeds: a review of the implications for aquaculture production

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Abstract

Sustainability concerns associated with protein sources and currently used fishmeal and plant-based meal have necessitated the quests for novel sustainable ingredients for use in aquafeeds. Yeasts have been proposed as sustainable ingredients particularly because of their potential to valorise non-food lignocellulosic biomass into valuable protein resources. Prior to now, extensive studies exist on the role of yeast cell wall components in modulating health responses of fish. However, research on its use as a major protein source in fish diets is still in its infancy. The current review collates, synthesises and discusses the prospects of five major yeast species as future protein ingredients with respect to their nutritional adequacy in fish. Nutritional quality of *Saccharomyces cerevisiae*, *Cyberlindnera jadinii*, *Kluyveromyces marxianus*, *Blastobotrys adeninivorans* and *Wickerhamomyces anomalus* and their use as replacement for fishmeal and soy protein in the diets of Atlantic salmon and rainbow trout are discussed based on three protein quality indices: chemical score, essential amino acid index and ideal protein concept based on the first limiting amino acids, methionine. The crude protein contents of yeast (40–55%) are lower than that of fishmeal, but comparable with soya bean meal. Compared to fishmeal, the different yeast species have favourable amino acid profiles, except for methionine, lysine, arginine and phenylalanine which are the frequently limiting essential amino acids in Atlantic salmon and rainbow trout. This review also presents future area of research and emphasise the need for large-scale production of yeast at competitive price to constitute a feasible replacement for fishmeal and soy protein in aquaculture.

Key words: amino acids, aquafeeds, nutritional values, protein quality, protein-rich ingredients, yeast.

Introduction

Aquaculture is the fastest-growing food production sector in the world. With 5.8% annual growth rate since 2010, aquaculture continues to surpass other food production sectors (FAO 2018). Sustained growth of aquaculture is necessary to meet the future demand for animal protein as a result of continuous increase in human population. However, availability of resources for aquafeed production is a major constraint expected to exacerbate the rapidly expanding aquaculture sector. Traditionally, fishmeal and fish oil have been the major sources of protein and lipids for intensive farming of carnivorous fish species (Tacon & Metian 2008). The stagnation in the forage fish output implies that continuous high inclusion of fishmeal and fish oil in the diets is no longer sustainable (Tacon & Metian 2008). In recent time, salmon farming has shown reduced dependence on marine ingredients by replacement with plant ingredients, particularly soy protein concentrate (Ytrestøyl *et al.* 2015). This is evident in the reduction in fish-in:fish-out ratio (FIFO) over the years, from 2.57:1 in 2000 to about 0.82:1 at the end of 2015 (IFFO 2017). A major reason for using processed soy products such as, soy protein concentrate is that saponins and other anti-nutritional constituents in conventional soya bean meal can cause distal intestine enteritis and consequently regressed growth in Atlantic salmon and rainbow trout (Van den Ingh *et al.* 1991; Iwashita *et al.* 2009; Chikwati *et al.* 2012; Krogdahl *et al.* 2015). The transition to plant-based ingredients also raises serious ethical and sustainability concerns. The use of more plant-based ingredients in aquafeeds may contribute to intensified crop production, imposing

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pressure on land and water use, energy, resource allocation and forest biodiversity (Pahlow *et al.* 2015; Fry *et al.* 2016). More importantly, the use of soy protein and other plant products in aquaculture reduces their availability for direct human consumption (Ytrestøyl *et al.* 2015). Thus, there is an emerging need for suitable and sustainable novel feed ingredients for aquaculture. More than ever, the quest for novel feed ingredients is gaining attention. At the forefront of this attention is microbial ingredients, particularly yeast, as potential feed ingredients.

One reason why yeasts are potential sustainable ingredients is their ability to convert low-value non-food biomass from forestry and agricultural industry into high-value feed with less dependence on arable land, water and changing climatic conditions (Anwar *et al.* 2014; Couture *et al.* 2019; Lapeña *et al.* 2020a; Lapeña *et al.* 2020b). Yeast cells contain appreciable crude protein (about 40–55%), and other bioactive components beneficiary to fish growth and development (Øverland *et al.* 2013; Hansen *et al.* 2019; Rawling *et al.* 2019; Vidakovic *et al.* 2020). Research on yeast products in fish diets have centred on their roles as nutritional supplements and functional supplements with beneficial effects on the immune responses and gut health in fish (Yilmaz *et al.* 2007; Torrecillas *et al.* 2012; Eryalçin *et al.* 2017). The cell walls represent 26–32% of the dry weight and contain mannan-oligosaccharides (MOS), β-glucan and chitin (Klis *et al.* 2002; Schiavone *et al.* 2014). Over the years, extensive scientific reviews have elucidated the health benefits of these cell wall components in various species, but little information exists on the role of yeast as macro-ingredient in fish feeds (Meena *et al.* 2013; Torrecillas *et al.* 2014). Therefore, this review aims at describing the potential of yeast as protein sources in fish feeds, particularly for Atlantic salmon and rainbow trout. Furthermore, this review focuses on *Saccharomyces cerevisiae* and four non-saccharomyces species that have been documented or are currently under investigation as aquafeed ingredients. The non-saccharomyces of interest are: *Cyberlindnera jadinii* (anamorph name *Candida utilis*), *Kluyveromyces marxianus*, *Blastobotrys adeninivorans* (synonym *Arxula adeninivorans*) and *Wickerhamomyces anomalus* (Øverland *et al.* 2013; Huyben *et al.* 2017; Hansen *et al.* 2019; Vidakovic *et al.* 2020; Lapeña *et al.* 2020a; Lapeña *et al.* 2020b).

Yeast as an efficient bio-converter of non-food biomass

Traditionally, molasses is used as principal raw material in the production of yeast. However, the surge in price and application of molasses in other industrial processes (CIBE 2017) has necessitated the needs for new substrate sources for yeast production. Because of serious environmental concerns such as biodiversity, water and land use, as well as, competition with human food, the first-generation feedstock (mainly food biomass) may be less desirable as substrates for yeast fermentation. Instead, second-generation feedstock, representing non-food biomass, is gaining increasing attention as carbon sources for yeast production. Second-generation feedstocks, such as lignocellulosic biomass, represent the most economical and renewable resources in the world for biofuel production (Anwar *et al.* 2014). Lignocellulosic biomass contains highly complex network of polysaccharides such as cellulose, hemicellulose and lignin, which are not easily hydrolysed by acid, alkaline or enzyme treatments. The main sources of lignocellulosic biomass are from the agricultural and forestry sectors. Yeast offers a great opportunity for conversion of highly non-hydrolysable lignocellulosic biomass into biofuel with tremendous industrial applications.

The presence of fermentable sugars as carbon sources is crucial for efficient yeast production. However, unlike molasses, lignocellulosic biomass first needs to be delignified and saccharified into fermentable sugars for yeast production. To obtain fermentable sugars for yeast fermentation, lignocellulosic biomass undergoes two major processing steps: pre-treatment and enzyme hydrolysis (Binder & Raines 2010; Anwar *et al.* 2014). Pre-treatment entails breaking down the highly complex polysaccharide structure of the lignocellulosic biomass, thereby disentangling them into lignin, cellulose and hemicellulose (Mosier *et al.* 2005; Binder & Raines 2010). In addition, pre-treatment also facilitates disruption of the crystalline structure of the cellulose and hemicellulose, making them more accessible before enzyme hydrolysis to monosaccharides. Methods commonly used for pre-treatment are physical, chemical or a combination of both methods (Mosier *et al.* 2005). Physical treatment uses mechanical milling, whereas chemical treatment mainly uses acid or alkaline treatment (Mosier *et al.* 2005). The choice of pre-treatment methods often depends on the nature and resistance of the biomass to enzymatic and microbial actions. Woody biomass requires more stringent pre-treatment conditions than non-woody biomass (Øverland & Skrede 2017). Enzyme hydrolysis occurs after pre-treatment to break down the biomass into fermentable sugars. It entails degrading the cellulose and hemicellulose into pentose and hexose sugars. The efficiency of enzymatic breakdown of cellulose is influenced by conditions such as temperature, time, pH, enzyme loading and substrate concentration (Horn *et al.* 2012). Figure 1 shows typical steps in production of yeast from molasses and lignocellulosic biomass.

Multi-functional values of yeast cell walls

The cell wall is an important component of the yeast cell architecture. It is vital for growth, shape, protection,

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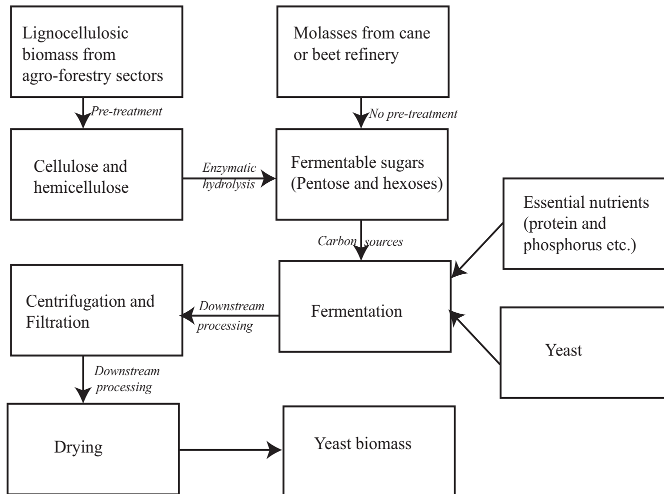

Figure 1 Fermentation process for converting low-value product into high-value yeast biomass (modified from Øverland and Skrede (2017)).

survival and morphogenesis of yeast. Generally, the cell wall represents 26–32% of the total dry weight of the cell (Fleet 1985; Nguyen *et al.* 1998; Klis *et al.* 2002). The cell wall principally contains about 85–90% polysaccharides and 10–15% protein (Nguyen *et al.* 1998; Schiavone *et al.* 2014). Glucan and mannan are the main polysaccharides, with small amounts of chitin. The cell wall structure of the extensively studied species *S. cerevisiae* typically contains 30–60% glucans, 25–50% of mannans and 5–10% of chitin (Fleet & Manners 1976; Fleet 1985; Schiavone *et al.* 2014). The mannan polysaccharides are in complex with the cell wall protein and are more correctly designated as mannoprotein. The chemical composition of the cell wall depends on the species and strains of yeast, fermentation substrates and the methods used for analysis (Papatryphon *et al.* 1999). The cell wall composition of yeast can be determined by chemical or enzymatic treatment or a combination of both methods, as previously highlighted by Magnelli *et al.* (2002) and Schiavone *et al.* (2014). These methods not only determine the content of total glucan, but also distinguish between the β-1,3 and β-1,6 glucan. Chemical analysis of yeast cell walls and separation into individual polysaccharide components continue to face further research aiming at producing well-refined, pure forms of these polysaccharides. Additionally, the current methods were developed for *S. cerevisiae* and there is possibility that further optimisation may be required for non-saccharomyces species.

In recent time, the use of derivatives from the yeast cell wall has become more prominent in the animal feed industry. This is in part due to governmental restrictions and elimination of prophylactic growth-promoting antibiotics in animal feeds within the European Union and United States. The ban of antibiotics in animal feeds consequently stimulated interest in using alternative products (including yeast derivatives) to support animal health and growth performance. There is evidence to show that dietary β-glucans enhance immune responses and survival of the host after a pathogen infection in fish, including Atlantic salmon (Robertsen *et al.* 1990; Bridle *et al.* 2005), rainbow trout (Siwicki *et al.* 2004; Guselle *et al.* 2007), European seabass (Bonaldo *et al.* 2007). Regardless of the health stimulating function performed by β-glucan, it seems to exert its mode of action in a dectin-1 dependent manner. Dectin-1 receptor is highly expressed on the surface of several immune cells such as dendritic cells, neutrophils, eosinophils, macrophages, monocytes and some T-cells (Volman *et al.* 2008). β-glucan binds to the dectin-1 receptor to activate NF-κB through intracellular signalling, which in turn leads to cytokine production, phagocytosis and respiratory burst (Volman *et al.* 2008). Yeast-derived β-glucans have also been used to adsorb or bind toxins, viruses and pathogenic bacteria (Volman *et al.* 2008).

Like β-glucan, MOS from yeast cell walls also exert beneficial and health stimulating effects in different animal species. Many reports have concluded that dietary inclusion of MOS can positively influence health and growth performance of fish, including Atlantic salmon (Refstie *et al.* 2010), rainbow trout (Staykov *et al.* 2007; Yilmaz *et al.* 2007), European sea bass (Torrecillas *et al.* 2011;

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Torrecillas *et al.* 2012) and rohu (Andrews *et al.* 2009). Furthermore, dietary MOS can be used to modulate gut morphology (Eryalçin *et al.* 2017; Schmidt *et al.* 2017) and to enhance skin mucous barrier function in fish (Micallef *et al.* 2017). The most recognised mechanism of action associated with MOS is its ability to bind to enteropathogenic bacteria, preventing host colonisation (Torrecillas *et al.* 2014). This is carried out by binding to the mannose specific lectin-type receptor (Type 1 fimbriae) present on the surface of enteropathogenic bacteria through its branched α-mannosides, thereby preventing adhesion to the surface glycoproteins of intestinal villi (Firon *et al.* 1983; Torrecillas *et al.* 2014; Rawling *et al.* 2019). Several studies have documented the positive effects of both β-glucan and MOS in fish, while others have shown no effects on many of the parameters studied as shown in Table 1. The inconsistencies observed across different experiments may be due to the molecular structure of β-glucan or MOS used, dose and time of feeding, fish species used, stage of growth, culture conditions and health status of fish (Torrecillas *et al.* 2014). Shelby *et al.* (2009) and Lokesh *et al.* (2012) indicated that the effects of these oligosaccharides are more apparent in fish challenged with infection, suggesting their potency during clinical conditions. Detailed reviews on the role of yeast-derived β-glucan and MOS, and their mode of action in fish have been previously provided by Meena *et al*. (2013), Torrecillas *et al*. (2014) and Shurson (2018).

Table 1 Summary of growth and health beneficial effects of yeast-derived β-glucan and mannan-oligosaccharides in fish compared with control diets (without β-glucan or mannan-oligosaccharides inclusions)

Parameters	Positive effects	No effects	Responses considered as positive effects per category
β-glucans†			
Growth rate	1	7	Increased weight gain Reduced feed intake Increased specific growth rate
Feed: Gain	0	8	Reduced feed conversion ratio Increased feed efficiency
Immune response	15	3	Increased survival rate Protection against infection Upregulation of pro-inflammatory cytokines Downregulation of anti-inflammatory cytokines Improved serum biochemistry
Mannan-oligosaccharides‡			
Growth rate	12	19	Increased weight gain Reduced feed intake Increased specific growth rate Increased nutrient absorption
Feed: Gain	6	18	Reduced feed conversion ratio Increased feed efficiency
Immune response	15	5	Increased survival rate Protection against infection Upregulation of pro-inflammatory cytokines Downregulation of anti-inflammatory cytokines Improved serum biochemistry Improved gut barrier function

†Adapted from Meena *et al*. (2013).
‡Adapted from Torrecillas *et al*. (2014).

Nutritional composition of common yeast of interest for aquaculture

Saccharomyces cerevisiae has been the most commonly used yeast species in aquaculture, particularly for its health stimulating effects in various fish species. However, in recent time, there has been an increased focus on non-saccharomyces species with potential values in aquaculture. The utilisation of different substrates influences the chemical composition of different yeast species. For instance, yeast species such as *S. cerevisiae* are strictly efficient at metabolising hexose sugars, whereas others are efficient fermenters of pentose sugars. However, the strict preference for a specific type of sugar, can be resolved through genetic engineering (Wahlbom *et al.* 2003; Attfield & Bell 2006) or using yeast that can co-ferment both hexose and pentose sugars (e.g. *C. jadinii* and *K. marxianus*) (Parajó *et al.* 1995; Yanase *et al.* 2010) or through co-culture of two yeast strains (Azhar *et al.* 2017). Furthermore, environmental conditions such as temperature, oxygen and pH often influence the nutritional composition of whole yeast cells (Halasz & Lasztity 1991).

The nutritional compositions of *S. cerevisiae* and some non-saccharomyces species are presented in Table 2. It is noteworthy to mention that this study considers inactivated yeast or autolysed dry yeast, but not yeast extracts in the calculation of nutritional composition of yeast. The reported crude protein content ranges from 38 to 52% for the five yeast species, although limited data were found for *K. marxianus*, *B. adeninivorans* and *W. anomalus*. Yeast crude protein contains considerable amounts of non-protein nitrogen in the form of nucleic acids, about 10–25% of crude protein depending on yeast species, growth media, the growth rate and the methods used for analysis (Halasz & Lasztity 1991; Rumsey *et al.* 1991b; Øverland *et al.* 2013; Lapeña *et al.* 2020a). In most monogastric animals, elevated concentrations of plasma uric acid due to high dietary nucleic acids interfere with normal protein, fat, carbohydrate and uracil metabolism (Rumsey *et al.* 1992). However, this is not the case in some fish, as salmonids synthesise considerable level of urate oxidase, and are thereby able to metabolise relatively high levels of nucleic acids (Kinsella *et al.* 1985; Rumsey *et al.* 1991b;

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Table 2 Nutritional composition (g/kg dry matter) of selected yeast species of commercial importance

	Saccharomyces cerevisiae†		*Cyberlindnera jadinii*‡		*Kluyveromyces marxianus*§		*Blastobotrys adeninivorans*¶		*Wickerhamomyces anomalus*¥	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Dry matter	939	27 (6)	943	29 (9)	943	5 (2)	948	5.6 (4)	943	7.5 (4)
Crude protein	501	102 (10)	463	66 (10)	531	28 (2)	382	8.4 (4)	528	1.2 (4)
Crude lipids	18	27 (8)	23	21 (10)	7	2 (2)	85	0.3 (4)	89	1.6 (4)
Ash	75	39 (9)	91	36 (10)	76	0 (2)	62	1.2 (4)	33	0.6 (4)
Gross energy	18	2 (6)	19	3 (5)	21	NA	22	0.2 (4)	23	0.1 (4)
Starch	46	33 (3)	37	0 (2)	8	NA	NA	NA	NA	NA
Nucleic acids	48	28 (4)	104	16 (2)	102	NA	NA	NA	NA	NA

Values in parenthesis are the number of studies used for calculating the mean and standard deviation for each yeast species.
Sources: †Chanda and Chakrabarti (1996), Pacheco *et al*. (1997), Cheng *et al*. (2004), Spark *et al*. (2005), Yamada and Sgarbieri (2005), Yalcin *et al*. (2011), Øverland *et al*. (2013), Kim *et al*. (2014), Vidakovic *et al*. (2016); ‡Valdivie *et al*. (1982), Martin *et al*. (1993), Chanda and Chakrabarti (1996), Olvera-Novoa *et al*. (2002), Rodŕguez *et al*. (2011), Øverland *et al*. (2013), Sharma *et al*. (2018), Hansen *et al*. (2019), Sharma (thesis, unpublished); §Revillion *et al*. (2003), Øverland *et al*. (2013); ¶&¥Lapeña *et al*. (2020b), Lapeña *et al*. (2020a) and unpublished data from in-house trials.
NA, not available.

Andersen *et al.* 2006). Nucleic acids may have protein-sparing effects and enhance immune responses and growth of epithelial cells in several fish species including salmonids (Øverland & Skrede 2017). Despite higher contents of nucleic acid, yeasts show comparatively similar composition of amino acids with fishmeal and soy protein, except for sulphur-containing methionine and cysteine, which are characteristically low in yeast (Tables 3 and S1). The amino acid compositions, as shown in Table 3, vary among the different yeast species. The data indicate that *S. cerevisiae* have higher content of methionine and cysteine, but lower content of lysine than the other yeast species. Similarly, *B. adeninivorans* has lower content of arginine compared to other yeast species. Glutamic acid is consistently high in all the yeasts considered. The variation in amino acids profile of yeasts can be attributed to difference in species and strains, substrate media used, culturing conditions, downstream processing and analytical methods used during the production process (Øverland *et al.* 2013).

Yeasts have relatively low lipid content, high ash content and moderate levels of carbohydrates (Halasz & Lasztity 1991; Øverland *et al.* 2013). The fatty acid composition is characterised mainly by unsaturated fatty acids (Halasz & Lasztity 1991; Brown *et al.* 1996). The carbohydrates are predominately polysaccharides, with low amounts of mono- and oligosaccharides except trehalose (Halasz & Lasztity 1991). Aside from these macronutrients, yeasts are moderate sources of other valuable components such as vitamins (mostly B-group vitamins), minerals and enzymes (Lapeña *et al.* 2020a). Mineral contents vary between the different yeast species; and is greatly influenced by the amounts of corresponding minerals in the growth media. For instance, yeasts grown in media containing considerable amount of calcium (whey, calcium lignosulfonate, sulphite waste liquor) are known to be high in calcium content (Halasz & Lasztity 1991). This ability of yeast to efficiently incorporate minerals present in the culturing media, is the mechanism behind the production of selenium (Se) yeast. Selenium yeast is a type of specialty yeast produced commercially and marketed as a highly bioavailable form of Se (selenomethionine) and has a unique role of improving antioxidant status of animals (Schrauzer 2006; Han *et al.* 2017; Wang *et al.* 2018).

Nutritional adequacy of yeast as a sustainable protein ingredient for salmonids

Protein quality indices using the amino acid profile of yeasts, fishmeal, soya bean meal and their corresponding requirements in Atlantic salmon and rainbow trout, as shown in Table 3 (with Table S1), form the basis of this section. Comparatively, the total essential amino acid contents of yeasts in general meet the amino acids requirements of Atlantic salmon and rainbow trout (Fig. 2a,b). The protein quality of yeasts and the conventional fishmeal and soya bean meal throughout this calculations, are evaluated based on the estimated digestible amino acid contents. There is paucity of information on protein and amino acid digestibility of yeasts in literature. From the few available studies, protein digestibility values of yeasts in different fish species vary from 40 to 90% depending on species and strains of yeast, as well as the type of downstream processing used after fermentation (Rumsey *et al.* 1990; Barrows *et al.* 2011; Øverland *et al.* 2013; Sharma *et al.* 2018). These values are mainly reported for *S. cerevisiae* and *C. jadinii*; there are no data on protein digestibility coefficient of

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Table 3 Average amino acid composition (g/16 g nitrogen) of selected yeast species of commercial importance

	Saccharomyces cerevisiae†	*Cyberlindnera jadinii*‡	*Kluyveromyces marxianus*§	*Blastobotrys adeninivorans*¶	*Wickerhamomyces anomalus*¥
Essential amino acids					
Arginine	4.3 (6)	5.1 (10)	4.1 (3)	2.3 (4)	4.7 (4)
Histidine	2.0 (6)	1.8 (10)	1.7 (3)	2.3 (4)	2.6 (4)
Isoleucine	4.3 (6)	4.1 (10)	4.0 (3)	4.3 (4)	5.0 (4)
Leucine	6.5 (6)	6.2 (10)	6.4 (3)	6.2 (4)	6.9 (4)
Lysine	6.4 (6)	6.9 (10)	6.8 (3)	6.7 (4)	6.9 (4)
Methionine	1.8 (6)	1.1 (10)	1.3 (3)	1.4 (4)	1.5 (4)
Phenylalanine	3.9 (6)	3.6 (10)	3.9 (3)	3.5 (4)	3.9 (4)
Threonine	4.4 (6)	4.6 (10)	5.0 (3)	3.7 (4)	4.6 (4)
Tryptophan	1.0 (6)	1.4 (6)	1.0 (1)	NA	NA
Valine	5.1 (6)	5.0 (10)	4.6 (3)	5.1 (4)	4.5 (4)
Non-essential amino acids					
Alanine	5.9 (6)	5.4 (7)	7.9 (3)	5.0 (4)	5.0 (4)
Aspartic acid	9.1 (6)	8.6 (6)	10.1 (3)	7.1 (4)	8.0 (4)
Glycine	4.2 (6)	3.8 (7)	4.1 (3)	3.9 (4)	4.2 (4)
Glutamic acid	12.5 (6)	12.1 (7)	13.3 (3)	10.8 (4)	11.0 (4)
Cysteine	1.3 (6)	0.8 (8)	0.6 (3)	0.6 (4)	0.7 (4)
Tyrosine	3.5 (6)	2.9 (7)	3.0 (3)	4.0 (4)	2.7 (4)
Proline	3.8 (6)	3.4 (6)	3.6 (3)	4.4 (4)	3.7 (4)
Serine	4.2 (6)	4.3 (7)	5.3 (3)	3.0 (4)	3.8 (4)

Values in parenthesis are the number of studies used for calculating the average for each yeast species.
Sources: †Pacheco *et al*. (1997), Cheng *et al*. (2004), Øverland *et al*. (2013), Kim *et al*. (2014), Vidakovic *et al*. (2016); ‡Prior *et al*. (1981), Valdivie *et al*. (1982), Martin *et al*. (1993), (Nigam 1998), Olvera-Novoa *et al*. (2002), Øverland *et al*. (2013), Sharma *et al*. (2018), Hansen *et al*. (2019), Sharma (thesis, unpublished); §Anderson *et al*. (1988), Øverland *et al*. (2013); ¶&¥Lapeña *et al*. (2020b), Lapeña *et al*. (2020a) and unpublished data from in-house trials.
NA, not available.

K. marxianus, *B. adeninivorans* and *W. anomalus*. For this reason, it becomes apparently impossible to compare the nutritional values of individual yeasts based on their specific protein and amino acid digestibility. Therefore, to bypass this limitation, the digestible amino acid contents of yeasts (i.e. *S. cerevisiae*, *C. jadinii*, *K. marxianus*, *B. adeninivorans and W. anomalus*) (presented in Table S2) used for all the necessary calculations were based on amino acid digestibility coefficient of 80% – the expected target digestibility coefficient for yeast to be able to nutritionally compete with the conventional ingredients. Furthermore, amino acid digestibility coefficients of 90% and 85% were used for fishmeal and soya bean meal, respectively, throughout this article (presented in Table S2) (Glencross *et al.* 2004; Barrows *et al.* 2011). Radar charts of digestible amino acids indicate that the contents of some amino acids in yeasts are below the requirements of Atlantic salmon and rainbow trout (Fig. 2c,d); these amino acids below the requirements of fish are otherwise referred to as limiting amino acids. To gain further insights into the limiting amino acids in the different yeast species, protein quality indices such as, chemical score, essential amino acids index (EAAI) and ideal protein concept are employed in this article.

Chemical score and EAAI

The protein value of ingredients can in principle be evaluated based on chemical scoring system proposed by Mitchell and Block (1946) and recently modified by Veldkamp and Bosch (2015) to quantify protein quality of novel feed ingredients. This method is used to determine the single essential amino acid in maximum deficit compared to a reference protein. Nine essential amino acids (excluding tryptophan), were used in calculating the chemical score and EAAI to test the concept of ideal protein based on the amino acid requirements of juvenile Atlantic salmon and rainbow trout (Table S1). Tryptophan was exempted because contents in most yeasts are scarcely reported in literature. As shown in Figure 2, digestible amino acids values are closer to Atlantic salmon requirements, compared to total amino acids values. Therefore, for each ingredient, chemical score was calculated from the ratio of each digestible amino acid and the corresponding requirements in Atlantic salmon and rainbow trout. The resultant ratios were then compared with fishmeal as the reference protein source. The chemical score for *S. cerevisiae*, *C. jardinii*, *K. marxianus*, *B. adeninivorans*, *W. anomalus*, soya bean meal and

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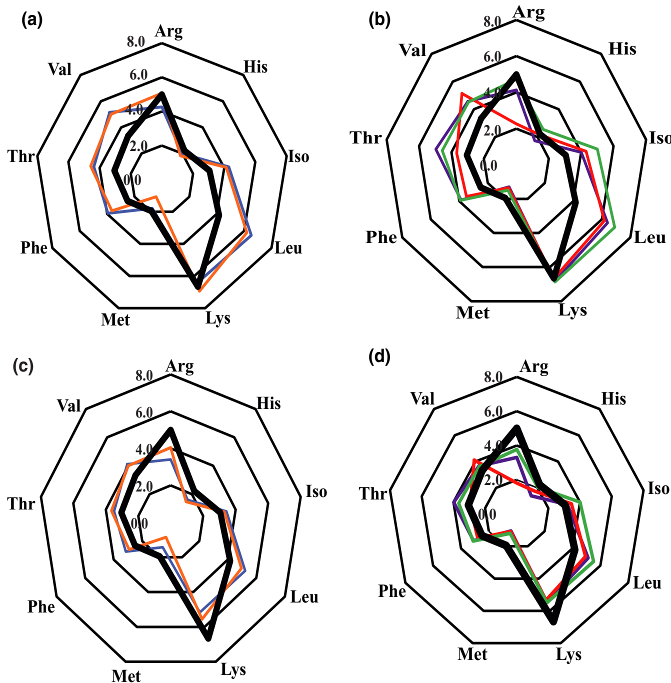

Figure 2 Radar plots (in g/16 g nitrogen) showing the comparison of total (a, b) and digestible amino acids (c, d) in the selected yeast species with the corresponding requirements in Atlantic salmon (similar trends were observed for rainbow trout, not presented to avoid repetition)[†,‡,§]. [†]The digestible amino acids content was calculated from the total amino acids and protein digestibility coefficient of 80% for all the yeast species in both fish species. [‡]SC, *Saccharomyces cerevisiae*; CJ, *Cyberlindnera jadinii*; KM, *Kluyveromyces marxianus*; BA, *Blastobotrys adeninivorans*; WA, *Wickerhamomyces anomalus*; AS, Atlantic salmon. [§]Arg, Arginine; His, Histidine; Iso, Isoleucine; Leu, Leucine; Lys, Lysine; Met, Methionine; Phe, Phenylalanine; Thr, Threonine and Val, Valine. All essential amino acids except tryptophan which values are missing for some yeast ingredients. (—) SC; (—) CJ; (—) KM; (—) BA; (—) WA; (—) AS.

fishmeal are shown in Table 4. The results indicated that *S. cerevisiae*, *C. jadinii*, *K. marxianus*, *B. adeninivorans and W. anomalus* had comparable chemical score with soya bean meal, but lower than fishmeal for both Atlantic salmon and rainbow trout. Veldkamp and Bosch (2015) considered chemical score as the measure of limiting amino acids. Methionine was the first limiting amino acid in most yeast species, except *B. adeninivorans* where arginine was the most limiting (Table 4).

A major limitation of chemical score is that it considers each amino acid as an individual entity, whereas all amino acids work in synchrony during protein synthesis. To sidestep this limitation, a model integrating all the nine essential amino acids (same as in chemical score) was used in estimating the EAAI. The EAAI was calculated according to the method proposed by Oser (1951) and recently used by Smith (2017) and Veldkamp and Bosch (2015), and presented in Equation (1). The EAAI method integrates all the essential amino acids into the nutritional evaluation of protein. The EAAI was defined by Veldkamp and Bosch (2015), as the adequacy between the concentration of all the essential amino acids in the dietary protein and the requirement of the target animal. A protein source completely matching the requirement of a target animal has an EAAI equals to 100, whereas those which amino acids profiles fall below the target animal requirement has EAAI less than 100. In this paper, the EAAI of *S. cerevisiae*, *C. jadinii*, *K. marxianus*, *B. adeninivorans*, *W. anomalus* and soya bean meal were reported relative to fishmeal as the reference protein source, as shown in Figure 3. Consistent with chemical score, the EAAI of *S. cerevisiae*, *C. jadinii*, *K. marxianus*, *B. adeninivorans*, *W. anomalus* and soya bean meal were lower than for fishmeal. Furthermore, *W. anomalus* showed the highest EAAI among the yeast candidates, whereas *B. adeninivorans* had the lowest value. Oser (1951) previously asserted that protein quality rating of an ingredient should be based on the contribution a protein makes in respect to all the essential amino acids rather than simply the first limiting amino acid, because each amino acid has its own specific peculiarity and are all

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Table 4 Chemical score of selected yeast species and reference protein ingredients for Atlantic salmon and rainbow trout†

	SC	CJ	KM	BA	WA	SBM	FM
Arginine	67.3	80.1	65.3	35.7	74.5	123.2	100.0
Histidine	74.2	68.0	64.8	81.3	95.0	105.3	100.0
Isoleucine	81.6	78.5	76.1	81.8	94.9	91.3	100.0
Leucine	75.8	72.0	74.4	71.7	80.2	96.0	100.0
Lysine	73.4	79.0	77.8	75.9	78.2	77.7	100.0
Methionine	53.2	31.6	38.5	40.3	44.3	43.9	100.0
Phenylalanine	86.1	79.7	84.7	76.4	85.7	115.0	100.0
Threonine	90.1	94.3	102.4	75.8	94.3	86.1	100.0
Valine	85.2	82.8	75.7	85.1	74.9	96.0	100.0

BA, *Blastobotrys adeninivorans*; CJ, *Cyberlindnera jadinii*; FM, Fishmeal; KM, *Kluyveromyces marxianus*; SBM, soya bean meal; SC, *Saccharomyces cerevisiae*; WA, *Wickerhamomyces anomalus*.

†First, the digestible content of each amino acids was calculated from the total amino acids and protein digestibility coefficients of 80%, 85% and 90% for yeast species, soya bean meal and fishmeal, respectively. The chemical score was then calculated as the ratio of these digestible amino acids and the corresponding requirements in Atlantic salmon and rainbow trout. The values presented are expressed relative to chemical score of fishmeal as the reference protein which is 100 and assumed to be ideal for Atlantic salmon and rainbow trout.

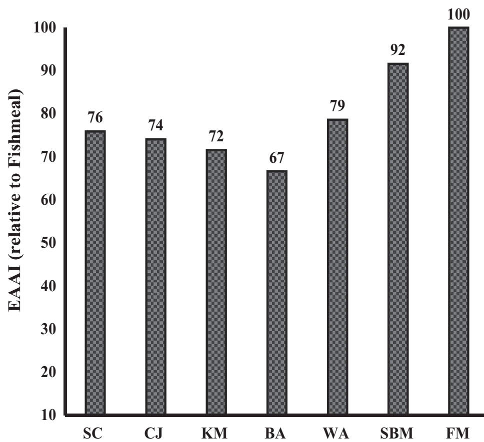

Figure 3 Essential amino acid index (EAAI) of selected yeast species and reference protein ingredients in both Atlantic salmon and rainbow trout†,‡. †The EAAI were calculated based on Equation (1) from the digestible amino acids content of each ingredient and their corresponding requirements in both target fish species. †The EAAI presented are expressed relative to fishmeal as the reference protein which is 100 and assumed to be ideal for Atlantic salmon and rainbow trout. ‡SC, *Saccharomyces cerevisiae*; CJ, *Cyberlindnera jadinii*; KM, *Kluyveromyces marxianus*; BA, *Blastobotrys adeninivorans*; WA, *Wickerhamomyces anomalus*; SBM, soya bean meal and FM, Fishmeal.

equally essential. Thus, EAAI give a true representation of nutritive value of an ingredient, compared to chemical score.

$$\mathrm{EAAI} = \sqrt[n]{\frac{\mathrm{aa1}}{\mathrm{AA1}} \times \frac{\mathrm{aa2}}{\mathrm{AA2}} \times \frac{\mathrm{aa3}}{\mathrm{AA3}} \ldots\ldots\ldots\ldots \frac{\mathrm{aa}n}{\mathrm{AA}n}} \quad (1)$$

Sources: Oser (1951), Veldkamp and Bosch (2015) and Smith (2017).

Where, aa is the percentage of each of the essential amino acids in observed protein source.

AA is the requirement of each of the essential amino acids in the target animals.

n is the total number of amino acids used in the calculation.

Ideal protein concept based on limiting methionine

In this paper, we have established through chemical score that methionine is the first limiting amino acid in the selected yeast species. However, from Table 4, it was evident that aside from methionine, there are other essential amino acids responsible for lower values of EAAI recorded for the selected yeasts compared to fishmeal. To deepen our knowledge further on these other amino acids, a multivariate statistical analysis was conducted on the levels of digestible amino acids in the selected yeast, soya bean meal and fishmeal and their corresponding requirements in both Atlantic salmon and rainbow trout. The levels of digestible arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine and valine of the ingredients were expressed as percentage of digestible methionine (Table S3), according to Faria-Filho *et al.* (2005). Likewise, the corresponding requirements of these amino acids in Atlantic salmon and rainbow trout were also expressed as percentage of methionine (Table S3). Linear discriminate function analysis (DFA) (Seron *et al.* 1998) was performed on these data to identify the amino acids (other than methionine) that better contribute to the differentiation of these ingredients from the amino acid requirements of Atlantic salmon and rainbow trout. Methionine (100%) was excluded because it was the basis for standardising the data and because our aim was to identify other amino acids responsible for the discrimination. The eight remaining amino acids were used as the predictor variables and were linearly combined to obtain three discriminant functions. The first two functions (function 1 = 43.4% and function 2 = 37.8%) explained 81.2% of the variation associated with the multivariate structure on the discriminant analysis function plot (Fig. 4). The discriminant power of the model was significant ($P < 0.001$) based on Wilk's Lambda test of significance. As expected, the scattered distribution on the DFA plot showed that amino acids from fishmeal was not clearly differentiated from the amino acid requirement of Atlantic salmon and rainbow trout (located on the left side of the quadrant), but was discriminated by function 1 from *S. cerevisiae*, *C. jadinii*, *K. marxianus*,

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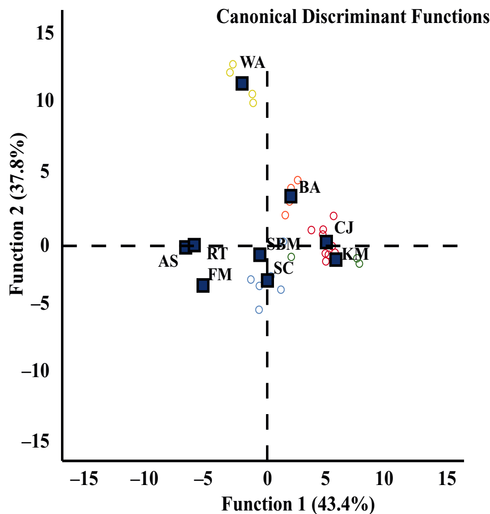

Figure 4 Plot showing the discrimination of the selected protein sources following discriminant function analysis (DFA) of their digestible essential amino acid profile from the corresponding amino acid requirements of Atlantic salmon and rainbow trout. SC, *Saccharomyces cerevisiae*; CJ, *Cyberlindnera jadinii*; KM, *Kluyveromyces marxianus*; BA, *Blastobotrys adeninivorans*; WA, *Wickerhamomyces anomalus*; FM, Fishmeal; SBM, soya bean meal; AS, Atlantic salmon and RT, rainbow trout. (◯) SC; (◯) CJ; (◯) KM; (◯) BA; (◯) WA; (◯) FM; (◯) SBM; (◯) AS; (◯) RT; (■) Group centroid.

B. adeninivorans and soya bean meal (located on the right side of the quadrant). The discriminant power of function 1 was highly influenced by lysine and phenylalanine as indicated by higher positive values of standardised coefficient of variables (Table S4). Function 2, on the other hand, discriminated the amino acid profiles of *W. anomalus* from fishmeal, Atlantic salmon and rainbow trout. Histidine, leucine and isoleucine were the amino acids responsible for the discrimination along function 2 (Table S4). Consistent with the results obtained with chemical score and EAAI, there was no clear discrimination between amino acid profiles of *S. cerevisiae*, *C. jadinii*, *K. marxianus*, *B. adeninivorans* and soya bean meal. Cross-validation of the discriminant model revealed that, among the yeasts, all data points were correctly assigned for *S. cerevisiae*, *B. adeninivorans and W. anomalus*. However, the model inaccuracy revealed that two data points for *C. jadinii* were wrongly classified for *K. marxianus*. The data suggest that apart from methionine, lysine and phenylalanine are also responsible for the variation between the amino acid profiles of selected protein sources (i.e. *S. cerevisiae*, *C. jadinii*, *K. marxianus*, *B. adeninivorans* and soya bean meal) and fishmeal, and their ability to match the amino acids requirements of Atlantic salmon and rainbow trout. On the contrary, histidine, leucine and isoleucine accounted for the discrimination observed with *W. anomalus*.

From this section, there are indications based on EAAI that *W. anomalus* has the best suited amino acids for Atlantic salmon and rainbow trout among all the yeast considered; whereas *B. adeninivorans* has the least suited amino acid profile. The yeasts *S. cerevisiae*, *K. marxianus* and *C. jadinii* are in-between. Furthermore, the chemical score, EAAI and ideal protein concept based on limiting amino acid used in this article are quick and inexpensive methods to support important conclusions on nutritional value of yeasts, especially on their amino acid (im)balance with respect to the requirements in target fish species. As such, with the emergence of different novel ingredients, these methods could be of valuable assistance in the feed industry for pre-screening of ingredients before delving into the actual fish trials. Despite the benefits accrued with these methods, they are confronted with certain limitations, which are briefly highlighted below.

Methodological constraints

Assumption of a single amino acid digestibility value for all yeasts adopted in this paper may lead to underestimation or overestimation of protein value. Similarly, the digestibility of individual amino acids present in yeasts could have provided the best estimate to predict their nutritional values. These two limitations were not catered for because of the paucity of information on protein and individual amino acid digestibility of the five considered yeasts, implying the need for future research. Taken into consideration the digestibility of protein and individual amino acids, therefore, becomes imperative when predicting the nutritional values of yeasts. Additionally, the chemical score and EAAI models endeavour to take into consideration all essential amino acids present in an ingredient. These methods, however, fail to consider practical scenarios when these yeasts are used in combination with non-target ingredients in typical compound feeds for fish. It is left to be seen whether ingredient–ingredient interaction between these yeasts and non-target ingredients will dampen and/or improve the nutritional quality of yeast covered in this review. Furthermore, the protein quality indices used in this report failed to take into consideration animal related factors, such as feed intake, passage rate, retention time, endogenous losses and rearing conditions which may have significant bearing on how different nutrients are utilised and metabolised by the different fish species. Moreover, other macronutrients (aside protein), micronutrients, antinutritional factors and feed processing conditions, which may positively or negatively impact the nutritional values of an ingredient are also not covered by these models.

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Nutritional values for different fish species

Despite the numerous studies available on the functional benefits of yeast cell wall derivatives in fish (Meena *et al.* 2013; Torrecillas *et al.* 2014), only few studies have considered yeast as a macro protein ingredient in fish feeds. Of the limited available studies, *S. cerevisiae* is the most widely studied as shown in Table 5, and this may be connected with its ubiquitous availability as by-products generated from many industrial processes, including beer, alcohol and bio-ethanol production. In fact, *S. cerevisiae* is regarded as the second most valuable by-product from brewing industry (Ferreira *et al.* 2010) and has potential as valuable raw material for different industrial applications, including feed for different fish species. A majority of studies in aquaculture have shown that *S. cerevisiae* (Table 5) could be used to partly replace fishmeal or soy protein without adverse effect on growth performance of aquatic species, such as Atlantic salmon (Øverland *et al.* 2013), rainbow trout (Huyben *et al.* 2017; Vidakovic *et al.* 2020), Artic charr (Vidakovic *et al.* 2016), catfish (Essa *et al.* 2011; Peterson *et al.* 2012), goldfish (Gumus *et al.* 2016), lake trout (Rumsey *et al.* 1990), Nile tilapia (Abass *et al.* 2018), sea bass (Oliva-Teles & Gonçalves 2001), shrimp (Guo *et al.* 2019) and sea bream (Fronte *et al.* 2019). In general, these studies showed positive responses even at high replacement level of fishmeal protein, except few where high inclusion of *S. cerevisiae* linearly depressed growth and nutrient utilisation in fish. Examples of these are in rainbow trout (Hauptman *et al.* 2014), Atlantic salmon (Øverland *et al.* 2013), Nile tilapia (Ozório *et al.* 2012), Southern African dusky kob (Madibana & Mlambo 2019) and Mirror carp (Omar *et al.* 2012). Fermentation media, yeast strain and post-fermentation processing, as well as fish species and diet formulation are factors that may be responsible for the decreased growth and nutrient utilisation with increasing levels of *S. cerevisiae* in some fish species (Øverland & Skrede 2017). Dietary supplementation of intact *S. cerevisiae* may also be used to modulate intestinal microbiota in fish, such as rainbow trout (Huyben *et al.* 2017) and Beluga sturgeon (Hoseinifar *et al.* 2011).

Limited studies have documented the use of non-saccharomyces yeasts as major protein ingredients in farmed fish (Table 6). *Candida* yeast, especially *C. jadinii*, has been used at different dietary inclusion levels in several species, including Atlantic salmon (Øverland *et al.* 2013; Hansen *et al.* 2019; Sahlmann *et al.* 2019), rainbow trout (Mahnken *et al.* 1980), Coho salmon (Mahnken *et al.* 1980) and shrimp (Babu *et al.* 2013). Similarly, studies have reported possible replacement of fishmeal protein with *K. marxianus* (Øverland *et al.* 2013), *Yarrowia lipolytica* (Hatlen *et al.* 2012), *Rhodotorula mucilaginosa* (Chen *et al.* 2019) and *W. anomalus* (Huyben *et al.* 2017; Vidakovic *et al.* 2020) in various fish species. In general, these studies have shown positive results on performance and overall health status of fish. Furthermore, yeast has been used as an abatement strategy to counteract distal intestine inflammation in Atlantic salmon (Grammes *et al.* 2013; Hansen *et al.* 2019). However, inconsistent responses have been observed on the ability of yeast to alleviate intestinal inflammation in Atlantic salmon. According to Grammes *et al.* (2013), *C. jadinii* supplemented at 20% dietary inclusion level counteracts soya bean meal induced enteritis in Atlantic salmon fed 20% soya bean meal-based diets during the seawater phase. On the contrary, in a recently published article *C. jadinii* addition did not counteract mild intestinal inflammation changes observed in Atlantic salmon reared in freshwater (Hansen *et al.* 2019). In a work by Grammes *et al.* (2013), *K. marxianus* and *S. cerevisiae* had little or no counteracting effect on intestinal inflammation in Atlantic salmon. Thus, the disparity in these results may be due to a number of factors, including yeast species and strain, fermentation media, yeast inclusion levels and rearing phase and age of fish. From the available studies, it is evident that different yeast species can be used as major protein ingredients in fish feeds. However, the optimal inclusion levels of many of these yeasts remain largely undetermined. Therefore, future research is warranted to unravel the optimal inclusion levels of yeasts for different aquaculture species.

Strategies to increase the utilisation of yeast in fish feeds

In spite of the documented nutritive values of yeasts in various fish species (Tables 5 and 6), the incorporation of yeast into commercial aquafeeds is currently constrained by a number of factors. These constraints and possible solutions to overcome them are discussed in the following part of this review.

Nutrient optimisation of yeast through diet formulation

Dietary crystalline amino acids supplementation could be a strategy to augment the imbalance of amino acids present in yeasts. However, post-prandial availability differs between these two classes of amino acids (i.e. the intrinsic amino acids in yeasts and crystalline amino acids); crystalline amino acids tend to be more readily available than intrinsic ones within the intestinal lumen (Berge *et al.* 1994; Yamamoto *et al.* 1998; Larsen *et al.* 2012). Therefore, through diet optimisation, an effective synchronisation strategy between the intrinsic and the crystalline amino acids is warranted in the future to improve dietary utilisation of yeasts as a major protein ingredients in fish feeds. The effects of feeding frequency on amino acid synchronisation and consequently on protein utilisation, are well-documented in fish, such as

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Table 5 Bibliographic review of research with *Saccharomyces cerevisiae* as macro-ingredient in aquaculture feeds

Fish	Duration	Experiment	Results	Reference
African catfish (*Clarias gariepinus*)	186 days	*S. cerevisiae* supplemented at 0–2% dietary inclusion levels	*S. cerevisiae* could be used to improve performance and profitability of African catfish	Essa *et al*. (2011)
Artic charr (*Salvelinus alpinus*)	99 days	Intact and extracted *S. cerevisiae* replacing 40% fishmeal protein	Intact and extracted *S. cerevisiae* could replace 40% fishmeal protein without compromising feed conversion ratio (FCR) in Artic charr	Vidakovic *et al*. (2016)
Goldfish (*Carassius auratus*)	84 days	Replacement of 0–45% dietary fishmeal protein with *S. cerevisiae*	Up to 45% replacement of fishmeal with *S. cerevisiae* improved performance of goldfish	Gumus *et al*. (2016)
Lake trout (*Salvelinus namaycush*)	84 days	six different preparations of *S. cerevisiae* supplementing 50% crude protein in the diets	*S. cerevisiae* could replace up to 50% crude protein in the diet without deleterious effect on growth performance and feed efficiency, optimal result was observed with disrupted yeast cell.	Rumsey *et al*. (1990)
Nile tilapia (*Oreochromis niloticus*)	51 days	*S. cerevisiae* supplemented at 0–40% inclusion level of the experimental diets	Above 15% inclusion level of *S. cerevisiae* linearly decreased growth performance and nutrient utilisation of Nile tilapia	Ozório *et al*. (2012)
Pacu (*Piaractus mesopotamicus*)	54 days	*S. cerevisiae* replacing 0–100% dietary fishmeal protein	50% replacement of dietary fishmeal in the diets of Pacu optimally improved feed efficiency and growth performance.	Ozório *et al*. (2010)
Sea bass (*Dicentrarchus labrax*)	84 days	Partial replacement of fishmeal protein with 0–50% *S. cerevisiae*	*S. cerevisiae* could partially replace up to 50% fishmeal protein in Sea bass, without adverse effect on performance and nutrient retention.	Oliva-Teles and Gonçalves (2001)
Thai Panga (*Pangasianondon hypophthalmus* × *Pangasius bocourti*)	252 days	*S. cerevisiae* substituting 0–75% dietary fishmeal protein	*S. cerevisiae* reduced fish performance, as reflected in significant lower weight gain and FCR compared to fishmeal control. Meat quality was, however, not affected by *S. cerevisiae* supplementation.	Pongpet *et al*. (2016)
Giant freshwater prawn (*Macrobrachium rosenbergii*)	90 days	*S. cerevisiae* replacing 0–60% fishmeal protein in diets of giant freshwater prawn reared in either a recirculating aquaculture system (RAS) or a biofloc system	It was possible to substitute 60% fishmeal protein with *S. cerevisiae* in giant freshwater prawn diets, especially for prawn reared in biofloc system	Nguyen *et al*. (2019)
Gilthead sea bream (*Sparus aurata*)	92 days	*S. cerevisiae* replacing 20% fishmeal protein (4.6% dietary inclusion level)	*S. cerevisiae* could partially replace 20% fishmeal protein without adverse effect on growth performance and gut morphology	Fronte *et al*. (2019)
Hybrid striped bass (*Morone chrysops* × *M. saxatilis*)	Trial 1 - 42 days; Trial 2 - 56 days	In both trials, yeast biomass represented 0–4% dietary inclusion levels	*S. cerevisiae* could be used to enhance growth, feed efficiency and disease resistance of hybrid striped bass	Li and Gatlin (2003)
Nile tilapia (*Oreochromis niloticus*)	84 days	*S. cerevisiae* replacing 0–100% fishmeal protein in diets of Nile tilapia reared in either a recirculating aquaculture system (RAS) or a biofloc system	*S. cerevisiae* could completely replace fishmeal protein in diets of Nile tilapia. Better results were observed in Nile tilapia reared in biofloc environment than in RAS system.	Nhi *et al*. (2018)
Pacific white shrimp (*Litopenaeus vannamei*)	42 days	*S. cerevisiae* replacing 0–24% fishmeal or soya bean meal protein	*S. cerevisiae* could be used as partial replacement for FM or SBM in shrimp diets, without deleterious effect on growth performance, protein retention efficiency and survival	Guo *et al*. (2019)
Pacific white shrimp (*Litopenaeus vannamei*)	56 days	Diets supplemented with 1% yeast hydrolysate or yeast biomass	1% inclusion of yeast hydrolysate or yeast biomass could improve growth performance, enhance innate immunity and strengthen resistance to ammonia nitrogen stress in shrimp.	Jin *et al*. (2018)
South African dusky kob (*Argyrosomus japonicus*)	42 days	Diets supplemented with 0–30% inactivated *S. cerevisiae*	At 5% inclusion level, *S. cerevisiae* that does not compromise growth and health of dusky kob. Growth depressed at dietary supplementation above 5%.	Madibana and Mlambo (2019)

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Table 5 (continued)

Fish	Duration	Experiment	Results	Reference
Beluga sturgeon (*Huso huso*)	42 days	*S. cerevisiae* supplemented at 0–2% dietary inclusion levels	*S. cerevisiae* could be used to improve growth performance and modulates intestinal microbiota, without detrimentally affecting haematological parameters of beluga sturgeon.	Hoseinifar *et al*. (2011)
Rainbow trout (*Oncorhynchus mykiss*)	70 days	Fishmeal protein was replaced with 0–60% *S. cerevisiae* or a mixture (70:30 biomass mix) of W. *anomalus* and *S. cerevisiae*	40% replacement of fishmeal protein with yeast caused no adverse effect on growth performance, nutrient digestibility or intestinal health of rainbow trout	Vidakovic *et al*. (2020)
Rainbow trout (*Oncorhynchus mykiss*)	70 days	Fishmeal protein was replaced with 0–60% *S. cerevisiae* or a mixture (70:30 biomass mix) of W. *anomalus* and *S. cerevisiae*	40% and 60% replacement of fishmeal protein with a mixture of W. anomalus and *S. cerevisiae* modulated the gut microbiota, while 20% replacement and diets with only *s. cerevisiae* had little or no effects in rainbow trout.	Huyben *et al*. (2017)
Nile tilapia (*Oreochromis niloticus*)	84 days	*S. cerevisiae* supplemented with 0–7% in diets.	*S. cerevisiae* enhanced fish tolerance to acute heat and hypoxia condition. It was concluded that *S. cerevisiae* could enhance the growth performance, stress resistance and disease resistance of Nile tilapia.	Abass *et al*. (2018)
Rainbow trout (Oncorhynchus mykiss)	63 days	Grain Distiller Dried Yeast (GDDY) replacing 0–100% fishmeal protein	Further replacement of fishmeal protein beyond 35% GDDY generally decreased fish performance.	Hauptman *et al*. (2014)
Mirror carp (*Cyprinus carpio*)	56 days	Yeast Protein Concentrate (YPC) replacing 0–50% fishmeal protein	YPC could replace half of fishmeal protein in mirror carp without depressing growth performance and health status of the fish. Optimal performance was observed with 15% and 20% replacement of fishmeal protein with YPC	Omar *et al*. (2012)
Channel catfish (*Ictalurus punctatus*)	62 days	NuPro® meal replacing 0–125% fishmeal	NuPro® could replace up to 100% fishmeal without adverse effect on performance of Channel catfish	Peterson *et al*. (2012)
Atlantic salmon (*Salmo salar*)	89 days	*S. cerevisiae* substituted 40% fishmeal protein	*S. cerevisiae* depressed growth performance and nutrient utilisation	Øverland *et al*. (2013)
Atlantic salmon (*Salmo salar*)	28 days	20% each yeast was used in combination with 20% SBM to investigate yeast potential in counteracting SBMIE. FM and SBM were, respectively, used as negative and positive controls	Histopathological examination of the distal intestine showed that *S. cerevisiae* could not be used to counteract SBMIE in Atlantic salmon	Grammes *et al*. (2013)

FM, fishmeal; SBM, soya bean meal; SBMIE, soya bean meal induced enteritis.

common carp (Nwanna *et al.* 2012), rainbow trout (Peragón *et al.* 1992; Barroso *et al.* 1999), channel catfish (Zarate *et al.* 1999) and Nile tilapia (Lanna *et al.* 2016). Therefore, the use of different feeding frequency in yeast diets supplemented with crystalline amino acids could be an interesting area of research in the future.

Hitherto, dietary enzyme supplementations have been used to improve nutritional values of feedstuff in fish (Castillo & Gatlin III 2015; Adeoye *et al.* 2016; Maas *et al.* 2018). This approach could also be used to increase nutrient digestibility and utilisation of yeast in fish. The yeast cell walls contain a complex network of polysaccharides that are unsusceptible to endogenous enzymes produced by aquaculture species. However, this challenge could be ameliorated by dietary supplementation with exogenous enzymes capable of degrading the yeast cell wall and enhance the utilisation of nutrients. Currently, there is a paucity of literature specifically on the role of exogenous enzymes to enhance nutritional value of yeast in various fish species. However, enzymes specific for yeast cell wall components such as mannanase, glucanase, chitinase and glucosidase are commercially available in the market. Therefore, the technical feasibility of unlocking the nutritional potential of various yeast species with these commercially available enzymes, either singly or as cocktail of enzymes could be an interesting area of research in the future.

Promoting increased nutrient digestibility through cost-effective downstream processing

Øverland and Skrede (2017) suggested that downstream processing of yeast after harvesting is imperative to preserve

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Table 6 Bibliographic review of research with non-saccharomyces as macro-ingredients in aquaculture feeds

Fish	Yeast species & duration	Experiment	Results	Reference
Black tiger shrimp (*Panaeus monodon*)	CA & 30 days	Diet contained 10% inclusion level of CA. Diets were given to shrimp at different frequencies (daily, once in three days, once in seven days and once in five days), followed by white spot syndrome virus (WSSV) challenge	CA administered once every 7 days could enhance protective ability of P. monodon against WSSV	Babu *et al*. (2013)
Atlantic salmon (*Salmo salar*)	CU & 56 days split into two periods: 0–28 days freshwater and 28–56 days in salt-water	CU supplemented at 25% dietary inclusion level. The diet was used in a crossover design between the freshwater and salt-water phases of the fish	Feeding yeast containing diets throughout the experiment improved fish performance compared to those receiving control diet. In addition, yeast significantly downregulated the secretion of IFNγ, TNFα, IL-1β, IL-8 and modulated the expression of aquaporin 8 (aqp8ab) superoxide dismutase (sod1) and major histocompatibility complex 1 (mhc1).	Sahlmann *et al*. (2019)
Atlantic salmon (*Salmo salar*)	CU & 48 days	CU supplemented at 30% dietary inclusion level	CU could be included in the diet of Atlantic salmon without negatively affecting weight gain and overall fish health status	Sharma *et al*. (2018)
Atlantic salmon (*Salmo salar*)	CU & 28 days	Graded levels of CU were used in combination with 40% soya bean meal to investigate the potential of CU to counteract SBMIE. FM and SBM based diets were used as the negative and positive controls, respectively.	CU supplementation supports fish performance but was unable to counteract the mild histology changes observed in the distal intestine of SBM fed fish.	Hansen *et al*. (2019)
Shrimp (*Litopenaeus vannamei*)	CU & 29 days	CU replacing 0–100% fishmeal on protein basis	CU could be used to replace up to 60% fishmeal protein without deleterious effect on shrimp performance	Gamboa-Delgado *et al*. (2016)
Atlantic salmon (*Salmo salar*)	CU, KM & 89 days	Each yeast substituted 40% fishmeal protein	CU and KM could replace 40% fishmeal protein without adverse effects on growth performance, nutrient digestibility and retention.	Øverland *et al*. (2013)
Atlantic salmon (*Salmo salar*)	CU, KM & 28 days	20% each yeast was used in combination with 20% SBM to investigate yeast potential in counteracting SBMIE. FM and SBM were, respectively, used as negative and positive controls	Histopathological examination of the distal intestine showed that CU could be used to counteract SBMIE in Atlantic salmon, whereas KM could not.	Grammes *et al*. (2013)
Rainbow trout (*Oncorhynchus mykiss*)	CU	CU replacing 0–35% fishmeal protein	CU could be used in rainbow trout's diet without dietary imbalance or significance loss of growth performance	Martin *et al*. (1993)
Coho salmon (*Oncorhynchus kisutch*)	C & 196 days	Candida yeast replacing 0–100% fishmeal protein	More than 25% replacement of fishmeal protein with Candida yeast depressed growth of Coho salmon. Methionine supplementation could be used to enhance performance at higher level of yeast inclusion.	Mahnken *et al*. (1980)

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Table 6 (continued)

Fish	Yeast species & duration	Experiment	Results	Reference
Rainbow trout (*Salmo gairdneri*)	C & 162 days	Candida yeast replacing 0–40% fishmeal protein	Candida yeast could replace up to 40% fishmeal protein without compromising performance and health status of rainbow trout	Mahnken *et al*. (1980)
Atlantic salmon (*Salmo salar*)	YL & 95 days	YL supplemented 0–30% dietary inclusion levels	Up to 20% dietary inclusion of YL did not compromise fish performance, but apparent digestibility of nutrients linearly declined with increased inclusion of yeast biomass. Yeast supplementation, however, increased the ratio of omega 3 (n-3) fatty acids in the fillet.	Hatlen *et al*. (2012)
Nile tilapia (*Oreochromis niloticus*)	RM & 56 days	RM supplemented at 0–1% dietary inclusion level	Dietary supplementation of RM could be used to enhance growth performance, nutrient composition, immune response and antioxidant capacity of Nile tilapia	Chen *et al*. (2019)
Rainbow trout (*Oncorhynchus mykiss*)	WA + SC & 70 days	Fishmeal protein was replaced with 0–60% mixture (70:30 biomass mix) of WA + SC	40% replacement of fishmeal protein with yeast caused no adverse effect on growth performance, nutrient digestibility or intestinal health of rainbow trout	Vidakovic *et al*. (2020)
Rainbow trout (*Oncorhynchus mykiss*)	WA + SC & 70 days	Fishmeal protein was replaced with 0–60% mixture (70:30 biomass mix) of WA + SC	40% and 60% replacement of fishmeal protein with a mixture of WA + SC modulated the gut microbiota, while 20% replacement and diets with only *s. cerevisiae* had little or no effects in rainbow trout.	Huyben *et al.* (2017)

C, *Candida* sp.; CA, *Candida aquaetxtris*; CU, *Candida utilis*; FM, fishmeal; KM, *Kluyveromyces marxianus*; RM, *Rhodotorula mucilaginosa*; SBM, soya bean meal; SBMIE, soya bean meal induced enteritis; WA + SC, *Wickerhamomyces anomalus* mixed with *Saccharomyces cerevisiae* in a 70:30, respectively biomass mix; YL, *Yarrowia lipolytica*.

valuable nutrients and bioactive components and to improve nutrient digestibility. The rigid cell walls of yeast limits accessibility of digestive enzymes to the intracellular contents and consequently affects utilisation of dietary yeast protein (Murray & Marchant 1986; Rumsey *et al.* 1990; Yamada & Sgarbieri 2005). To the authors' knowledge, this was first investigated by Rumsey *et al.* (1991a) and showed that cell wall disruption improved protein and energy digestibility of brewers' yeast cells, yeast extract and yeast protein isolate compared to intact cells. Other authors have shown that partial or complete disruption of yeast cell walls enhance nutrient digestibility and overall utilisation in Atlantic salmon (Hansen *et al*. 2021), shrimp (Zhao *et al.* 2017) and Arctic charr (Langeland *et al.* 2016). The treatments for rupturing the yeast cell walls range from chemical, enzymatic, physical, to mechanical methods (Nasseri *et al.* 2011; Lapeña *et al.* 2020b). Chemical rupturing can be done by exposing the cell walls to acid or alkaline treatments or a combination of both methods (Schiavone *et al.* 2014). Enzymatic hydrolysis can be performed by autolysis, with the aid of endogenous enzymes encapsulated by the yeast cell walls, or by exogenous enzymes targeting the specific layer of the cell walls (Schiavone *et al.* 2014; Hansen *et al*. 2021). Mechanical disintegration of the cell wall can be done either by crushing, crumbling, grinding, pressure homogenisation or ultrasonification (Nasseri *et al.* 2011; Hansen *et al*. 2021). Cost-effectiveness and intended use of the final yeast products should be of paramount concern while making decisions on the choice of downstream processing to be used. Some downstream methods may be excessively harsh to preserve the bioactive components prevalent on the surface of the cell walls. Therefore, a well-structured balance should be maintained when producing yeast products with nutritional and health beneficial values.

Manipulating the protein quality of yeast through genetic engineering

Research has shown that efforts to increase protein content of yeasts through manipulation of the fermentation media seems to produce minimal improvement, as observed by

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Lapeña *et al.* (2020b) and Lapeña *et al.* (2020a) when yeast quality were optimised by using different fermentation media and growing conditions. Therefore, it becomes imperative to devise other means for increasing protein content and improving the protein quality of yeast. Genetic engineering has potential as a tool for production of high-protein novel yeast strains. Traditionally, novel production strains have been developed by mutagenesis (Guthrie & Fink 2002), breeding (Walker 1998) and evolutionary engineering (Francis & Hansche 1972). More recently, there are different attempts to manipulate the metabolic pathways in order to favour the protein secretion process in yeasts (Tang *et al.* 2015; Bao *et al.* 2017). According to Chiang (2004), metabolic engineering has the potential to develop novel biosynthesis pathways to produce new molecules or existing products that are traditionally made by expensive and complex chemical synthesis routes. Understanding the underlying mechanism behind the protein secretory pathway and its interaction with other cellular processes is key to stimulating protein secretion, and concomitantly protein production in yeast (Huang *et al.* 2017; Wang *et al.* 2019). Improved fermentation capacity and balancing of amino acids in *S. cerevisiae* yeast were achieved by tuning many other cellular processes, particularly energy metabolism (Huang *et al.* 2017). Wang *et al.* (2019) identified nine different genes with functions in cellular metabolism, protein modification and degradation, as well as cell cycle, which upon silencing improved protein production in engineered *S. cerevisiae* cells. Although the two previously cited reports focused on the use of yeast as cell factories to enhance production of specific protein (α-amylase in this case), we suggest that such methodology may be replicated to improve overall protein production of yeast. As such, research into genetic engineering using Crispr technology, gene editing, gene insertion and other forms of advanced techniques should be given utmost attention going forward, in order to create high-quality genetically modified yeast strains that can compete nutritionally with the conventional protein sources in fish feeds.

Increase investment portfolio for yeast production

An additional important constraint hindering the use of yeast as a major protein ingredient in fish feed is limited market availability in terms of quantity needed for commercial aquafeeds. To be considered as viable replacement for conventional fishmeal and soy protein, an alternative protein source must, apart from being nutritionally adequate, be commercially available with consistent supply to the end users. To our knowledge, yeasts are currently not economical as major protein ingredients in aquafeed. However, due to the potential sustainability of such ingredients, large corporate players in the yeast industry, such as Lallemand® (https://www.lallemand.com/), Phileo-Lesaffre® (https://phileo-lesaffre.com/en/) and emerging start-ups like Arbiom® (https://arbiom.com/), as well as Research Centres like Foods of Norway (https://www.foodsofnorway.net/) and others are investing in upscaling and optimising the production process for many yeast species. It is therefore, expected that constraints associated with availability and price will be resolved in the near future.

Impacts on environmental sustainability

Responsible sourcing is crucial to the contribution of feed ingredients to the overall sustainability index of most fish feed industries, and concomitantly fish farms. In this regard, the competitiveness of yeast as a major protein ingredient in fish feeds compared to conventional protein sources depends on its overall environmental contributions to the feed industry. Therefore, for better understanding of environmental impacts attributable to yeast as fish protein ingredients, there is need for holistic life cycle assessment of the process involved during production. Life cycle assessment is an analytical technique used to measure the overall environment impacts within all stages of a product lifecycle. This methodology is not alien to the currently used feed ingredients by the aquaculture industry (Pelletier *et al.* 2009; Henriksson *et al.* 2013; Henriksson *et al.* 2017; Smárason *et al.* 2017; Silva *et al.* 2018; Couture *et al.* 2019). Indeed, several of the formerly mentioned studies have documented the environmental footprint of various ingredients constituting the compound feeds, however, the environmental costs of yeast as potential major fish feed ingredient is conspicuously missing in literature. One major sustainability benefit of microbial products is that they are produced in a closed/controlled environment (fermenters) with strict biosecurity as opposed to GMO crops in open field. To our knowledge, only one study has conducted a direct comparison between the environmental impacts of yeast and that of conventional ingredients in fish feeds (Couture *et al.* 2019). In this study, attributional life cycle assessment (ALCA) was used to document the environmental benefits of replacing soy products with yeast in the diets of Atlantic salmon based on seven resource use and emission indicators: climate change impacts, acidification, freshwater eutrophication, marine eutrophication, land occupation, water consumption and primary production requirement. The authors first compared the environmental impacts of soy protein concentrate and yeast protein concentrate at the level of meal, and subsequently extended the model to measure the impacts when these ingredients are incorporated into two different complete feeds (with other non-target ingredients) of Atlantic salmon (Couture *et al.* 2019). At the level of meal, yeast protein concentrate exhibited drastically lower impacts in all

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categories compared to soy protein concentrate. The author, however, further observed that the environmental benefits accrued with the yeast are dampened by high impacts from the non-target ingredients used in the complete feeds (Couture *et al.* 2019). This implies that a proper combination of ingredients with less environmental footprint is needed to achieve more sustainable aquafeeds, indicating that diversifying alternative protein sources in modern fish diets is likely to be the way forward. Although the results of this assessment showed a potential of yeast to provide better environmental performance than conventional feed resources, more study is needed in the future to substantiate this claim.

Regulation/legislation for use of yeast in animal feeds

The European Commission (EC) Regulation No 68/2013 on the catalogue of feed materials, classified yeast under products obtained by fermentation using micro-organisms, but in which the micro-organisms have been inactivated before use as animal feed (Commission Regulation (EC) 2013). Commission Regulation (EC) 1829/2003 guides the authorisation of genetically modified feed and food materials (Commission Regulation (EC) 2003). This regulation aimed to ensure high protection of human life and health, animal health and welfare, environment and consumer interests in relation to genetically modified food and feed (Commission Regulation (EC) 2003). Currently under these guidelines, only inactivated *S. cerevisiae* and *C. jadinii* among the yeast reviewed are allowed for use as macro-ingredients in feed within the EU. Similarly, these same yeasts are listed as GRAS (Generally Recognised as Safe Substances) under the Food and Drug Administration (FDA) Code of Federal Regulations (21 CFR), indicative of their authorisation as macro-ingredients in the feeds. In contrast, *K. marxianus, B. adeninivorans* and *W. anomalus* are currently unauthorised for use as major feed sources in both the EU and the US. However, it is important to state that *K. marxianus* and *W. anomalus* are listed under qualified presumption of safety biological agents catalogue of European Food Safety Association and listing them in the catalogue of feed material should not be an issue (https://zenodo.org/record/3828466#.Xu2_gGgzbLY). Research in the area of nutritional values, toxicology, safety (to both recipient animals and man), as well as environmental impacts of these three aforementioned yeasts are currently ongoing in different parts of the world. Therefore, dossier application seeking for their authorisation as novel feed ingredients is warranted in the future. It is of note to mention that Commission Regulation No 258/97 (Commission Regulation (EC) 1997) detailed the established procedures for submitting the dossier application for novel food and food ingredients in the EU.

Concluding remarks and future research consideration

With respect to the opinions expressed in this review article, the use of yeast as a sustainable protein ingredient in fish feed appear as technically feasible. Yeast is efficient in converting non-food lignocellulosic biomass to valuable products. Yeasts contain lower crude protein and lipids compared to conventional fishmeal. The amino acid compositions of five yeasts under study are comparable with the fishmeal meal and soy protein currently used in aquafeeds, except for methionine, arginine, lysine and phenylalanine, which are the most frequently limiting essential amino acids for juvenile Atlantic salmon and rainbow trout. Genetic modification or improved nutrient digestibility through exogenous enzymes supplementation and the use of cost-effective downstream processing could be a feasible approach to improve the overall protein quality in yeast. For yeast to become competitive with fishmeal and soy protein in aquafeeds, there is a need for additional investment in large-scale production and at affordable costs for feed manufacturers and fish farmers. Finally, of the five yeast species considered in this article, only *S. cerevisiae* and *C. jadinii* are currently allowed for use in animal feeds under the existing EU and US legislations. In the future, more concerted efforts should be dedicated at reviewing the existing legislations to accommodate more yeasts that are found to be safe for fish, environment and for human consumption of the final products.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Amino acid (g/16g nitrogen) compositions of fishmeal and soybean meal, and their corresponding requirements in Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*).

Table S2. Digestible amino acid contents in g/16g nitrogen (mean values) of selected yeasts, fishmeal, and soybean meal.

Table S3. Ideal amino acid profiles (mean values) of selected yeast species, fishmeal and soybean meal relative to digestible methionine and their corresponding requirements in Atlantic salmon and rainbow trout.

Table S4. Summary of standardized coefficient of variables and variance structure described by the discriminant function analysis (DFA).

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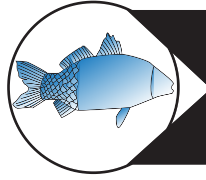

Paper II

scientific reports

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Impact of down-stream processing on functional properties of yeasts and the implications on gut health of Atlantic salmon (*Salmo salar*)

Jeleel Opeyemi Agboola[1]✉, Marion Schiavone[2,4,6], Margareth Øverland[1]✉, Byron Morales-Lange[1], Leidy Lagos[1], Magnus Øverlie Arntzen[3], David Lapeña[3], Vincent G. H. Eijsink[3], Svein Jarle Horn[3], Liv Torunn Mydland[1], Jean Marie François[4], Luis Mercado[5] & Jon Øvrum Hansen[1]✉

Yeasts are becoming popular as novel ingredients in fish feeds because of their potential to support better growth and concomitantly ensure good fish health. Here, three species of yeasts (*Cyberlindnera jadinii*, *Blastobotrys adeninivorans* and *Wickerhamomyces anomalus*), grown on wood sugars and hydrolysates of chicken were subjected to two down-stream processes, either direct heat-inactivation or autolysis, and the feed potential of the resulting yeast preparations was assessed through a feeding trial with Atlantic salmon fry. Histological examination of distal intestine based on widening of lamina propria, showed that autolyzed *W. anomalus* was effective in alleviating mild intestinal enteritis, while only limited effects were observed for other yeasts. Our results showed that the functionality of yeast in counteracting intestinal enteritis in Atlantic salmon was dependent on both the type of yeast and the down-stream processing method, and demonstrated that *C. jadinii* and *W. anomalus* have promising effects on gut health of Atlantic salmon.

Future growth of salmon farming is highly dependent on sustainable feed ingredients that meet the nutritional needs and improve overall health status of fish, at low environmental cost. The growth of the salmon sector imposes demands on feed resources like wild fish stocks, which are under pressure[1,2]. This has led to a change in the salmon feed composition, from being mainly based on marine ingredients towards the use of more plant-based ingredients[3]. Studies have shown that high dietary inclusion of plant ingredients such as soybean meal (SBM)[4–10], pea protein concentrate[11], faba bean[12,13] and corn gluten meal[14], is associated with a condition widely known as SBM induced enteritis (SBMIE) in fish, including Atlantic salmon, rainbow trout and sea bass.

Microbial ingredients such as yeasts[15,16], bacterial meal[17–19] and microalgae[15] have been shown to counteract SBMIE in Atlantic salmon. However, question remains on whether this effect is primarily due to the intrinsic properties of microbial biomass itself, the type of processing or the combination of both. Øverland and Skrede[20] suggested that down-stream processing of yeast after harvesting is imperative to preserve valuable nutrients and bioactive components, and to improve nutrient digestibility in fish. Previously, chemical, enzymatic, physical, and mechanical treatments have been used to enhance the nutritional and functional values of yeasts for various applications[21–24]. Different down-stream processing strategies have shown varying impacts on the integrity and nutritional values of yeast[21,23]. While down-stream processing may increase accessibility to contents of the yeasts cells, methods such as cell crushing using a microfluidizer may be excessively harsh, leading to alteration in bioavailability of the bioactive components[25,26]. Having this in mind and considering cost-effectiveness in terms of energy savings, scalability and commercialization, autolysis was selected as the down-stream processing method in the present study.

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Yeast species[b]	*Cyberlindnera jadinii*		*Blastobotrys adeninivorans*		*Wickerhamomyces anomalus*	
	Inactivated	Autolyzed	Inactivated	Autolyzed	Inactivated	Autolyzed
Dry matter[c]	94.0 ± 0.01	92.4 ± 0.10	95.3 ± 0.05	94.3 ± 0.11	94.9 ± 0.00	93.6 ± 0.20
Cell wall polysaccharides (% dry mass)[c]						
α-glucan	2.0 ± 0.04	1.3 ± 0.06	0.7 ± 0.48	0.3 ± 0.01	0.3 ± 0.03	0.3 ± 0.01
β-glucan	20.4 ± 1.71	16.0 ± 1.30	12.3 ± 1.61	10.1 ± 0.92	11.1 ± 0.88	9.6 ± 0.71
Mannan	10.9 ± 0.58	11.5 ± 0.77	10.2 ± 0.80	8.7 ± 0.57	17.8 ± 1.36	16.7 ± 0.91
Chitin	1.1 ± 0.14	1.9 ± 0.08	2.2 ± 0.08	2.1 ± 0.29	2.0 ± 0.24	2.7 ± 0.33
Other components (% dry mass)[c]						
Crude protein	45.6 ± 0.05	47.6 ± 0.09	38.9 ± 0.07	37.4 ± 0.09	52.8 ± 0.05	52.8 ± 0.21
Crude lipids	6.0 ± 0.00	6.2 ± 0.00	8.6 ± 0.00	8.5 ± 0.00	8.8 ± 0.00	9.1 ± 0.02
Ash	7.8 ± 0.04	8.1 ± 0.01	6.1 ± 0.02	6.3 ± 0.00	3.3 ± 0.00	3.2 ± 0.04
Sum of analyzed components[c]	93.8	92.7	78.9	73.4	96.2	94.4

Table 1. Composition of yeast cells with and without autolysis treatment, after the drying process[a]. [a]Cell wall thickness (nm): Inactivated *Cyberlindnera jadinii* = 95.9, autolyzed *C. jadinii* = 80.9, inactivated *Blastobotrys adeninivorans* = 103.6, autolyzed *B. adeninivorans* = 62.2, inactivated *Wickerhamomyces anomalus* = 161.4 and autolyzed *W. anomalus* = 116.5. [b]Composition of ref-*Cyberlindnera jadinii* (% dry weight): α-glucan = 0.9, β-glucan = 15.0, mannans = 9.4, chitin = 1.9, dry matter = 93.2, crude protein = 58.1, crude lipids = 7.0 and ash = 5.4. This is the same yeast used in Grammes et al.[15]. [c]Amounts of α-glucans, β-glucans and mannans of the cell wall are mean value ± SD from triplicate analyses; whereas chitin, dry matter, crude protein, crude lipids and ash were from duplicate analyses. [d]Sum of analyzed components is equal to sum of the cell wall polysaccharides and other components in the yeast ingredients.

Autolysis is a slow process during which cell membrane permeability increases and endogenous lytic enzymes such as proteases, β-glucanases and chitinases are activated within the yeast cells[27,28], leading to lysis of the intracellular components of the cell. Autolysis can be induced at low pH or high temperature[21,23,29–32]. Hernawan and Fleet[29] reported that the ultrastructure and content of yeast cell wall polysaccharides could be modified through autolysis. In addition, using atomic force microscopy (AFM), Schiavone, et al.[32] have shown that autolysis can be used to enhance the adhesive property of mannoprotein present on yeast cell wall. However, to our knowledge, no study has reported the relationship between changes induced by autolysis and the effectiveness of yeast in modulating gut health in Atlantic salmon fry.

The current study was designed to investigate whether the ability of yeast to counteract enteritis is linked to either the type of yeast, with its associated cell wall properties, or to the down-stream processing used during yeast production, or a combination of both factors. To address this question, we used three different non-*Saccharomyces* yeasts, namely *Cyberlindnera jadinii* (anamorph name *Candida utilis*), *Blastobotrys adeninivorans* (synonym *Arxula adeninivorans*) and *Wickerhamomyces anomalus* produced at laboratory scale. The yeasts were selected based on their ability to utilize hydrolyzates of wood and meat co-products, their high growth rate and high protein content as well as their low production of side products such as alcohol[22,33]. Yeasts were subjected to direct inactivation or autolysis and their functionality as feed ingredient was tested using 5% inclusion levels in diets for Atlantic salmon fry. The impact of the down-stream processing on the yeast cells and the impact of the yeast cells on salmon performance were assessed using a variety of methods.

Results

Production of yeast. The three types of yeast were produced by fermentation at 20 or 200 L scale using a growth medium based on wood-derived sugars and a hydrolysate of by-products from chicken[22]. The yeast cells were harvested and washed before either spray-drying directly or autolysis followed by spray-drying. Table 1 shows compositional data for the various yeast preparations. The contents (% of dry mass) of the cell wall components β-glucan, mannan, and chitin ranged between 9.6% and 20.4%, 8.7% and 17.8%, and 1.1% and 2.7%, respectively. The total glucan content of *C. jadinii* was 40–80% higher compared to *B. adeninivorans* and *W. anomalus*. On the other hand, the mannan and chitin contents of *W. anomalus* were 30–40% and 17–60% higher compared to *C. jadinii* and *B. adeninivorans*, respectively. Autolysis reduced the glucan content by 20%, 13% and 18% for *C. jadinii, W. anomalus* and *B. adeninivorans,* respectively. There was no reduction in mannan content after autolysis for *C. jadinii,* whereas the mannan contents of the other yeasts were reduced by 5 to 15%. The chitin content was lower in autolyzed yeasts compared to inactivated yeasts, except for *B. adeninivorans*.

The contents of crude protein, crude lipids and ash were mostly unaffected by autolysis (Table 1). *W. anomalus* had the highest crude protein (52–53%) and crude lipids content (8–9%) compared to *C. jadinii* (45–48% for crude protein and 6–6.2% for crude lipids) and *B. adeninivorans* (37–39% for crude protein and 8.5–8.6% for crude lipids). The ash contents ranged from 3–8%. The sum of detected compounds showed values close to 100% for *C. jadinii* (93.2%) and *W. anomalus* (95.3%), whereas this value was clearly lower for *B. adeninivorans* (76.1%), suggesting that *B. adeninivorans* contained components that were underestimated and/or undetected by our analysis (Table 1).

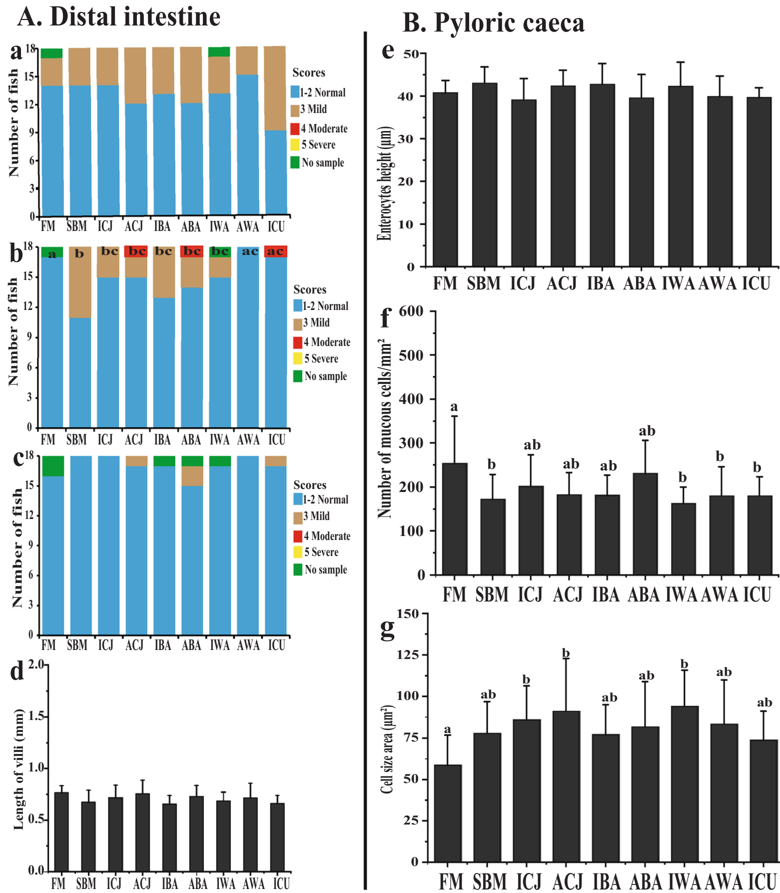

Figure 1. Morphological and histopathological changes in the distal intestine (**A**) and pyloric caeca (**B**) of Atlantic salmon fry fed FM-based diet or SBM-based diets with yeasts. The histological scores were obtained through a semi-quantitative scoring system measuring changes in three morphological parameters: (**a**) loss of supranuclear vacuoles in absorptive enterocytes; (**b**) widening of the lamina propria in mucosal folds; (**c**) increase of connective tissue between base of folds and stratum compactum; and measurement of villi length (**d**). Each parameter (**a**–**c**) was given a score of "**1**–**2**" representing normal morphology; "**3**–**4**" mild and moderate enteritis; whereas "**5**" denotes severe enteritis. For changes in pyloric caeca, the enterocyte height (**e**), the number of mucous cells/mm^2 mucosal area (**f**) and average mucous cell size (**g**) in the mucosal area are presented. Groups with different letters (**a**–**c**) above the bar charts are significantly different ($P < 0.05$). The **green bar** represents the number of missing fish samples. The diets are: **FM**-fishmeal-based; **SBM**-Soybean meal-based; 7 other diets containing 40% SBM and 5% of inactivated *Cyberlindnera jadinii* (**ICJ**), autolyzed *C. jadinii* (**ACJ**), inactivated *Blastobotrys adeninivorans* (**IBA**), autolyzed *B. adeninivorans* (**ABA**), inactivated *Wickerhamomyces anomalus* (**IWA**), autolyzed *W. anomalus* (**AWA**) and ref-*C. jadinii* (**ICU**).

Fish growth performance. To assess the impact of the yeasts on SBMIE in Atlantic salmon fry, a feeding trial was conducted where fish were raised from an average initial weight of 5 to 25 g during the experimental period (Supplementary Fig. S3a). During this period, no mortality or abnormal behaviour were observed. There were no significant differences in feed intake, biomass gain and SGR ($P > 0.05$) between the various dietary treatments (Supplementary Fig. S3b, d). The FM fed fish had lower FCR compared to the other dietary treatments (Supplementary Fig. S3c).

Morphology and histopathological changes in fish. Fish fed with the FM diet showed normal distal intestine morphology, whereas fish fed the SBM diet developed mild signs of SBMIE (Fig. 1b and Supplementary Fig. S2a, b). Considering widening of the lamina propria, fish fed the AWA or ICU diet showed only mild signs of SBMIE that were not statistically different ($P > 0.05$) from the FM treatment (Fig. 1b). Thus, based on

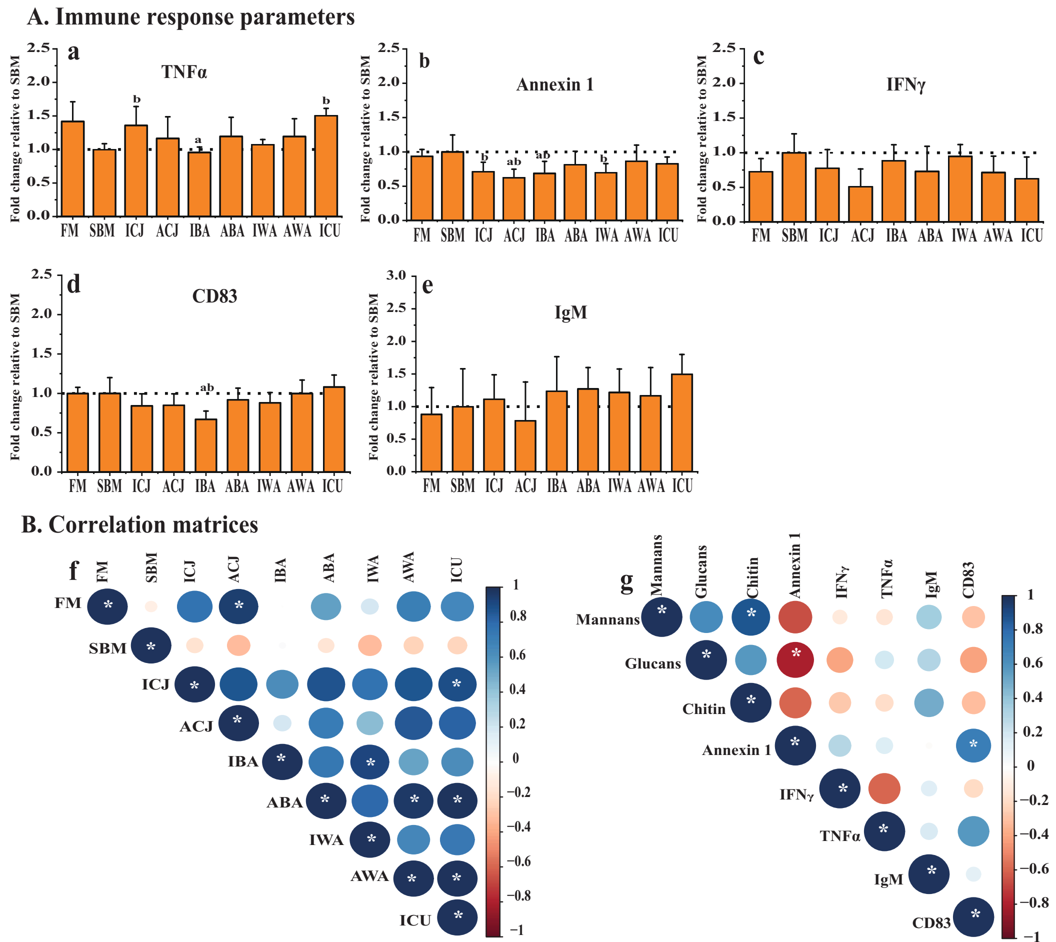

Figure 2. Immune responses (**A**) of Atlantic salmon fry fed soybean meal-based diets with yeasts. Protein expression values for distal intestine (**a**–**e**) were obtained by indirect ELISA and are expressed as fold-change relative to the value obtained for the SBM group. Correlation matrices between the immune markers, and calculated mannan, glucan and chitin intake are shown in panel (**B**). Graph (**f**) shows the correlation between all the experimental diets using five immunological markers (**TNFα**, **Annexin 1**, **IFNγ, CD83 and IgM**). Graph (**g**) shows the correlation between the calculated average daily intake of glucan, mannan and chitin, and the previously mentioned immune markers. The average daily intake of glucans, mannans and chitin were calculated from average dry matter daily feed intake and the composition of the respective cell wall components in each yeast (Table 1). For the correlation matrices, **positive** correlations are displayed in **blue** and **negative** correlations in **red** color; both the **color intensity** and the **size of the circle** are proportional to the correlation coefficients. The diets are: **FM**-fishmeal-based; **SBM**-Soybean meal-based; 7 other diets containing 40% SBM and 5% of inactivated *Cyberlindnera jadinii* (**ICJ**), autolyzed *C. jadinii* (**ACJ**), inactivated *Blastobotrys adeninivorans* (**IBA**), autolyzed *B. adeninivorans* (**ABA**), inactivated *Wickerhamomyces anomalus* (**IWA**), autolyzed *W. anomalus* (**AWA**) and ref-*C. jadinii* (**ICU**). The letters **a** and **b** directly above the bar charts (**a**–**e**) denote treatment(s) with a statistical difference ($P < 0.05$) compared to the fishmeal and soybean meal control groups, respectively. Correlations (**f, g**) with significant values at $P < 0.05$ are shown with *.

this parameter, the AWA and ICU diets led to suppression of SBMIE in the SBM control diet. Fish fed either the ACJ ($P = 0.072$) or IWA ($P = 0.067$) diets showed mild signs of SBMIE in the distal intestine and showed a tendency to be statistically distinguishable from the SBM group (Fig. 1b). Fish fed either ICJ, IBA or ABA were not statistically different from the SBM control. When considering changes in supranuclear vacuoles (Fig. 1a) and connective tissue (Fig. 1c) in absorptive enterocytes, there were no differences between the diets. Morphometric measurements of villi length showed there was no significant difference among the diets (Fig. 1d). Similarly, there was no significant variation in the morphological measurement of the enterocyte height of the pyloric caeca among the diets (Fig. 1e). Also, the number and size of mucous cells in the mucosal area of pyloric caeca were significantly different between the diets (Fig. 1f, g).

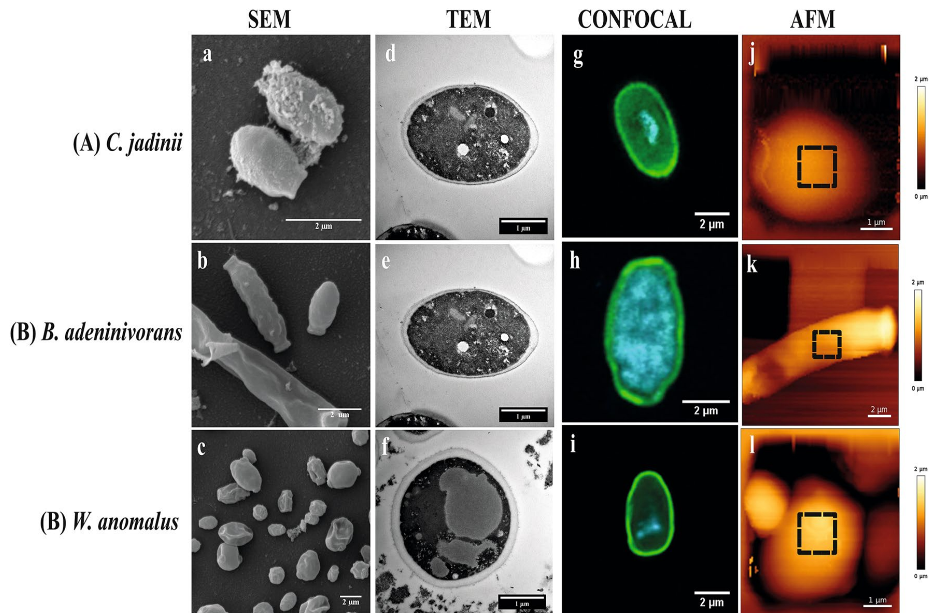

Figure 3. Cell surface architecture of three inactivated yeast species grown on sugars from lignocellulosic biomass. The pictures show Scanning Electron Microscopy (**SEM**; **a**–**c**), Transmission Electron Microscopy (**TEM**; **d**–**f**), Confocal microscopy (stained with concanavalin A-FITC for mannan) (**g**–**i**) and Atomic Force Microscopy (**AFM**; height) (**j**–**l**) micrographs of *Cyberlindnera jadinii* (**panel A**), *Blastobotrys adeninivorans* (**panel B**) and *Wickerhamomyces anomalus* (**panel C**). The SEM and TEM micrographs were taken on yeast creams (before drying), whereas the confocal and AFM micrographs were taken on dried yeast samples, as described in the 'Material and Methods'. The dotted squares on the AFM height micrographs represent the spots where mapping was done for determination of the Young modulus and measurement of adhesion events, as described in 'Material and Methods'.

Changes in immune response parameters. Measurements of protein expression of five different markers indicative of immunological response in the distal intestine showed significant differences between the FM and SBM control diets for TNFα and Annexin 1 (Fig. 2a, b). There was a significant increase ($P < 0.05$) in the level of TNFα in fish fed the ICJ and ICU diets compared to the SBM control (Fig. 2a). In addition, fish fed the yeast diets ACJ and IBA showed significantly reduced levels of Annexin 1, relative to the SBM control (Fig. 2b). No significant differences were observed in the level of CD83 for all the experimental diets, except for the IBA diet, which gave a significantly lower CD83 level compared to both the FM and SBM controls (Fig. 2d). No significant differences were detected in the levels of IFNγ and IgM (Fig. 2c, e).

Relationships among all diets based on the five immune markers, showed a positive and significant correlation ($P < 0.05$) between the FM control diet and the ACJ diet (Fig. 2f). Also, several of the experimental diets (ICJ/ICU, IBA/IWA, ABA/AWA, ABA/ICU and AWA/ICU) showed significant positive correlations ($P < 0.05$) (Fig. 2f). Furthermore, we examined possible correlations between cell wall components of yeasts consumed by the fish and the measured immune parameters. The daily intake of glucans, mannans and chitin was calculated from average daily feed intake (dry matter) and the presence of the respective cell wall components in each yeast (Table 1). Based on these calculations, fish fed ICJ/ACJ consumed the highest amounts of glucans (3–4 mg per day), whereas fish fed IWA/AWA had the highest intake of mannans (3–3.2 mg per day) (Supplementary Table S3). The results of the correlation analysis showed a negative and significant relationship between Annexin 1 expression in the distal intestine and glucan intake (Fig. 2g).

Yeast ultrastructure and cell wall composition. SEM micrographs (Figs. 3 and 4) showed that *C. jadinii* and *W. anomalus* have ovoid-like shape, whereas *B. adeninivorans* has a rod-like shape. The inactivated yeasts (Fig. 3a–c) appeared to have smooth surfaces with no wrinkles, whereas the autolyzed yeasts (Fig. 4a–c) appeared shrivelled and partly broken, seemingly releasing their intracellular contents. These observations were further confirmed by the TEM micrographs, which showed that the intracellular compartment in inactivated cells (Fig. 3d–f) is compact, with visible organelles, whereas autolyzed cells showed a destroyed intracellular structure (Fig. 4d–f). Measurements of cell wall thickness showed that, among the three inactivated yeasts, *W. anomalus* had the thickest cell wall (ca. 160 nm), followed by *B. adeninivorans* (ca. 104 nm) and *C. jadinii* (ca. 96 nm), which both had considerably thinner cell walls (Table 1). Autolysis reduced the cell wall thickness of all the three yeasts, but the extent of this reduction varied. *B. adeninivorans* was mostly affected by autolysis,

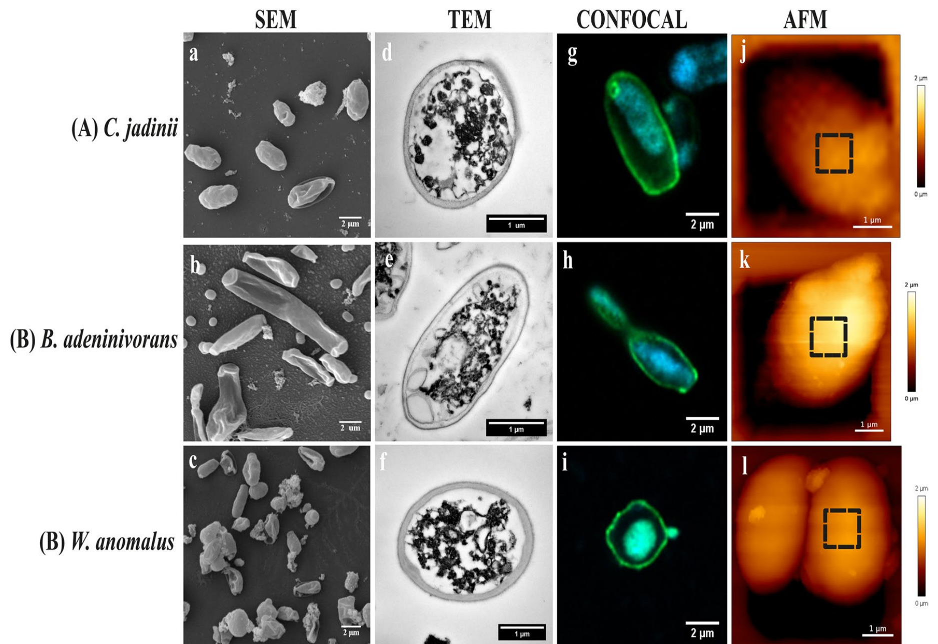

Figure 4. Cell surface architecture of three autolyzed yeast species (50 °C for 16 h) grown on sugars from lignocellulosic biomass. The pictures show Scanning Electron Microscopy (**SEM**; **a**–**c**), Transmission Electron Microscopy (**TEM**; **d**–**f**), Confocal microscopy (stained with concanavalin A-FITC for mannan) (**g**–**i**) and Atomic Force Microscopy (**AFM**; height) (**j**–**l**) micrographs of *Cyberlindnera jadinii* (**panel A**), *Blastobotrys adeninivorans* (**panel B**) and *Wickerhamomyces anomalus* (**panel C**). The SEM and TEM micrographs were taken on yeast creams (before drying), whereas the confocal and AFM micrographs were taken on dried yeast samples, as described in the 'Material and Methods'. The dotted squares on the AFM height micrographs represent the spots where mapping was done for determination of the Young modulus and measurement of adhesion events, as described in 'Material and Methods'.

showing a reduction of nearly 40% in cell wall thickness. For *W. anomalus* and *C. jadinii* the reductions were 28% and 16%, respectively (Table 1). Confocal and AFM imaging showed that yeasts were able to retain their shape and structure after the drying process. After the spray-drying process (Fig. 3g–l), yeasts regained their shape like yeast creams i.e. before drying (Fig. 3a–f) and appeared smooth with thicker and intact intracellular layers. In comparison, autolyzed dry yeasts appear roughened and possess thinner and hollow intracellular layers (Fig. 4g–l).

Yeast surface properties determined by AFM. The elasticity of the yeast cells was determined by measuring the Young modulus using AFM with a silicon nitrite cantilever. *C. jadinii* exhibited the lowest Young modulus, 254 ± 12 kPa, while *B. adeninivorans* showed an intermediate value of 509 ± 7 kPa and *W. anomalus* showed the highest value (1126 ± 29 kPa) (Fig. 5a–c). The distributions were bimodal for ICJ and ABA, indicating that the cell elasticity is not homogenous among the cell population of these species. However, for all the three yeasts, autolysis reduced the Young modulus (Fig. 6a–c) and this effect was most pronounced for *B. adeninivorans*. This implies that cell permeability was modified during autolysis in the three yeast species.

The experiments with ConA-functionalized tips at the surface *vs* the force needed to break the interaction are presented in Fig. 5d–l and Fig. 6d–l. The adhesion frequency for inactivated yeast was 65%, 86% and 52%, for *C. jadinii*, *B. adeninivorans* and *W. anomalus,* respectively (Fig. 5j–l). Autolysis decreased the adhesion frequency for *C. jadinii* (22%) and *B. adeninivorans* (29%), but led to a minor increase in adhesion for autolyzed *W. anomalus* (57%) (Fig. 6j–l). The unbinding force or adhesion force of the interaction between the ConA-tip and the yeast cell surface was estimated to be in the range of 44–76 pN and there were no clear trends regarding the effect of autolysis on this force (Figs. 5j–l and 6j–l).

The length of mannoprotein unfolded (nm) differed between the yeast species and declined upon autolysis for *C. jadinii* and *B. adeninivorans*, but not for *W. anomalus*, which had the highest length of mannoprotein unfolded to begin with (Figs. 5g–i; 6g–i). The length of mannoprotein unfolded for inactivated *C. jadinii* and *B. adeninivorans* were around 70% and 20%, lower compared to inactivated *W. anomalus*, respectively. For autolyzed yeasts, the length of mannoprotein unfolded were 78% and 55% lower in *C. jadinii* and *B. adeninivorans*, compared to *W. anomalus*, respectively. Based on adhesion frequency and length of mannoprotein unfolded, it appeared that *B. adeninivorans* was most significantly affected by autolysis. The AFM experiments with ConA-functionalized

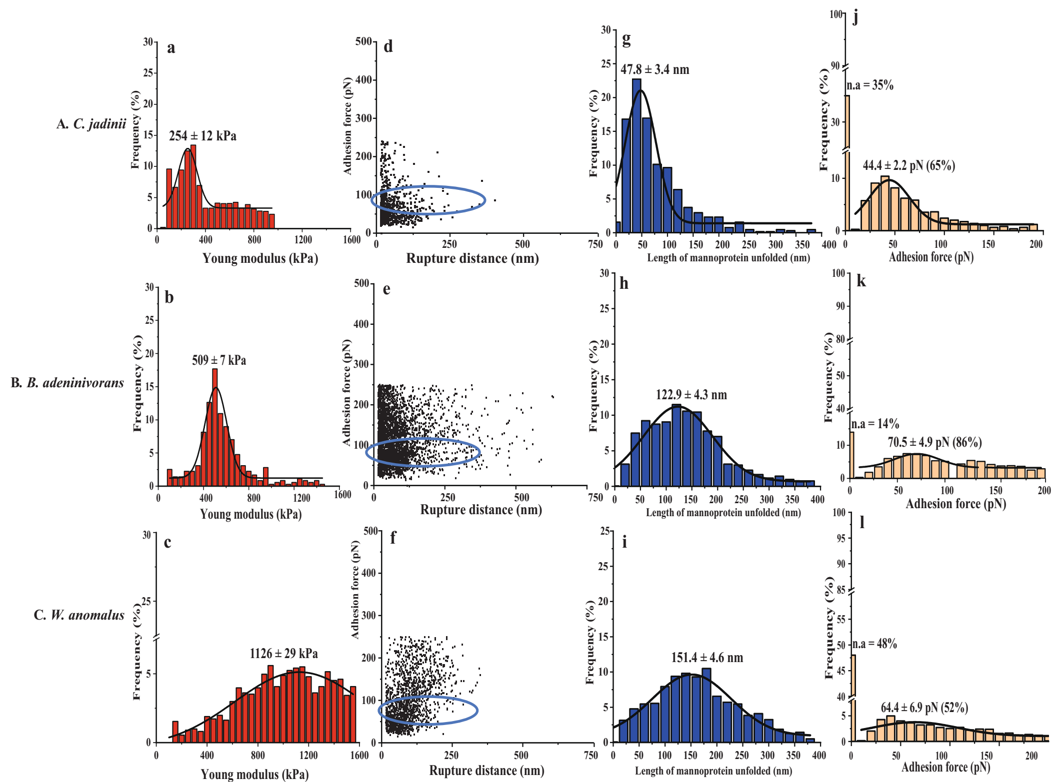

Figure 5. Probing cell wall architecture of three inactivated yeast species (**A**–**C**) with Atomic Force Microscopy, using naked tips (**a**–**c**) or tips functionalized with (mannan-binding) concanavalin A (**d**–**l**). The graphs show the distribution of the Young modulus (**a**–**c**), the relationship between adhesion force and rupture distance (**d**–**f**), the distribution of the length of mannoprotein unfolded (**g**–**i**), and the frequency of adhesion events with varying adhesion forces (**j**–**l**). In panels **j**–**l, n.a**. stands for non-adhesion. Rows **A**, **B** and **C** show results for *Cyberlindnera jadinii*, *Blastobotrys adeninivorans* and *Wickerhamomyces anomalus*, respectively. The data were obtained from 3 cells (3072 curves were analyzed with JPK data processing software before fitting Gaussian curves on the distribution). The **blue circle** highlights the rupture distance at the adhesion force for ConA.

tips also provided insight into the rupture distance, as shown in Figs. 5d–f and 6d–f. The rupture distance ranged from 0–400 nm for *C. jadinii* and *B. adeninivorans*, with a numerically wider distribution towards larger lengths for the autolyzed yeasts (Figs. 5d, e and 6d, e). In contrast, the rupture distance for *W. anomalus* ranged from 0–300 nm and became only slightly larger after autolysis (Figs. 5f and 6f).

Confocal micrographs (Figs. 3g–i; 4g–i), confirmed the expected specificity of ConA for mannans, as shown by the localized green coloration along the most exterior part of the yeast cell walls, where mannoproteins are predominately expected. Potential correlations between chemical composition of the cell wall and AFM-probed cell surface properties are presented in Supplementary Fig. S4. The data showed that there are significant positive correlations ($P < 0.05$) between mannan content, cell wall thickness and Young modulus. Also, length of mannoprotein unfolded and adhesion force showed positive, but insignificant correlations ($P > 0.05$) with the mannan contents of the yeasts. In contrast, there was a significant negative correlation between glucans and the AFM-derived parameters.

Discussion

The current study shows that all the three yeasts could be used at 5% dietary inclusion level without compromising the performance of Atlantic salmon fry. Feed intake and biomass gain of fish were not affected by the dietary treatments; thus, we can assume that the observed differences in health-related parameters are mainly due to dietary treatments and not to differences in fish weight or feed consumption. The present study provides insight into the ability of the three yeast species to counteract SBMIE in Atlantic salmon fry, with particular focus on the effect of yeast autolysis.

Although dietary exposure to SBM is known to induce SBMIE in the seawater phase of the Atlantic salmon[4,9,15], the effects are less severe during the freshwater phase[16,34,35]. In the current freshwater experiment, the histological findings were in accordance with previous studies performed in juveniles[16,34,35], with only mild

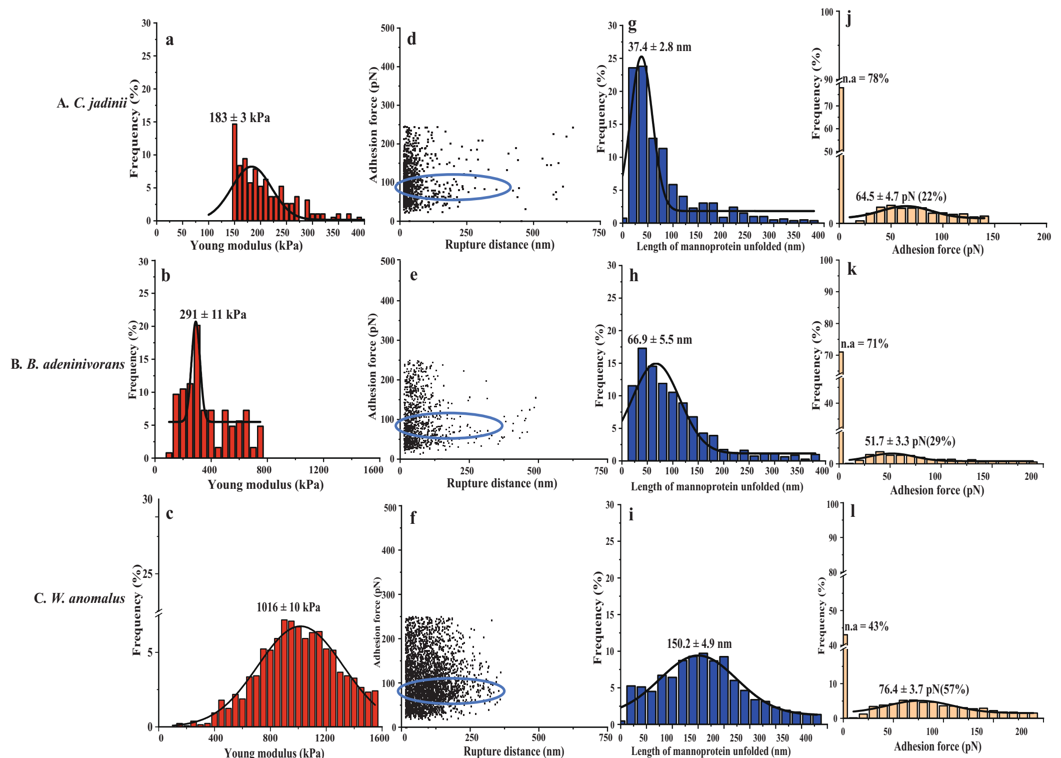

Figure 6. Probing cell wall architecture of three autolyzed yeast species (**A**–**C**) with Atomic Force Microscopy, using naked tips (**a**–**c**) or tips functionalized with (mannan-binding) concanavalin A (**d**–**l**). The graphs show the distribution of the Young modulus (**a**–**c**), the relationship between adhesion force and rupture distance (**d**–**f**), the distribution of the length of mannoprotein unfolded (**g**–**i**), and the frequency of adhesion events with varying adhesion forces (**j**–**l**). In panels **j**–**l, n.a**. stands for non-adhesion. Rows **A**, **B** and **C** show results for *Cyberlindnera jadinii*, *Blastobotrys adeninivorans* and *Wickerhamomyces anomalus*, respectively. The data were obtained from 3 cells (3072 curves were analyzed with JPK data processing software before fitting Gaussian curves on the distribution). The blue circle highlights the rupture distance at the adhesion force for ConA.

SBMIE symptoms detected. It has been suggested that the immaturity of intestinal functions may be responsible for the mild inflammatory changes observed in juveniles, compared to post-smolt salmons[34,35]. Despite mild symptoms, our results show that both AWA and ICU were efficient in alleviating SBMIE, as demonstrated by changes associated with widening of lamina propria, similar to those fed the FM control diet. ACJ and IWA had smaller non-significant effects on preventing SBMIE, while ICJ, IBA and ABA had no effects. These observations are in agreement with an earlier study of Grammes, et al.[15], which showed that inactive dry *C. jadinii* and *Kluyveromyces marxianus* can be used to mitigate SBMIE in Atlantic salmon reared in seawater. Interestingly, the same yeast (ICU) used in the study of Grammes, et al.[15], showed similar effect in our study, reinforcing our choice of a positive control. Our data also showed that the health beneficial effects of yeast depend on the type of yeast and the processing condition used after harvest.

Although the aetiology of SBMIE has been linked to the saponin content of SBM[4,6], the exact mechanism of action is still debatable. Hitherto, amino acid and fatty acid metabolism, T-cell mediation, intestinal dysbiosis, and immune responses have been linked to SBMIE in Atlantic salmon[5,15,17,36,37]. Studies have shown a consistent abundancy of enteropathogenic bacteria[15,37] and revealed that NOD-like receptors[15] and Toll-like receptors[7] are activated in fish suffering from SBMIE. Based on this information, we propose two possible pathways through which AWA, ICU, IWA and ACJ could alleviate SBMIE in the present experiment.

The first proposed mode of action is activation of the immune system by yeast cell wall components. In higher vertebrates, β-glucan exerts its mode of action by binding to dectin-1 receptors expressed on the surface of several innate immune cells such as dendritic cells, neutrophils, eosinophils, macrophages, monocytes and some T-cells[38]. It has been shown that the dectin-1 receptor synergizing with Toll-like receptors can modulate the production of TNFα in mice[39]. Similarly, the involvement of mannan in immune system activation has been reported in literature[40,41] Mannan activates the immune system through C-type lectin receptors such as mannose receptors, dectin-2, dectin-3, galectin-3 and Toll-like receptors present in several immune cells[40,41]. In addition, a previous study has shown that loss of mannan in mutant yeasts reduces the levels of TNFα and IL-6 in human monocytes, which demonstrates the importance of mannan as an inducer of cytokine production in immune

cells[42]. However, in fish, the presence of the dectin-1 receptor, along with the entire superfamily V of C-type lectin receptors, is still debatable[43]. Our results showed that only fish fed ICJ, ACJ, AWA and ICU had increased TNFα levels compared to SBM. The other yeast treatments did not promote increased production of TNFα compared to the SBM control group. TNFα is a pro-inflammatory cytokine, involved in an early stage of the immune response and has a key role during the inflammatory process by regulating the proliferation, migration and phagocytic activity of leukocytes[44]. Increased levels of TNFα may be related to the health beneficial effect of yeast in functional feeds, as reported in previous study[45].

Increased TNFα production was counterbalanced by reduced Annexin 1 production in FM/ICJ/ICU fed fish, indicating that the immune responses were normalized in fish fed these diets. Our results showed that Annexin 1 production reduced in fish fed ICJ, ACJ, IBA, IWA, AWA and ICU diets, compared to SBM fed fish. Annexin 1 is an important marker for anti-inflammatory responses and has protective properties in the gut, as indicated previously being up-regulated in distal intestine of fish suffering from SBMIE[46]. Similarly, it has been reported that Annexin 1 was up-regulated during response to inflammatory bowel disease in humans[47], which resemble SBMIE in fish, as reported in previous literature[15,48] The positive correlation in immunological responses between the FM, ACJ, AWA and ICU diets suggested that the ability of ACJ, AWA and ICU to counteract SBMIE was linked to immune responses. Furthermore, the difference between ICJ-ACJ and IWA-AWA, in alleviating SBMIE, may be linked to accessibility of specific immune receptors (dectin-1, dectin-2 etc.) with yeast cell wall components[40,42].

The second mechanism of action by which yeasts may counteract intestinal enteritis is through binding of its mannans with mannose-specific lectin-type receptors of enteropathogenic bacteria, thereby preventing adhesion of these bacteria to the surface glycoproteins of intestinal villi[49]. This is supported by our AFM experiment with ConA, which allow us to determine the adhesive (binding) capacity of mannan molecules on the surface of the yeast cells. The specificity of ConA for mannans was confirmed by the immunofluorescence analysis in this experiment, which is in contrast to the earlier approach where D-mannose was used to antagonize the surface of the AFM functionalized tip[50,51]. The binding capacity of yeast could be associated with the amount of mannan present on its cell wall. Our results showed that *W. anomalus* contained the highest amount of mannan, which may account for the improved protection against SBMIE, compared to the other yeasts.

The adhesion frequency influences the binding capacity of yeast cells and give an indication of distribution and accessibility of the mannoproteins on the surface of the yeast cell wall. High adhesion frequency suggests that mannoproteins are more accessible for interaction. In this study, adhesion frequency was reduced with autolysis in *C. jadinii* and *B. adeninivorans* yeast, but slightly increased in *W. anomalus*. This difference may explain the improved protection of AWA against SBMIE, compared with IWA. There is an indication that the length, type and flexibility of mannoprotein unfolded[52] and the branching and structure of its α-mannoside residues[49,53] may contribute to the adhesive properties of the yeast cell. In the present study, the length of mannoprotein unfolded ranged between 45–150 nm for the three yeasts and was higher than those observed for different strains of *S. cerevisiae*[52]. The difference in length of mannoprotein unfolded is an indication that the length of the mannan chains that made up the cell wall protein differed among the three yeasts, with IWA/AWA having the longest. Positive correlation between mannan content and length of mannoprotein unfolded indicates that mannan composition is linked to the stretching of mannoprotein on the surface of the yeast cells. Furthermore, the rupture distance gives useful information on the flexibility and extension of the mannoproteins[32,52]. The rupture distance ranged from 0–400 nm in *C. jadinii* and *B. adeninivorans*, with a slightly wider distribution towards larger lengths for the autolyzed yeasts. In contrast, rupture distance in *W. anomalus* ranged from 0–300 nm, and became slightly longer due to autolysis. The variation in rupture distance suggested that anchorage of mannoprotein differed among the yeasts[52]. This may indicate that the health beneficial effects of AWA were linked to its capability to adhere better with enteropathogenic bacteria, compared to the other yeasts.

Data from our AFM and cell wall thickness measurements indicated that *W. anomalus* had the highest mannoprotein levels, increased adhesion frequency when autolyzed and had the largest cell size compared to the other yeasts. Thus, the accessibility of mannoproteins can be a decisive factor for the protective effect of *W. anomalus* against SBMIE in Atlantic salmon fry. Moreover, the fact that the autolysis process increased this effect can be due to alteration of the cell wall surface, as shown by the change in the rupture distance. This observation support previous assertion of Firon, et al.[49], who argued that the relationship between mannan concentration and pathogen adhesion is not always direct, indicating that involvement of other factors such as length, type and flexibility of mannoproteins, are key to the binding capacity of yeast. Although the present study indicates positive effects of AWA and ICU on SBMIE, further in vivo experiments, using the SBMIE model with Atlantic salmon in seawater is warranted to study the effect of different yeast strains and down-stream processing on gut health. Likewise, validation of these results with similar yeasts produced under industrial scale is recommended in the future. Furthermore, the diets in this trial were produced with cold-pelleting processing which differs from extrusion processing; however, in future studies, extruded diets are recommended to document the applicability of these yeasts in commercial salmon production.

In conclusion, this study demonstrates that the yeast strains *C. jadinii* and *W. anomalus* showed the most promising effect on gut health of Atlantic salmon, as demonstrated by histological changes based on widening of lamina propria, as well as changes in immune response parameters. Furthermore, processing by autolysis improved the health beneficial effect of the *W. anomalus*. Our data show that *C. jadinii* and *W. anomalus*, which has shown high productivity in previous fermentation studies, have potential for reducing SBMIE in Atlantic salmon. The results also showed that the amounts, length and accessibility of cell wall components (β-glucans and mannoproteins) could be decisive factors for the protective effects of yeast against SBMIE in Atlantic salmon fry. The functionality of yeast in counteracting intestinal enteritis in Atlantic salmon fry is dependent on the yeast species and the down-stream processing used during yeast production.

Materials and methods

Yeast production and processing. The yeasts *C. jadinii* and *B. adeninivorans* were cultivated in a company demonstration plant at 200 L scale (Biorefinery Demo, Borregaard AS, Sarpsborg, Norway), using a medium composed of enzymatic hydrolyzates of pre-treated spruce wood (*Picea abies*)[54] and hydrolyzates of chicken by-products (Norilia, Oslo, Norway), as described in Lapeña, et al.[22] *B. adeninivorans* was cultivated for 18.5 h in batch mode, while *C. jadinii* was cultivated for 42 h in fed-batch fermentation mode with the addition of wood sugars, urea, KH_2PO_4, $CaCl_2 \cdot 2H_2O$, $MgSO_4 \cdot 7H_2O$ and NaCl (See supplementary Fig. S1). *W. anomalus* yeast was cultivated in 20 L scale according to the protocols described in Lapeña, et al.[33] For washing, the yeasts were separated by centrifugation and re-suspended in the same volume of 7 °C deionized water in a 30 L EINAR bioreactor system (Belach Bioteknik, Sweden), equipped with a helical impeller. The washed yeasts were then again centrifuged to obtain yeast creams with 12.5%, 5.5% and 15% dry matter contents for *C. jadinii* (CJ), *B. adeninivorans* (BA) *and W. anomalus* (WA), respectively. Half of these microbial biomasses were dried and heat-inactivated by spray-drying using a SPX 150 MS (SPX Flow Technology, Denmark AS) spray-dryer with inlet and outlet temperatures of 180 °C and 80 °C, respectively. The spray-dryer was fitted with a co-current nozzle and the pump speed was set to auto and stabilized at around 35% of maximum speed of the pump. The other half of the yeast creams underwent autolysis by incubating the creams at 50 °C for 16 h in a 30 L EINAR bioreactor system, with constant stirring at 50 rpm using a helical impeller, followed by spray-drying using the same conditions as for the untreated yeast. Dried yeasts were kept at 4 °C until use.

Formulation and production of fish feeds. Nine experimental diets were produced in this experiment. The diets were as follows: a fishmeal (FM) control; a diet with 40% SBM as a positive control; 6 treatment diets containing 40% SBM and 5% yeast ingredients [inactivated CJ (ICJ), autolyzed CJ (ACJ), inactivated BA (IBA), autolyzed BA (ABA), inactivated WA (IWA) and autolyzed WA (AWA)], respectively. An extra control diet containing 40% SBM and 5% of a reference preparation of *C. jadinii* (ICU) already described for its ability to counteract enteritis[15] was also used in this trial. The feed formulation is as presented in supplementary Table S1. The diets were formulated to have a similar ratio of digestible protein to digestible energy, and to meet the nutrient requirements of Atlantic salmon as recommended by NRC[55]. To meet fish amino acid requirements, crystalline lysine and methionine were added to the diets due to the high inclusion of plant-based ingredients. All dry ingredients were mixed in a Spiry 25 dough mixer (Moretti Forni, Mondolfo, Italy). Gelatin was mixed in cold water and heated up to 60 °C in a microwave oven before mixing with dry ingredients and fish oil using the same mixer as above. The mash was cooled down to room temperature prior to cold-pelleting using a P35A pasta extruder (Italgi, Carasco, Italy). The pellets were dried (to about 91% dry matter content) in small experimental dryers at approximately 60 °C drying temperature and stored at 4 °C prior to feeding.

Fish management and feeding. The fish experiment was conducted at the Fish Laboratory of Norwegian University of Life Sciences (NMBU, Ås, Norway), which is an experimental unit approved by the National Animal Research Authority, Norway (Permit No. 174). The experimental procedures were performed in accordance with the institutional and national guidelines under the applicable laws and regulations controlling experiments with live animals in Norway (regulated by the "Norwegian Animal Welfare Act" and "The Norwegian Regulation on Animal Experimentation" derived from the "Directive 2010/63/EU" on the protection of animals used for scientific purposes). The study was carried out in compliance with the ARRIVE guidelines.

In total, 1215 Atlantic salmon fry with an average start weight of 5.71 ± 0.05 g were sorted, batch weighed and randomly distributed into 27 fiberglass tanks (80 L) equipped with automatic feeders. Each tank was randomly stocked with 45 fish. Each diet was fed to triplicate tanks, 20% in excess based on feed consumption in each tank. Feeding was done twice a day with automatic feeders, and uneaten pellets were collected after each feeding from the outlet water settling on a screen for each tank. Daily feed intake was calculated from the dry weight of the feed given and the dry weight of recovered uneaten pellets, adjusted for feed recovery rate from fish tanks. Feeds were kept under refrigerated conditions (4 °C) throughout the experiment. Fish were exposed to a 24 h light regime and recirculated freshwater with an average temperature of 15.0 °C. The water flow was standardized to about 6 L min^{-1}, and the oxygen content of the outlet water was kept within 8.2–10.1 mg L^{-1}. The experiment lasted for 37 days, after which the fish were counted and grouped weighed to estimate the growth performance.

Sampling procedure for fish tissue. For tissue sampling, six fish per tank were randomly selected, anesthetized with metacaine (MS-222; 50 mg L^{-1} water) and killed with a sharp blow to the head. The individual body weight of each fish was recorded and included in the total tank mean. Distal intestine and pyloric caeca tissues were collected from each fish and further processed, as described below. The distal intestine was opened longitudinally, the content was removed and the tissue was carefully divided into two parts: one part was fixed in 10% phosphate-buffered formalin for 24 h before storage in 70% ethanol until further processing for histological analysis; the second part was immediately snap-frozen in liquid nitrogen and stored at −80 °C for indirect enzyme-linked immunosorbent assays (ELISA). Pyloric caeca were treated in the same way as the distal intestine samples for histological analysis.

Morphometric and histological examination of fish tissues. Formalin-fixed distal intestine and pyloric caeca samples were dehydrated in ethanol, equilibrated in xylene and embedded in paraffin using standard histological techniques. Longitudinal sections of approximately 6 μm in thickness were prepared. The sections were stained with hematoxylin, eosin and Alcian blue 8 GX. Changes in villi length were captured using a DMLS light microscope (Leica Microsystems, Wetzlar, Germany) equipped with a Leica E3 digital imaging camera and LAS EZ v4.9 software. Randomly selected villus of 18 distal intestine tissues from each dietary group

(at least 80 measurements per group) was measured from the stratum compactum to the tip of the fold by ImageJ software. For histological evaluation, changes associated with intestinal tissues were blindly evaluated with a focus on the characteristic changes known for SBMIE in Atlantic salmon[9]. The histological scores were obtained through a semi-quantitative scoring system measuring changes in three morphological parameters: loss of supranuclear vacuoles in absorptive enterocytes; widening of lamina propria in mucosal folds; and increase of connective tissue between the base of folds and stratum compactum[9]. Each parameter was given a score of 1–5, where 1–2 represents normal morphology; 3–4 mild and moderate enteritis; and 5 for severe enteritis (Supplementary Fig. S2a, b). To measure changes associated with pyloric caeca, the longitudinal enterocytes area was selected (image 20×) and measured from the base to the apex. Measurement of enterocyte height was performed using the Easy Scan software. The total number and average mucous cell size in the caeca mucosal area were measured using the ImageJ software. The number of mucous cells was counted per 1 mm^2 of the mucosal area (Supplementary Fig. S2c–e).

Indirect ELISA of distal intestine tissues. Immunological parameters were analyzed using the distal intestine samples by indirect ELISA[56]. Briefly, distal intestine samples from nine fish per treatment were homogenized using metal beads and lysis buffer (Tris 20 mM, NaCl 100 mM, Triton X-100 0.05%, EDTA 5 mM, and protease inhibitor cocktail 1×, pH = 7.2). Subsequently, the homogenate was centrifuged at 12,000×g for 25 min at 4 °C. The supernatant containing soluble proteins was stored at −20 °C until use. The protein concentration was quantified using the BCA protein assay kit (Thermo Fisher Scientific) following the manufacturer's instructions. Then, each sample was diluted in carbonate buffer ($NaHCO_3$ 60 mM, pH 9.6) and seeded (in duplicate) in a 96-well plate (Maxisorp, Thermo Fisher Scientific) at 50 ng μL^{-1} (100 µL) for overnight incubation at 4 °C. After blocking with 5% Block solution (Bio-Rad) diluted in PBS, for 2 h at 37 °C, the plates were incubated for 90 min at 37 °C with the first antibody (Supplementary Table S2). Then, the second antibody-HRP (Thermo Fisher Scientific), at 1:7000 dilution, was added, followed by incubation for 1 h at 37 °C. Finally, the chromogenic substrate 3,3′,5,5′-tetramethylbenzidine (Invitrogen) was added (100 µL) followed by incubation for 30 min at room temperature. The reaction was stopped with 50 µL of 1 N sulfuric acid and absorbance at 450 nm was measured using a Spectramax microplate reader (Molecular Devices).

Chemical analysis of yeast and fish feeds. The yeasts and diets were analyzed for dry matter by drying to constant weight at 105 °C (ISO 6496), for crude protein (N × 6.25) using CHNS Elemental Analyzer (Vario El Cube Elemental Analyzer system GmbH, Hanau, Germany), for crude lipid by Accelerated Solvent Extractor (ASE200, Dionex, California, USA) (ISO 6492) and for ash by incineration at 550 °C (ISO 5984). Gross energy content was determined using an adiabatic bomb calorimeter (Parr 1281; Parr Instruments, Moline, IL, United States), according to ISO (1998).

Calculations for fish growth parameters. The average biomass gain, feed conversion ratio (FCR), and specific growth rate (SGR) were calculated according to the equations presented in Agboola, et al.[57]. Briefly, the biomass gain was calculated as the difference between the average final weight and the average initial body weight of fish per tank. The FCR was calculated as the ratio between average feed consumption per day and average biomass gain per day. The SGR was calculated as logarithm differences between average final and initial weight of fish divided by the experimental duration.

Morphology and ultrastructure of the yeast cells. The ultrastructure of yeast cells was examined using a scanning electron microscope (SEM) and a transmission electron microscope (TEM). For each yeast, the SEM and TEM samples were taken before and after the autolysis process, i.e. before spray-drying. Three yeast samples per treatment were prepared according to the procedure described in Straume, et al.[58] for SEM and TEM imaging. Samples for SEM were coated with Pt-Pl and examined in a Zeiss EVO 50 EP (Zeiss International, Germany) scanning electron microscope at an accelerating voltage of 15 kV in the secondary emission mode. The sections for TEM were examined in a FEI Morgagni 268 (FEI, USA) transmission electron microscope, and photographs were recorded with a VELETA camera. The imaging was performed at the Imaging Centre, Faculty of Biosciences, Norwegian University of Life Sciences (NMBU). The cell wall thickness was obtained by measuring the length of five random locations on the cell wall surface of twenty TEM micrographs of each yeast using ImageJ.

Cell surface properties of yeast as determined by AFM. Atomic force microscopy (AFM) measurements were done following the protocol described in Schiavone, et al.[32] and Schiavone, et al.[50]. Experiments were carried out with a Nanowizard III atomic force microscope (Bruker-JPK Instruments). The spring constants of each MLCT cantilever (Bruker) were determined using the thermal noise method[59] and were found to be in the range of 10–20 pN nm^{-1}. Yeast sample preparation was done by re-suspending the dry yeast mass in sodium acetate buffer (18 mM CH_3COONa, pH 5.2, 1 mM $CaCl_2$ and 1 mM $MnCl_2$) and immobilized on polydimethylsiloxane (PDMS) stamps, as described in Dague, et al.[60]. 100 µL of yeast suspension was deposited on the PDMS stamps by convective/capillary assembly. Using bare AFM tips, AFM heights (expressed in nm) were recorded in Quantitative Imaging mode[61] with a maximal force of 1 nN, at 20 °C in buffer solution. Elasticity of cells was determined from 3072 force curves recorded in force volume mode at an applied force to the surface of 0.5 nN and speed of approach and retraction of 2 µm s^{-1}. Elasticity histograms were generated by analyzing the force-distance curves according to the Hertz model described in Schiavone, et al.[52], with an indentation of 50 nm and considering a conical tip geometry with half-opening angle α of 0.31 rad and a Poisson ratio ν of 0.5.

To probe cell surface polysaccharides, AFM tips were functionalized with Concanavalin A (ConA) from *Canavalia ensiformis* (Sigma-Aldrich, L7647) via a silicon nitride dendritip as described in Jauvert, et al.[62]. To analyze the stretching of polysaccharides at the surface of the cell, elongation forces were stretched using the worm-like chain model introduced in Bustamante, et al.[63], which describes the polymer as a curved filament. The contour length from this model represents the length of mannoprotein unfolded. At least three cells were analyzed for each treatment, representing a total of 3072 force curves for each treatment. The force curves were analyzed with the JPK data processing software (JPK BioAFM, Bruker Nano, Germany). All specific adhesion peaks were considered for the histograms, which were generated using the Origin 2020 software (OriginLab Northampton, MA, USA). A Gaussian distribution curve fitted on the histogram was used to determine the maximal values of Young modulus, length of mannoprotein unfolded and adhesion force for each yeast group.

Quantification of yeast cell wall polysaccharides. The total polysaccharide content of the yeast cell wall was estimated without prior cell wall isolation according to the protocol described by François[64]. Briefly, the yeast samples were hydrolyzed with sulphuric acid and the released sugar monomers (mannose, *N*-acetylglucosamine and glucose) were quantified by high-performance anion-exchange chromatography with pulsed amperometric detection as described in Dallies, et al.[65] and Hansen, et al.[23]. The content of β-glucan in the yeast samples was determined using a Megazyme kit (reference K-YBGL) and α-glucan was calculated as the difference between total glucan and β-glucan.

Immunofluorescence analysis of yeast for determining mannan specificity for ConA. Approximately 200 mg of each spray-dried yeast was fixed with 10% formalin for 30 min at room temperature in Eppendorf tubes. Thereafter, the sample was centrifuged at 1000×g for 5 min at 4 °C and re-suspended in PBS. For fluorescence detection of mannan with ConA lectin, the sample was blocked for 1 h at room temperature with PBS containing 1% bovine serum albumin. Subsequently, the sample was incubated with 5 mg mL^{-1} of ConA-conjugated FITC (Sigma-Aldrich) for 1 h at room temperature in the dark. The samples were then gently layered on slides and allowed to dry for 10 min, before mounting in the Vectashield Medium (Vector Lab). Between all the steps of this procedure, the samples were washed in PBS. The slides were analyzed using a Zeiss LSM800 confocal microscope (Zeiss International, Germany).

Statistical analysis. Fish performance, morphometric, histological and immune parameters were analyzed using the SPSS statistical software package version 26 (IBM Institute, Armonk, NY, USA). Fish performance, morphometric and immune response data were tested for treatment effects using one-way ANOVA. Significance difference ($P < 0.05$) between means for fish performance and morphometric data were detected using the Tukey HSD test, whereas, for immune response parameters, Dunnett's multiple comparison test was used for detecting significant differences. Data from morphometry measurements (villi length) was tested for normality by the Shapiro–Wilk test and homogeneity of variance using Levene's test. Data from the histological evaluation were analyzed using a non-parametric Kruskal–Wallis test by ranks followed by Dunn's multiple comparison test. Significance was set at $P < 0.05$. The tank effect was considered for all parameters and found to have no influence on the statistical analyses. Correlation coefficients between the diets using five immune markers were examined using corrplot package in R. Likewise, correlations between dietary intake of yeast cell wall components and immune markers were determine using the same R package. Also, the correlations between cell wall components and AFM data were examined using the corrplot package in R (CRAN: http://cran.r-project.org/package=corrplot).

Data availability

The datasets generated during and/or analysed during this study are available upon reasonable request from the corresponding authors.

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Author contributions

J.O.A., M.Ø., M.Ø.A., V.G.H.E., S.J.H., L.T.M. and J.Ø.H. conceptualized and formulated ideas the for the experiment. All authors contributed to the design of methodology for the experiments. J.O.A., M.S., B.M.L., L.L., D.L. and J.Ø.H. performed the experiments, as well as analyzed and interpreted the data. J.O.A. wrote the original draft of the manuscript. M.Ø., V.G.H.E., S.J.H. and L.T.M. acquired project funding for the experiments. J.Ø.H., M.Ø.A., M.Ø., and J.M.F. supervised planning and execution of all research activities. All authors critically reviewed the manuscript and gave the final approval for submission.

Competing interests

The authors declare no competing interests.

Additional information

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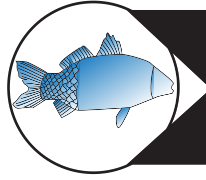

Paper III

frontiers in Immunology

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The Spleen as a Target to Characterize Immunomodulatory Effects of Down-Stream Processed *Cyberlindnera jadinii* Yeasts in Atlantic Salmon Exposed to a Dietary Soybean Meal Challenge

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Aquaculture feeds have changed dramatically from being largely based on fishmeal (FM) towards increased use of plant protein sources, which could impact the fish's immune response. In order to characterize immunomodulatory properties of novel functional ingredients, this study used four diets, one based on FM, a challenging diet with 40% soybean meal (SBM), and two diets containing 40% SBM with 5% of *Cyberlindnera jadinii* yeast exposed to different down-stream processing conditions: heat-inactivated (ICJ) or autolysation (ACJ). The immunomodulatory effects of the diets were analyzed in the spleen of Atlantic salmon after 37 days of feeding, using a transcriptomic evaluation by RNA sequencing (RNA-seq) and the detection of specific immunological markers at the protein level through indirect Enzyme-linked Immunosorbent Assay (indirect ELISA). The results showed that SBM (compared to FM) induced a down-regulation of pathways related to ion binding and transport, along with an increase at the protein level of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNFα) and interferon gamma (IFNγ). On the other hand, while ICJ (compared to FM-group) maintain the inflammatory response associated with SBM, with higher levels of TNFα and IFNγ, and with an upregulation of creatine kinase activity and phosphagen metabolic process, the inclusion of ACJ was able to modulate the response of Atlantic salmon compared to fish fed the SBM-diet by the activation of biological pathways related to endocytosis, Pattern recognition receptor (PPRs)-signal transduction and transporter activity. In addition, ACJ was also able to control the pro-inflammatory profile of SBM, increasing Interleukin 10 (IL-10) levels and decreasing TNFα production, triggering an immune response similar to that of fish fed an FM-based diet. Finally, we suggest that the spleen is a good candidate to characterize the immunomodulatory effects of functional ingredients in Atlantic salmon. Moreover, the inclusion of ACJ in fish diets, with the ability to control inflammatory processes, could be considered in the formulation of sustainable salmon feed.

Keywords: ***Salmo salar*, transcriptomics, ELISA, secondary lymphoid organ, inactivated yeast, autolysed yeast**

INTRODUCTION

In aquaculture, the relationship between nutrition and immune system has been recognized as an important part of the fish production process, due to the maintenance of high production densities continuously faces challenges related to multi-stressor conditions such as infectious diseases and suboptimal nutrition (1). In addition, energy and nutrients provided by the feed are essential to maintain an optimal immune function (2). Future growth in aquaculture depends on feed ingredients that are capable of meeting nutritional needs and of improving the overall health of the fish (3).

The dietary composition of the salmon feed has shifted from marine ingredients such as fishmeal towards increased use of plant ingredients (4, 5). However, high inclusion of plant ingredients such as soybean meal, pea proteins and faba bean in diets for Atlantic salmon (*Salmo salar*) can have adverse effects on growth performance and fish health (6–9). In fish, it has already been described that these plant ingredients, due to an imbalanced nutritional composition, content of fiber and antinutritional factors (ANFs) (10, 11), can induce significant changes in gut-microbiome that affect the mucosal immunity, reducing its protective capacity or causing its overreaction, by increasing the secretion of antimicrobial peptides, immunoglobulins and muc-like proteins (3, 12–14). Considering this, solvent extracted soybean meal (SBM) has been used as a dietary challenge to study the impact of alternative ingredients and functional feed components on gut health. SBM has high level of ANFs, which can disrupt intestinal homeostasis and induce inflammation in the distal intestine, commonly referred to as SBM-induced enteritis (SBMIE) (6, 11, 15).

In recent years, novel microbial ingredients (MI), including bacteria and yeast, are gaining increasing interest as replacement for plant-based diets for salmonids (16, 17). Moreover, these ingredients have other properties beyond their nutritional values such as modulators of fish's immune response (3, 18–22) through components that can be detected as microbial-associated molecular patterns (MAMPs) by pattern recognition receptors (PRRs) in the fish (3, 23, 24).

Furthermore, Grammes et al. (19) reported that the use of MI in feeds could counteract intestinal inflammation in Atlantic salmon. Nevertheless, feeding is a long-term process and its modulation capacity may not only occur locally in the intestine, but also systemically in active immune organs such as the spleen, since immunity has a wide range of cellular and molecular components that can act in an integrated and systemic way. In fish, the spleen has been considered the primordial secondary lymphoid organ with a key role in the antigen presentation processes and lymphocyte activation, promoting humoral immunity, by the cellular coordination of dendritic cells (DC) and with the specific induction of T cell proliferation (25, 26). In addition, in salmonids such as rainbow trout (*Oncorhynchus mykiss*), it has already been described that fish fed functional diets (with the inclusion of *Lentinula edodes*) were able to regulate the acute inflammatory profile in the spleen, reducing possible harmful responses after a LPS-challenge (27). This could be because splenic antigen-presenting cells (APC) would polarize T cells towards regulatory phenotypes, which are important to control the immune responses and in the maintenance of fish homeostasis (28).

Based on this background, the present study proposes the evaluation of the spleen response, as a target organ for the characterization of immunomodulatory effects of down-stream processed *Cyberlindnera Jadinii* in Atlantic salmon exposed to a dietary SBM challenge. To meet this goal, our methodology combines a transcriptomic evaluation by RNA sequencing (RNA-seq) with the specific detection of immunological markers at the protein level by indirect Enzyme-linked Immunosorbent Assay (indirect ELISA). This is in order to increase the knowledge about the modulation of the immune response in Atlantic salmon fed MI.

MATERIALS AND METHODS

Experimental Design

Atlantic salmon with an average starting weight of 5.71 ± 0.06 g were sorted (at an initial stocking density of 3.21 kg/m^3) into 100 L replicated tanks exposed to a 24 h-light regime and recirculated fresh water (15°C). Oxygen content of the water was measured throughout the experiment and was maintained at an average of 9.5 ± 0.5 mg L^{-1}. Moreover, ammonia nitrogen in the recirculating system was kept below the toxic level for the fish, and no mortality or abnormal behavior in any of the fish was recorded in the experimental period.

In each tank, fish were fed for 37 days using one of the four experimental diets: fishmeal diet as a control diet (FM), 40% soybean meal diet as a challenging diet (SBM), and two diets with 40% SBM and 5% inclusion of *C. jadinii* after different downstream processes: heat-inactivated (ICJ) or autolysed (ACJ). *C. jadinii* yeast used in this experiment was produced by fed-batch fermentation using wood sugars as carbon source according to Lapeña et al. (29).

The diet composition was described in **Table 1** and each diet was formulated to meet the nutrient requirement of salmon and contain similar ratio of digestible protein to digestible energy (30). All diets were fed in 20% of excess based on the feed consumption of fish in each tank.

After the 37-day feeding period, six fish were randomly selected, anesthetized using metacaine at 50 mg L^{-1} and killed with a sharp blow to the head per tank. The individual body weight of each fish was recorded. The final weight per dietary group was 26.01 g ± 0.30 (FM), 24.50 g ± 1.11 (SBM), 24.33 g ± 0.66 (ICJ), 23.53g ± 1.46 (ACJ). No significant differences were detected in the final weight of the fish among dietary groups.

For this study, the spleen of 40 fish was obtained (10 fish per dietary group from duplicated tanks). Then, for each dietary group, six spleen samples were stored frozen in liquid nitrogen (-80°C) until protein extraction and four spleen samples were immediately suspended in RNAlater and stored overnight in the refrigerator, and then kept at -80 °C until total RNA extraction.

The fish experiment was carried out in the Fish Laboratory of Norwegian University of Life Sciences (Ås, Norway) in

TABLE 1 | Formulation and nutritional composition of experimental diets according to Agboola et al. (3).

	FM	SBM	ICJ	ACJ
Fishmeal[a]	433.4	161.4	158.4	158.4
Soybean meal[b]	0	400	400	400
Wheat gluten meal[c]	170	136	111	111
Yeast	–	–	50	50
Potato starch[d]	120	90	68	68
Fish oil[e]	130	130	130	130
Gelatin[f]	60	60	60	60
Cellulose	80	–	–	–
MCP[g]	0	10.0	10.0	10.0
Premix[h]	5.0	5.0	5.0	5.0
L-lysine[i]	–	3.0	3.0	3.0
DL-Methionine[j]	–	3.0	3.0	3.0
Choline chloride[k]	1.5	1.5	1.5	1.5
Yttrium oxide[l]	0.1	0.1	0.1	0.1
Diet composition (analyzed)				
Dry matter	924.3	906.5	899.8	914.3
Crude protein	496.6	477.8	474.1	479.4
Crude lipids	191.5	166.5	162.5	171.0
Ash	71.5	60.0	63.5	64.9
Gross Energy (MJ kg^{-1})	21.6	20.9	20.9	21.2
DP : DE[m]	23.0	22.9	22.7	22.6

[a]LT fishmeal, Norsildmel, Egersund, Norway; [b]Soybean meal, Denofa AS, Fredrikstad, Norway; [c]Wheat gluten, Amilina AB, Panevezys, Lithuania; [d]Lygel F 60, Lyckeby Culinar, Fjälkinge, Sweden; [e]NorSalmOil, Norsildmel, Egersund, Norway; [f]Rousselot 250 PS, Rousselot SAS, Courbevoie, France; [g]Monocalcium phosphate, Bolifor MCP-F, Oslo, Norway Yara; [h]Premix fish, Norsk Mineralnæring AS, Hønefoss, Norway. Per kg feed; Retinol 3150.0 IU, Cholecalciferol 1890.0 IU, α-tocopherol SD 250 mg, Menadione 12.6 mg, Thiamin 18.9 mg, Riboflavin 31.5 mg, d-Ca-Pantothenate 37.8 mg, Niacin 94.5 mg, Biotin 0.315 mg, Cyanocobalamin 0.025 mg, Folic acid 6.3 mg, Pyridoxine 37.8 mg, Ascorbate monophosphate 157.5 g, Cu: CuSulfate 5H_2O 6.3 mg, Zn: ZnSulfate 151.2 mg, Mn: Mn(II)Sulfate 18.9 mg, I: K-Iodide 3.78 mg, Ca 1.4 g; [i]L-Lysine CJ Biotech CO., Shenyang, China; [j]Rhodimet NP99, Adisseo ASA, Antony, France; [k]Choline chloride, 70% Vegetable, Indukern SA., Spain; [l]Y_2O_3. Metal Rare Earth Limited, Shenzhen, China. [m]DP : DE, digestible protein: digestible energy ratio. Calculated using internal digestibility values of various ingredients.

FM, fishmeal-based; SBM, Soybean meal-based, ICJ, 40% SBM and 5% of inactivated C. jadinii; ACJ, 40% SBM and 5% of autolyzed C. jadinii (ACJ). Diet formulation and composition are expressed in g kg^{-1} unless otherwise stated.

accordance with the institutional (Permit No. 174) and national regulations for control of live animal experiments in Norway (Norwegian Animal Welfare Law and Norwegian Animal Experimentation Regulations derived from Directive 2010/63/EU).

RNA-Seq

Total RNA was extracted from sixteen spleen samples (four per dietary group from duplicated tanks), using the RNeasy Mini Kit (Qiagen) following the supplier's instructions. Then, each RNA sample was quantified using a NanoDrop TM 8000 spectrophotometer (Nanodrop Technologies). Later, RNA integrity was determined using Agilent Bioanalyzer 2100. All samples showed a RNA integrity number (RIN) ≥ 8. Library preparation and RNA-seq were performed by the Norwegian Sequencing Center (UiO, Norway), using TruSeq Stranded mRNA library prep and Illumina HiSeq 4000 System (150 bp paired-end RNA sequencing).

RNA-seq data analysis was performed according to Håkenåsen et al. (31). Raw reads were cleaned by BBDuk (v34.56) to trim/remove low quality reads, adapter sequences and PhiX (Illumina spike-in) using: ktrim = r, k = 23, mink = 11, hdist = 1, tbo, tpe, qtrim = r, trimq = 15, maq = 15, minlen = 36, forcetrimright = 149. Thereafter, cleaned reads were aligned to *Salmo salar* genome ICSASG_v2 (RefSeq assembly accession: GCF_000233375.1) by HISAT (v2.1.0). Fragments mapping were counted using featureCounts (v1.4.6-p1) and differentially expressed genes (DEGs) were estimated between diets using SARTools R package (v1.7.3). Significant DEGs were determined when the adjusted p value (padj) was < 0.05.

To characterize differentially expressed genes, functional classification was performed using Gene Ontology (GO) analysis by g:Profiler (32). To achieve this, *Salmo salar* genome database (Ensembl) and gene IDs (Entrezgene_ACC) from significant DEGs list were used. GO categories (g:SCS threshold 0.05) were displayed in $-\log_2(p)$. In addition, EnrichmentMap v3.3 (33) in Cytoscape v3.81 (34) was used with default settings to visualize all diet comparisons in a single network of GO terms.

To further understand gene biological functions, significant DEGs and their expression values were used for Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Enrichment Analysis among dietary groups (by clusterProfiler v3.16.1 package in R). Enriched pathways were selected (pvalueCutoff = 0.05) and displayed as $-\log_2(p)$. Enrichment maps were obtained using emapplot (Enrichplot package v.1.8.1 in R).

Detection of Immunological Markers

For the characterization of the immune response in the spleen of Atlantic salmon after a dietary challenge, biomarkers at the protein level were evaluated in six fish samples per dietary group (from duplicated tanks). Each sample was homogenized using metal beads and RIPA lysis buffer with protease inhibitor cocktail (1x). Then, the samples were centrifuged and total proteins were quantified from the supernatants using the Bicinchoninic acid protein assay kit (Pierce). Thereafter, indirect ELISA was performed following Morales-Lange et al. (35). Briefly, each sample was diluted in carbonate buffer (60 mM $NaHCO_3$ pH 9.6) and seeded by duplicate in a 96-well plate (Nunc) at 50 ng μL^{-1} (100 μL) for overnight incubation (4°C). Next day, the plates were washed three times with PBS-Tween20 (PBST 0.2%) and incubated with 200 μL of blocking solution (per well) for 2 h at 37°C (Pierce Clear Milk Blocking Buffer 1x). After successive washes with PBST 0.2%, 100 μL of the primary antibody (**Table 2**) was incubated for 90 min at 37°C and later, a secondary antibody diluted 1:5000 (goat anti mouse IgG-HRP or mouse anti rabbit IgG-HRP) was incubated per well during 60 min at 37°C. Chromagen substrate 3,3′,5,5′-tetramethylbenzidine single solution (TMB, Thermofisher) was added (100 μL) and incubated for 20 min at room temperature (in dark). All reactions were stopped with 50 μL of 1 N sulfuric acid and finally the plates were read at 450 nm in a SpectraMax microplate reader. (Molecular Devices).

Results from indirect ELISA were expressed in fold change relative to SBM. GraphPad Prism 8 was used to display the data and calculate means, standard deviations, one-way ANOVA and Tukey's test for multiple comparisons between diets.

Furthermore, Corrplot package in R (41) was used to make correlations among diets. All significant differences were determined when p value was <0.05.

RESULTS

Transcriptomics

DEGs number per diet comparison showed different patterns among groups (**Table 3**). The highest differentiated gene expression occurred when fish fed FM were compared to those fed SBM (313 down-regulated, 448 up-regulated). A lower number of DEGs were observed in ACJ-group compared with both FM (230 DEGs down-regulated, 163 DEGs up-regulated) and SBM (95 DEGs down-regulated, 51 DEGs up-regulated). Moreover, in ICJ-fed fish, few numbers of DEGs relative to both FM (seven down-regulated, 21 up-regulated) and SBM (21 down-regulated and four up-regulated) were detected.

The comparison between the two diets with *C. jadinii* (ACJ and ICJ) only showed four down-regulated and three up-regulated DEGs. Complete list of significant DEGs along with the name of each gene is attached in Supplementary File 2. In addition, RNA-seq raw data is available in Gene Expression Omnibus database (GEO-NCBI: GSE174262).

Gene Ontology

DEG classification by Gene Ontology using three categories (molecular function, biological processes and cellular components) showed 26 overrepresented GO terms (18 upregulated and 8 downregulated) in FM compared to SBM (FM|SBM, **Figure 1**). The analysis showed that the up-regulated terms in FM were mainly associated with ion binding, transporter and metabolic activity, while down-regulated GO terms were related to semaphorin activity, biological adhesion and cell adhesion. The same analysis comparing ICJ with both control diets showed only overrepresented GO terms (up-regulated) for ICJ compared to FM (ICJ|FM, **Figure 2A**). In this case, ICJ showed seven significant GO terms related to phosphagen metabolic and biosynthetic process. In addition, when comparing ACJ with FM (ACJ|FM, **Figure 2B**), the results showed one GO term up-regulated in ACJ (carbon-carbon lyase activity). On the other hand, the comparison between ACJ and SBM (ACJ|SBM, **Figure 2C**) showed two down-regulated terms (associated to intrinsic apoptotic signaling pathway) and 11 up-regulated terms in ACJ. Interestingly, the up-regulated terms observed in ACJ compared to SBM were similar to when FM was compared to SBM (molecular binding and gas transporter activity). The analysis between ACJ and ICJ did not show differentially significant GO terms.

By grouping the GO terms detected (from different diet comparisons) in a network (**Figure 3**), we observed that FM and ACJ (compared to SBM) share similarities associated with the up-regulation of tetrapyrrole binding, oxygen transport, oxygen carrier activity, hemoglobin complex, heme binding, gas transport, molecular carrier activity, oxygen binding and cytosol. Furthermore, it was possible to determine that FM compared to SBM (FM|SBM) was related to ICJ compared to FM (ICJ|FM) through ion binding.

TABLE 2 | Primary antibodies for indirect ELISA.

Marker	Source	Type	Dilution	Reference
CD3	Mouse	Monoclonal	1:400	(36)
CD4	Rabbit	Polyclonal	1:500	(37)
CD83	Rabbit	Polyclonal	1:200	(38)
IFNγ	Mouse	Polyclonal	1:400	(3)
IgD	Mouse	Monoclonal	1:400	(39)
IgM	Mouse	Monoclonal	1:400	(39)
IL-10	Mouse	Polyclonal	1:400	(40)
MHC II	Mouse	Polyclonal	1:400	(38)
TNFα	Mouse	Polyclonal	1:400	(3)
ZBTB46	Mouse	Polyclonal	1:400	**Supplementary Figure 1**

TABLE 3 | Significant differentially expressed genes (DEGs) per diet-comparison.

Diet-comparison	Downregulated	Upregulated
FM|SBM	313	448
ICJ|FM	7	21
ICJ|SBM	21	4
ACJ|FM	230	163
ACJ|SBM	95	51
ACJ|ICJ	4	3

KEGG Pathway Analysis

Pathway analyses according to KEGG showed that DEGs from FM compared with SBM (FM|SBM, **Figure 4A**) were related to five activated KEGG terms: biosynthesis of cofactors, peroxisome, herpes simplex virus 1 infection, carbon metabolism and metabolic pathways. Using these data, the association network showed that four of the five KEGG terms (except herpes simplex virus 1 infection) were related (**Figure 4B**). Moreover, DEGs from ICJ, compared to FM (ICJ|FM, **Figure 4C**), showed only a significant activation of metabolic pathways. The comparison between ICJ and SBM did not show any significant KEGG terms.

In **Figure 5A**, DEGs from ACJ compared to FM (ACJ|FM) showed the activation of 3 KEGG terms: arginine and proline metabolism, ECM-receptor interaction and phagosome, and the suppression of salmonella infection-pathway. However, the analysis of interactions from these pathways did not show close relationships (**Figure 5B**).

DEGs comparison between ACJ and SBM (ACJ|SBM) showed six KEGG terms significantly overrepresented in ACJ: herpes simplex virus 1 infection, endocytosis, cellular senescence, Toll-like receptor signalling pathway, C-type lectin receptor signalling pathway and salmonella infection (**Figure 5C**). Enrichment maps using these KEGG terms showed that all pathways were associated in a cluster (**Figure 5D**). Regarding the comparison between ACJ and ICJ, no significant KEGG terms were determined.

Immunological Markers

The detection of immunological markers by indirect ELISA showed lower levels of Cluster of differentiation 83 (CD83) in fish fed FM compared to SBM (0.88-fold, **Figure 6A**). Moreover,

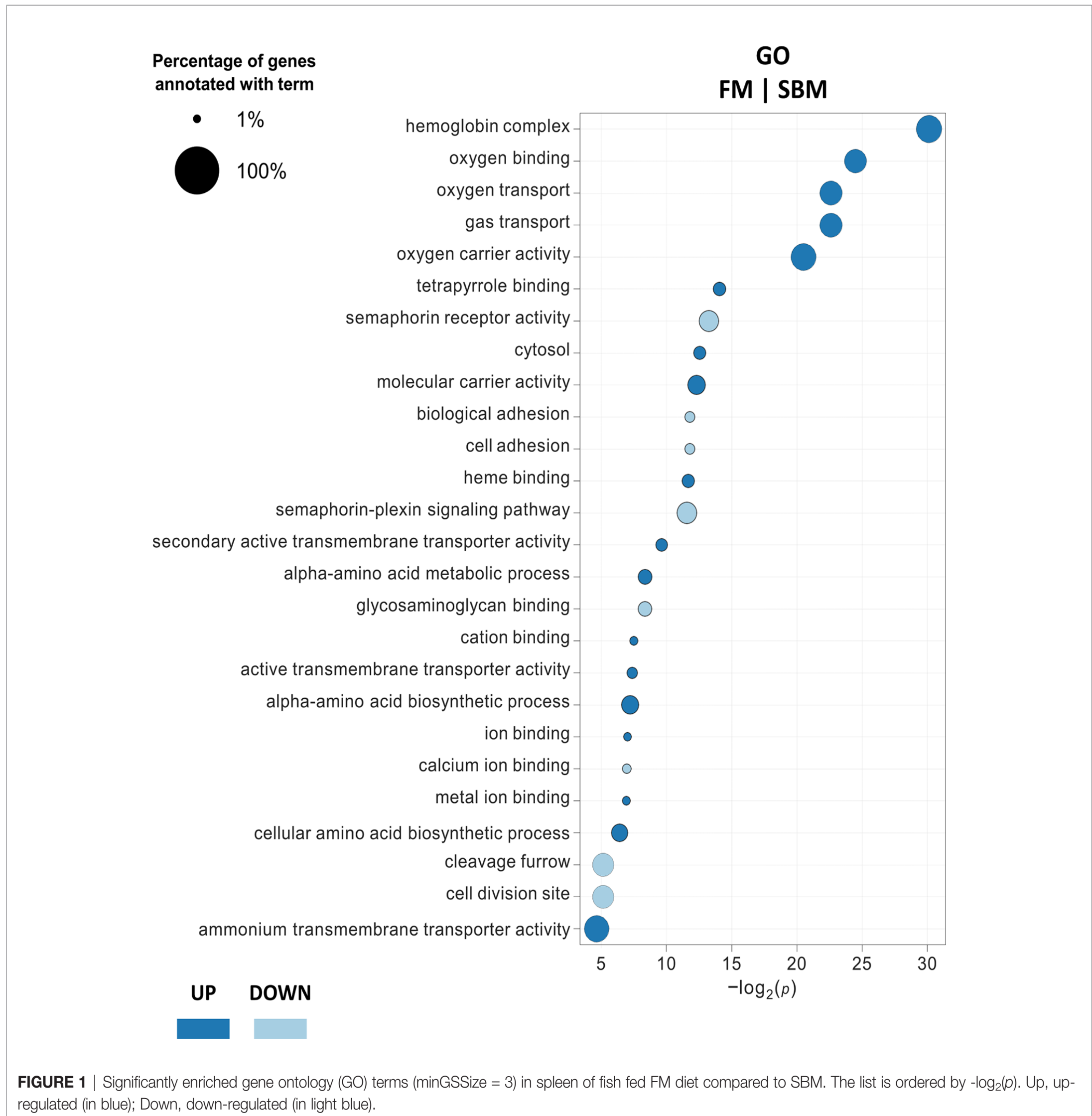

FIGURE 1 | Significantly enriched gene ontology (GO) terms (minGSSize = 3) in spleen of fish fed FM diet compared to SBM. The list is ordered by -$\log_2(p)$. Up, up-regulated (in blue); Down, down-regulated (in light blue).

an increased production of major histocompatibility complex class II (MHC II) was detected in fish fed both diets with *C. jadinii* inclusion (ICJ= 1.16-fold and ACJ= 1.10-fold, respectively) compared to FM (0.78-fold). In addition, the level of ZBTB46 (Zinc finger and BTB domain-containing protein 46) decreased in fish fed ICJ (0.64-fold) and ACJ (0.62-fold) diets, compared to both FM (1.00-fold) and SBM-fed fish (1.00-fold).

Also in **Figure 6**, only Cluster of differentiation 4 (CD4) was significantly different among diets (**Figure 6B**). It was higher in fish fed ACJ-diet (1.19-fold) compared to FM diet (0.66-fold). Cluster of differentiation 3 (CD3), Immunoglobulin D (IgD) and Immunoglobulin M (IgM) did not show significant differences between groups.

The level of cytokines in SBM-diet group (**Figure 6C**) showed a higher level of interferon gamma (IFNγ: 1.00-fold) and tumor necrosis factor alpha (TNFα: 1.00-fold) compared to FM (0.46-fold and 0.40-fold, respectively). A similar behavior to SBM-fed fish was detected in ICJ-diet group, where both pro-inflammatory cytokines showed an increase in their protein levels (IFNγ= 1.42-fold, TNFα= 1.33-fold) compared to FM. On the other hand, the cytokine values from ACJ-diet group, compared with SBM (**Figure 6C**), showed a reduction of TNFα levels (0.37-fold) and an increase in the availability of interleukin 10 (IL-10: 1.88-fold).

Finally, the correlation of all these immunological markers between the different diets (**Figure 6D**) showed a significant

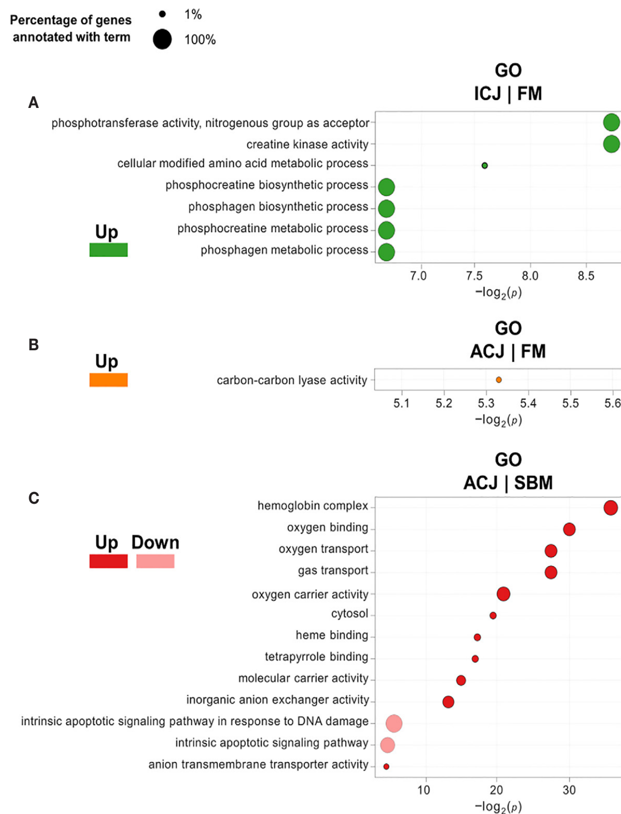

FIGURE 2 | Significantly enriched gene ontology (GO) terms (minGSSize = 3) in spleen of fish fed *C. jadinii* diets compared to control diets (FM and SBM). The list is ordered by -$\log_2(p)$. **(A)** GO terms in ICJ compared to FM. Up, up-regulated (in green). **(B)** GO terms in ACJ compared to FM. Up, up-regulated (in orange). **(C)** GO terms in ACJ compared to SBM. Up, up-regulated (in red); Down, down-regulated (in pink).

positive correlation among FM and ACJ diets (0.64). Correlations between other diets did not show significant results.

DISCUSSION

The proposal to consider the spleen as a target candidate for the characterization of immunomodulatory effects of down-stream processed *C. jadinii* in Atlantic salmon has been based on the fact that in fish the spleen has already been considered as the primordial secondary lymphoid organ and it has a central role in the systemic immune response through the coordination of the innate and adaptive immunity by the antigen presentation process (26). In Atlantic salmon, our results at the transcriptional level have shown that this organ also coordinates aspects related to molecular binding, transporter activity, receptor signalling pathways, cellular and metabolic processes, among others, which have already been described as important functions of the spleen in another salmonid specie such as rainbow trout (42).

On the other hand, from the use of different diets, we were able to detect that fish fed SBM diets, compared to FM, showed a down-regulation of GO and KEGG terms linked to ion binding, peroxisome, metabolic and transport-associated pathways. These results are also similar to those reported in intestine of salmon fed SBM diets. In intestine, in addition to inducing an inflammatory profile, SBM decreases barrier functions through the down-regulation of genes associated with iron-binding proteins, detoxification, transport and metabolic processes (43, 44).

The data also showed that the inclusion of SBM in the diets could induced systemic effects in Atlantic salmon that can be detected in the spleen after 37 days of feeding. Furthermore, the results at the protein level showed that SBM also elicits inflammatory responses in the spleen by increasing the production of cytokines such as TNFα and IFNγ, and inducing the availability of CD83. In higher vertebrates, CD83 is a molecule expressed mainly by mature dendritic cells and acts as an immuno-regulator protein by delivering co-stimulatory signals, which can trigger T helper cell-mediated responses that increase TNFα and IFNγ (45, 46). In fish,

FIGURE 3 | Significant networks of enriched GO terms related to comparisons between different diets. FM|SBM: Up (up-regulated in blue), Down (down-regulated in light blue). ICJ|FM: Up (up-regulated in green). ACJ|FM: Up (up-regulated in orange). ACJ|SBM: Up (up-regulated in red), Down (down-regulated in pink).

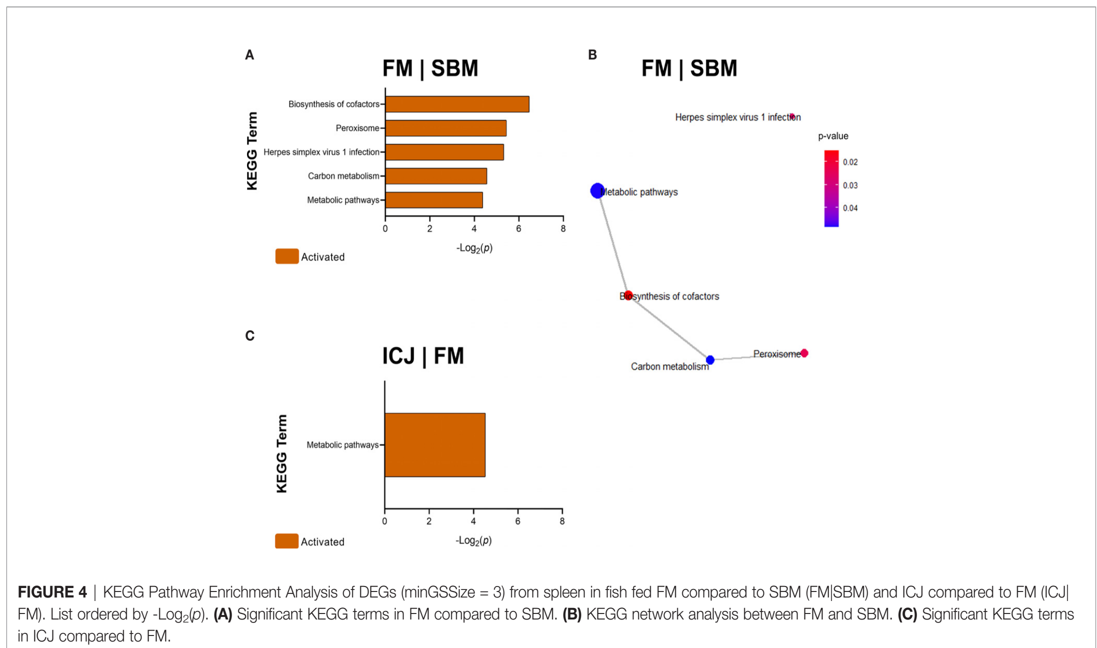

FIGURE 4 | KEGG Pathway Enrichment Analysis of DEGs (minGSSize = 3) from spleen in fish fed FM compared to SBM (FM|SBM) and ICJ compared to FM (ICJ|FM). List ordered by -Log_2(*p*). **(A)** Significant KEGG terms in FM compared to SBM. **(B)** KEGG network analysis between FM and SBM. **(C)** Significant KEGG terms in ICJ compared to FM.

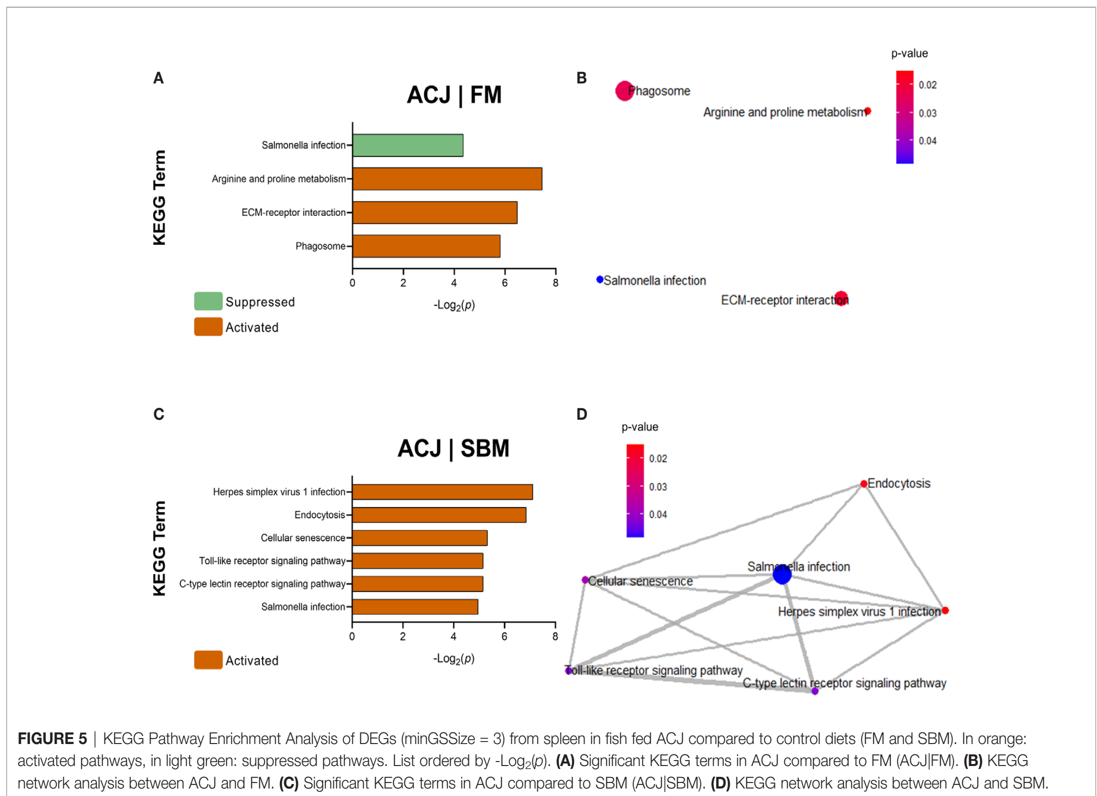

FIGURE 5 | KEGG Pathway Enrichment Analysis of DEGs (minGSSize = 3) from spleen in fish fed ACJ compared to control diets (FM and SBM). In orange: activated pathways, in light green: suppressed pathways. List ordered by -$Log_2(p)$. **(A)** Significant KEGG terms in ACJ compared to FM (ACJ|FM). **(B)** KEGG network analysis between ACJ and FM. **(C)** Significant KEGG terms in ACJ compared to SBM (ACJ|SBM). **(D)** KEGG network analysis between ACJ and SBM.

both cytokines have a key role in the inflammatory process by activating macrophages/phagocytes, thereby enhancing their phagocytic and antimicrobial activity (47).

Regarding the differences between ICJ and ACJ compared to control diets, we hypothesize that the down-stream processing of *C. jadinii* yeasts by autolysis before feed manufacture might explain the observed differences in responses. Different down-stream processes after the yeast is harvested may influence its nutritional value and the accessibility of its cell wall components (16). This could modulate the immune response of Atlantic salmon by exposing fish to different types or amount of MAMP's from *C. jadinii*, such as β-glucan, mannans, chitin and nucleic acids. Inactivated yeasts have already been described as having smooth surfaces without wrinkles, while autolysed yeasts are partially broken and wrinkled, releasing their intracellular content, exposing bioactive components (3, 22), which can be detected by PRRs from the fish, triggering a different immune response (23, 24).

The autolysis process has been reported to modify the nanomechanical properties of the yeast cell wall (without altering its chemical composition), increasing branching and availability of reactive molecules (48). Furthermore, previous works have described the effect of autolysis on the digestibility of yeast in Atlantic salmon, highlighting the importance of down-stream processing when using MI as protein source in feed production (3, 22).

When comparing ACJ with FM, the data showed activated KEGG terms such as phagosome and arginine and proline metabolism. These pathways are connected to the immune response through processes linked to the ability of cells to engulf solid particles to form internal vesicles (49) and with the maintenance of homeostasis, regulating the antioxidant activity in fish (50, 51). On the other hand, the inclusion of inactivated *C. jadinii* in SBM diets did not show marked differences compared to SBM diets. In fact, it seems to maintain a similar inflammatory profile of SBM compared to FM, with increased levels of TNFα and IFNγ. Regarding TNFα, this trend had already been reported in the intestine of Atlantic salmon fed ICJ (3). It is interesting to note that in fish both molecules (TNFα and IFNγ) would be capable of activating M1 macrophages, due to these cytokines can stimulate phagocytosis and the pro-oxidative process to destroy the potential aggressor (52), which would increase the inflammatory pattern of SBM.

On the contrary, the use of ACJ seems to control and regulate the inflammation caused by SBM. When comparing ACJ to SBM, we observed a similar pattern with the one observed among the comparison of FM with SBM. Both showed GO terms associated with molecular binding and transport. Moreover, ACJ was also able to up-regulate KEGG terms associated to endocytosis and signalling pathways of PRRs. We propose that the increase of IL-10 in ACJ contributed to the reduction of the

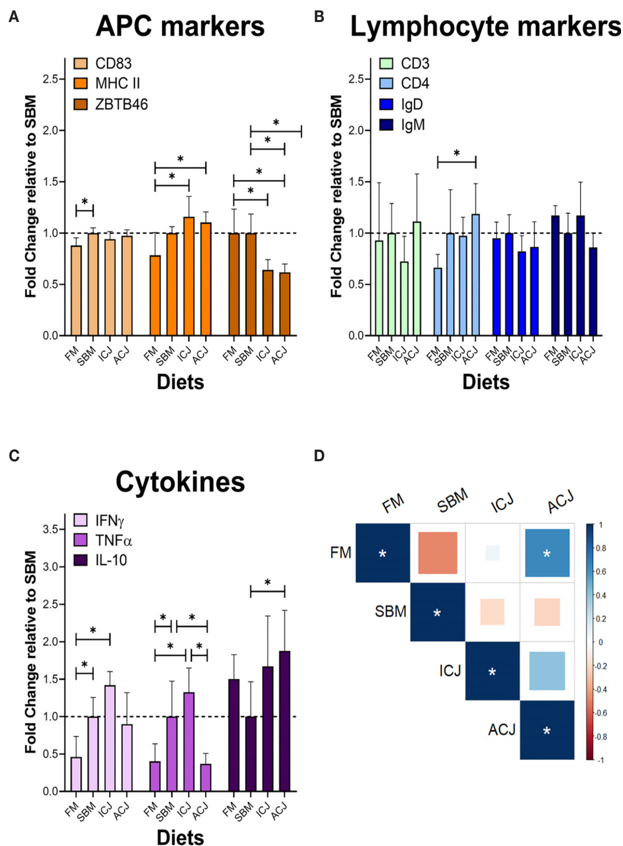

FIGURE 6 | Protein detection of immunological markers in spleen by indirect ELISA. **(A)** Antigen-presenting cell (APC) markers. **(B)** Lymphocyte markers. **(C)** Cytokines. **(D)** Correlation between diets using the data from different immunological markers (Degrees of freedom = 8). In **(A–C)** * shows significant differences among dietary groups ($p < 0.05$). In **(D)** * significant correlation ($p < 0.05$).

inflammatory profile associated with SBM, controlling the production of TNFα. In fish, IL-10 is a cytokine that acts as a suppressor and exerts a conserved role in dampening inflammatory responses (47). Furthermore, the cytokine profile in ACJ suggests a modulation of M1/M2 response, which has been reported as conserved in fish (53). While M1 macrophages increases the robustness of the immune response, M2 macrophages act as repair cells, capable of controlling tissue damage caused by both pathogens and the action of the immune system itself, through a phenotype regulated by molecules such as Transforming growth factor beta (TGFβ) and IL-10 (52, 54).

Additionally, Piazzon et al. (55) have reported that the regulatory activities of IL-10 would not only be associated with immunosuppression and M2 phenotype, but could also be related to the maintenance of memory cells over time. However, further studies must be conducted to better understand this relationship. In our work, ACJ was also able to increase CD4 levels compared to FM, nevertheless, its mechanism of action is not possible to explain with the present results. CD4 is the most characterized marker for T lymphocytes, which govern immune responses through specific antigen recognition and subsequent secretion of effector and regulatory cytokines (37). In rainbow trout, it has been described that cross-talk between activated splenocytes can induce an increase in Forkhead box P3 (FOXP3) (28), which is the transcriptional factor associated with polarization of naive T cells to Treg (56).

The positive correlation observed between FM and ACJ suggest a proportional immune response in these diets, which has also been described in the gut of Atlantic salmon (3). Moreover, in rainbow trout, the inclusion of β-glucans derived from fungi (*Lentinula edodes*) was able to control the acute inflammatory response in the spleen, reducing potential harmful responses for the fish (27).

It is also interesting that fish fed diets with *C. jadinii* showed higher levels of MHC II compared to the FM diet, in addition to a

lower level of ZBTB46 when compare to the FM and SBM diets. MHC II is a protein involved in the antigen-presentation of peptides derived from exogenous proteins to $CD4^+$ T-cells (57). On the other hand, ZBTB46 is a transcriptional factor that inhibits the maturation of APCs in higher vertebrates (58). In salmonids, this molecule has been described in rainbow trout (59). Furthermore, in Atlantic salmon, the modulation of ZBTB46 has been reported in spleen-APCs induced with IFNγ (38). Considering this background, the results in this study suggest an activation of APCs. However, in fish, APCs are still poorly described, and their detection and characterization should be studied deeper in future works to understand their role in the modulation of the immune response by functional diets. Despite this, we propose that the differential activation of APCs in diets with *C. jadinii* compared to SBM (with higher level of CD83, but without other modulated APC markers) would be due to leukocytes from which APCs progress are not a homogeneous subpopulation (38). Moreover, in mammals, APCs could be functional at different stages of maturity, depending on the cytokine environment in which they are found (60, 61).

In summary, we recommend the spleen as a target organ for characterization of immunomodulatory effects of down-stream processed *C. jadinii* in Atlantic salmon exposed to a dietary SBM challenge. Furthermore, our findings contribute to establish a baseline for the study of other novel ingredients that are capable of regulating the immune system of fish, without compromising nutritional parameters. The results from this study suggest that autolysis should be considered when formulating salmon feeds with *C. jadinii* as a functional ingredient with the ability to regulate inflammatory processes.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by Norwegian University of Life Sciences in accordance with the institutional and national regulations for control of live animal experiments in Norway.

AUTHOR CONTRIBUTIONS

The study was conceived by BM-L and JOA with key inputs from JØH, LL, and MØ. The experiments and data analysis were performed by BM-L, JOA, and OØ. LM, and LL were in charge of the production and obtaining of antibodies used in this study. The funds for this investigation were acquired by LL, LTM and MØ. BM-L drafted the manuscript with substantial contributions from all other authors. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.708747/full#supplementary-material

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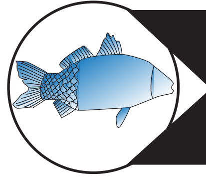

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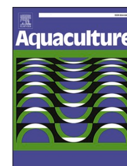

Yeast as a novel protein source - Effect of species and autolysis on protein and amino acid digestibility in Atlantic salmon (*Salmo salar*)

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ABSTRACT

Yeasts are gaining increasing attention as alternative protein sources in fish feeds. The nutritional value of yeast depends on cultivation conditions, yeast species and processing conditions used after harvesting. The objective of the current study was to evaluate the effect of autolysis on apparent digestibility coefficients (ADCs) of crude protein and amino acids (AA) of different yeasts species in Atlantic salmon (*Salmo salar*). Three yeast species (*i.e. Cyberlindnera jadinii*, *Blastobotrys adeninivorans* and *Wickerhamomyces anomalus*) produced from hydrolysates of pre-treated wood and chicken products were used. After harvesting, each yeast was either directly heat-inactivated with spray-drying or autolyzed at 50 °C for 16 h followed by spray-drying. The treatments consisted of a high-quality fishmeal-based reference diet and six test diets containing 30% of each of the yeast product and 70% of the reference diet. The results showed that protein and AA digestibility differed among the yeast species and that the effect of autolysis on nutrient digestibility was inconsistent among the three yeast species. The ADCs of protein in inactivated yeasts were 63%, 72%, 66% in *C. jadinii, B. adeninivorans* and *W. anomalus*, respectively. Autolysis increased the ADCs of protein by 12% and 9% in *C. jadinii* and *W. anomalus*, respectively, while it remained unchanged for *B. adeninivorans*. The ADCs of lysine were 67%, 79% and 72% in inactivated *C. jadinii, B. adeninivorans* and *W. anomalus*, respectively. Autolysis improved the ADCs of lysine by 15%, 7% and 13% in *C. jadinii, B. adeninivorans* and *W. anomalus*, respectively. The ADCs of methionine in inactivated yeasts was 47% in *C. jadinii*, 81% in *B. adeninivorans* and 74% in *W. anomalus*. After autolysing the yeasts, the ADC of methionine improved by 26% and 4% in *C. jadinii* and *B. adeninivorans,* respectively, while it slightly reduced by 2% in *W. anomalus*. Data from regression analyses showed that digesta viscosity, digesta dry matter and nitrogen solubility are important determinants of protein digestibility of yeasts in fish. In addition, cell wall porosity as demonstrated by nitrogen solubility test, had a larger impact on nutrient digestibility of yeasts compared to the cell wall thickness. In conclusion, the digestibility of protein and AA of yeasts in Atlantic salmon depends on type of yeasts and down-stream processing applied after harvesting. Also, the particular *in vitro* digestibility method used in the current study did not adequately reflect the protein digestibility of yeasts in Atlantic salmon.

1. Introduction

The application of yeasts as aquafeed resources can be traced back to previous decades (Austreng, 1978; Mahnken et al., 1980; Matty and Smith, 1978; Rumsey et al., 1990). In recent time, yeast and its cell wall components have become more prominent as immunostimulants in aquaculture. Yeast derived β-glucans and mannan oligosaccharides (MOS) have been used to enhance immune responses, health and growth performance in different fish species (Meena et al., 2013; Torrecillas et al., 2014). Yeast can also serve as an alternative protein source in fish feed when included in moderate levels. The crude protein content in yeasts range from 40 to 60% (on dry basis), and has a favourable amino acid (AA) profile, except for sulphur-containing methionine which is often limiting when used as major protein ingredient in fish feeds (Agboola et al., 2020; Mahnken et al., 1980; Oliva-Teles and Gonçalves, 2001). These attributes qualify yeasts as potential high-quality protein

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resources for aquaculture (Agboola et al., 2020; Glencross et al., 2020; Øverland and Skrede, 2017). The use of yeasts as major protein ingredients in fish feeds is, however, less reported in literature (Agboola et al., 2020). Studies have shown that moderate inclusion level (up to 20%) of yeast in practical fish feeds, support growth performance in fish species, such as Atlantic salmon (Øverland et al., 2013), rainbow trout (Dabrowski et al., 1980; Huyben et al., 2017; Mahnken et al., 1980; Vidakovic et al., 2020), Artic charr (Vidakovic et al., 2016) and European sea bass (Oliva-Teles and Gonçalves, 2001).

Despite the available knowledge on the nutritional values of yeasts, data on their nutrient digestibility in fish are scarce. Digestibility values are crucial for obtaining accurate matrix values for different ingredients in feed formulation as diets are formulated based on digestible nutrients rather than chemical composition of ingredients (Glencross, 2020). Thus, the first step towards promoting yeasts as major ingredients in fish feeds, is assessing their digestibility values in different fish species. To the authors' knowledge, only few studies have documented the nutrient digestibility of yeasts in fish (Hansen et al., 2021; Langeland et al., 2016; Rumsey et al., 1991b; Sharma et al., 2018; Vidakovic et al., 2020; Øverland et al., 2013). Majority of these studies reported the digestibility values on diet level, except for few studies (Hansen et al., 2021; Langeland et al., 2016; Rumsey et al., 1991b; Sharma et al., 2018), where the ADC values were reported for yeasts.

Although nutrient digestibility of yeasts in fish is scarce in literature, there are numerous studies on digestibility of other microbial ingredients such as microalgae (Agboola et al., 2019; Bélanger et al., 2021; Burr et al., 2011; Gong et al., 2020; Hart et al., 2021; Sarker et al., 2020; Teuling et al., 2017; Teuling et al., 2019; Tibbetts et al., 2017) and bacterial meal (Skrede et al., 1998; Storebakken et al., 1998; Øverland et al., 2006). In general, these studies stated that the rigid cell wall is the main reason for the lower digestibility and nutrient bioavailability of microbial ingredients in fish. This is relevant because yeasts like other microbial ingredients, also contains rigid cell wall layer that might impede their digestibility in fish (Rumsey et al., 1991b). This concern was first investigated by Rumsey et al. (1991b), where protein digestibility of *Saccharomyces cerevisiae* in rainbow trout improved by 35% after mechanical homogenization. Recently, Hansen et al. (2021) further tested this hypothesis and observed that different down-stream processing (DSP) of *S. cerevisiae* lead to increased protein digestibility in Atlantic salmon. The protein digestibility of yeasts increased by 60 and 45% in Atlantic salmon when processed by autolysis (at 50 °C for 16 h) and microfluidizer (mechanical homogenization), respectively. However, there is currently insufficient knowledge about the impact of the various processing methods on nutrient digestibility of non-*Saccharomyces* yeasts in literature. Based on these observations by Hansen et al. (2021), we have selected autolysis as our preferred DSP in this current study.

Thus, the objective of this study was to investigate the effect of species and DSP on nutrient digestibility of yeasts in Atlantic salmon. Three non-*Saccharomyces* yeast species, *C. jadinii* (CJ), *Blastobotrys adeninivorans* (BA) and *Wickerhamomyces anomalus* (WA) were used in this experiment. Additionally, the study tested the hypothesis that cell wall thickness and viscosity of yeasts are two limiting factors to the nutrient digestibility of yeast in fish. This was tested using nitrogen solubility, flow cytometry, and viscosity tests. The study also examined whether *in vitro* digestibility method could predict protein digestibility of yeasts in Atlantic salmon.

2. Materials and methods

2.1. Fermentation and processing of yeasts

The yeasts were produced and processed as previously described (Agboola et al., 2021). Briefly, *C. jadinii*, *B. adeninivorans* and *W. anomalus* were fermented using substrates containing hydrolysates from pre-treated spruce wood (*Picea abies*) and chicken products (Lapeña et al., 2020a; Lapeña et al., 2020b). The protein-rich enzymatic hydrolysates from chicken and turkey cut-offs were provided by BIOCO AS (Hærland, Norway) and were kept at 4 °C until further use (Lapeña et al., 2020a). The poultry hydrolysates contained 50.4% dry matter and 44.4% protein, according to product specifications (Lapeña et al., 2020a). After harvesting, the phase containing the yeast was resuspended in water (1:1, *v*/v) and washed one time with tap water to remove remaining residues from the fermentation broth using two-phase separator. Thereafter, yeasts were centrifuged to obtain yeast paste (5–15% dry matter (DM) contents). Each yeast paste was divided into two halves in which one half was directly inactivated using spray-drying (150 MS, SPX Flow Technology, Denmark). The other half was autolyzed by incubating at 50 °C for 16 h in 30 L EINAR bioreactor system (Belach Bioteknik, Sweden), with constant stirring at 50 rpm using a helical impeller. The autolysis was followed by drying using the same spray-dryer as previously mentioned. The inlet and outlet temperatures of the spray-dryer were set at 180 °C and 80 °C, respectively. The resulting test ingredients (yeasts) from the two DSP were: inactivated CJ (ICJ), autolyzed CJ (ACJ), inactivated BA (IBA), autolyzed BA (ABA), inactivated WA (IWA) and autolyzed WA (AWA).

2.2. Digestibility trial

The fish trial was performed in May/June 2020 at the Fish Laboratory of Norwegian University of Life Sciences (NMBU, Ås, Norway), which is an experimental unit approved by the National Animal Research Authority, Norway (permit no. 174). All fish were handled under the applicable laws and regulations guiding experiments with live animals in Norway (regulated by the "Animal Welfare Act" and "The Norwegian Regulation on Animal experimentation" derived from the "Directive 2010/63/EU on the protection of animals used for scientific purposes").

2.2.1. Diets formulation

The control feed consisted of 100% reference diet (REF; Table 1) and formulated to meet or exceed the nutrient requirements for pre-smolt Atlantic salmon (NRC, 2011; Prabhu et al., 2019). Six test diets (ICJ,

Table 1
Formulation of experimental diets fed to juvenile Atlantic salmon (g/kg).

	Reference diet	Test diets
Fishmeal[a]	480	336
Wheat gluten meal[b]	130	91
Gelatinized potato starch[c]	120	84
Fish oil[d]	151	105.7
Mineral and vitamin premix[e]	6.5	4.55
Gelatin[f]	110	77
Yttrium oxide[g]	0.15	0.105
Choline chloride[h]	2.35	1.645
Yeast[i]	0	300

[a] LT fishmeal, Norsildmel, Egersund, Norway.
[b] Wheat gluten, Amilina AB, Panevezys, Lithuania.
[c] Lygel F 60, Lyckeby Culinar, Fjälkinge, Sweden.
[d] NorSalmOil, Norsildmel, Egersund, Norway.
[e] Premix fish, Norsk Mineralnæring AS, Hønefoss, Norway. Per kg feed; Retinol 3150.0 IU, Cholecalciferol 1890.0 IU, α-tocopherol SD 250 mg, Menadione 12.6 mg, Thiamin 18.9 mg, Riboflavin 31.5 mg, d-Ca-Pantothenate 37.8 mg, Niacin 94.5 mg, Biotin 0.315 mg, Cyanocobalamin 0.025 mg, Folic acid 6.3 mg, Pyridoxine 37.8 mg, Ascorbate monophosphate 157.5 g, Cu: CuSulfate $5H_2O$ 6.3 mg, Zn: ZnSulfate 151.2 mg, Mn: Mn(*II*)Sulfate 18.9 mg, I: K-Iodide 3.78 mg, Ca 1.4 g.
[f] Rousselot® 250 PS, Rousselot SAS, Courbevoie, France.
[g] Y_2O_3. Metal Rare Earth Limited, Shenzhen, China.
[h] Choline chloride, 70% Vegetable, Indukern S.A., Spain.
[i] ICJ – inactivated *Cyberlindnera jadinii*; ACJ – autolyzed *C. jadinii*, IBA – inactivated *Blastobotrys adeninivorans*; ABA – autolyzed *B. adeninivorans*; IWA – inactivated *Wickerhamomyces anomalus*; AWA – autolyzed *W. anomalus*.

ACJ, IBA, ABA, IWA and AWA) consisting of 70% REF diet and 30% inactivated or autolyzed yeasts from the three species were also formulated. Yttrium oxide (Y_2O_3) was included as an inert marker for determination of nutrient digestibility (Table 1). The chemical composition of the seven experimental diets are presented in Supplementary Table S1. The REF part of the experimental diets was mixed with a concrete mixer. For the test diets, dried yeast was mixed with the REF part of experimental diets using a Spiry 25 mixer (Moretti Forni, Mondolfo, Italy). Gelatin was used as a binder by mixing in cold water, then heated up to 60 °C in a microwave oven before mixing with dry ingredients using the same Spiry 25 mixer. After mixing, the mash was cooled down to room temperature, followed by cold-pelleting using a P35A pasta extruder (Italgi, Carasco, Italy). The wet pellets were dried (to about 90% DM contents) at Center for Feed Technology, NMBU using small experimental dryers at 60 °C for about 45 min and stored at 4 °C until the start of the fish trial.

2.2.2. Management and feeding of fish

At the start of the experiment, a total of 1050 pre-smolt Atlantic salmon were sorted, batch-weighed and randomly allocated into 21 fiber glass tanks (300*L*) equipped with automatic belt feeders. There were 50 fish with an average initial weight of 46 ± 0.6 g in each tank. The seven experimental diets were randomly assigned to all tanks in triplicate. During the first week of the experiment, fish were fed 1.5% of their body weight to benchmark the tank with the lowest feed intake. Subsequently, the fish were fed restrictively under a pair-feeding regime as described previously in Nordrum et al. (2000). Briefly, the fish tank with lowest feed intake determined the amount of feed distributed to all tanks the next day. Feed was provided 6 h a day between 8:00 and 14:00 h using automatic belt feeders delivering feed every 12 min. Uneaten pellets were sieved after each feeding from the outlet water settling on a screen for each tank (Shomorin et al., 2019). Daily feed intake was estimated from the dry weight of the feed supplied and the dry weight of the recovered uneaten feed, adjusted for feed recovery rate for each tank. All fish were kept under a 24 h light regime and recirculated freshwater with an average temperature of 15.0 °C and water flow of 8 L min^{-1} during the experimental period. The oxygen content of the outlet water was within 7–8 mg L^{-1} throughout the experimental period. The experiment lasted for 42 days. On day 35 and 42, all fish were anesthetized with metacaine (MS-222; 50 mg L^{-1} water) and stripped for feces. Feces from both days were pooled by tank and stored in −20 °C before freeze-drying. On day 42, 10 fish per tank were randomly selected, anesthetized and killed with a sharp blow to the head. Digesta from distal intestine of each fish was pooled by tank in an Eppendorf tube and further stored in −20 °C until analysis for viscosity. All fish per tank were weighed at the end of the trial.

2.2.3. Chemical analyses

The dried feeds, yeasts and feces were ground prior to chemical analysis. The DM content was determined by drying the samples at 104 °C until a constant weight was achieved (ISO 6496). The nitrogen (N) and sulphur (S) contents were analyzed by CHNS Elemental Analyzer (Vario El Cube Elemental Analyzer system GmbH, Hanau, Germany). The crude protein was calculated as N × 6.25. The AA contents were analyzed according to Commission Regulation (EC) No 152/2009 using a Biochrom 30 AA Analyzer (Biochrom Ltd. Cambridge, UK). Ash content was determined using a muffle furnace by incineration at 550 °C according to ISO 5984. Total phosphorus (P) was analyzed using a commercial spectrophotometric kit (PH8328, Randox laboratories, County Antrim, UK) after combustion and acid digestion according to Commission Regulation (EC) No 152/2009. The yttrium, calcium (Ca), zinc (Zn), magnesium (Mg), potassium (K) and iron (Fe) were determined using a microwave plasma atomic emission spectrometer (MP-AES 4200, Agilent Technologies, USA) after acid decomposition in a microwave digestion system (Start D, Milestone Srl, Italy).

2.2.4. Growth parameters

Biomass gain of fish was calculated as the difference between average initial weight and average final body weight of fish per tank. The feed conversion ratio (FCR) was calculated on DM feed intake using equation (a).

$$FCR = \frac{Average\ feed\ intake\ per\ day}{Average\ biomass\ gain\ per\ day} \quad (a)$$

Where average feed intake per day (g) was calculated on DM basis and average biomass gain per day (g) was used as-is.

The specific growth rate (SGR) was calculated following equation (b).

$$SGR = \frac{(ln(average\ final\ fish\ weight) - (ln(average\ initial\ fish\ weight))}{Duration\ of\ the\ trial} \times 100 \quad (b)$$

Fish survival (%) was calculated as the ratio of final and initial number of fish, multiplied by 100.

2.2.5. Apparent digestibility calculations

Apparent digestibility coefficients (ADCs) of nutrients in the diets were calculated using equation (c) (Cho and Slinger, 1979).

$$ADC_{diet}\ (\%) = \left(1 - \left[\frac{Yt_{diet}}{Yt_{faeces}}\right] \times \left[\frac{Nutrient_{faeces}}{Nutrient_{diet}}\right]\right) \times 100 \quad (c)$$

Where Yt_{diet} is the content of yttrium in the diets and Yt_{feces} is the content of yttrium in the feces. $Nutrient_{diet}$ and $Nutrient_{feces}$ represent the content of nutrient in the diet and feces, respectively. The ADCs of nutrients in the test ingredients (*i.e.* yeasts) were calculated according to equation (d) (Bureau and Hua, 2006).

$$ADC_{ingredients}\ (\%) = ADC_{testdiet} + (ADC_{testdiet} - ADC_{refdiet}) \times \left(\frac{0.7 \times Nutrient_{refdiet}}{0.3 \times Nutrient_{testingr}}\right) \quad (d)$$

Where $ADC_{testdiet}$ is the ADC of nutrients in the test diet and $ADC_{refdiet}$ is the ADC of nutrients in the reference diet. $Nutrient_{refdiet}$ and $Nutrient_{testingr}$ denote the nutrient content in the reference diet and test ingredients, respectively. For both equation (c) and (d), yttrium and nutrient contents in the yeasts, feeds and feces were expressed in g/kg DM.

2.3. Viscosity of yeasts, diets and digesta

Viscosity of yeasts, diets and digesta were determined according to the protocol described in Svihus et al. (2000). For yeasts and diets, approximately 1 g of ground samples were mixed with 10 mL of milli-Q water and incubated in a shaking water bath at 25 °C for 30 min. Subsequently, the suspended yeasts and diet, as well as digesta tubes were centrifuged for 10 min at 12000 ×*g*. After centrifugation, the supernatant of each sample was measured in duplicate using the absolute viscosity (centipoise (cP)) by a Brookfield LVDV-II+ cone/plate viscometer (Brookfield Engineering Laboratories, Stoughton, USA). The pellet was oven-dried at 104 °C according to ISO 6496, and used for the determination of digesta DM after correcting for the initial sample weight.

2.4. Effects of processing on cell wall integrity

2.4.1. Yeast size distribution

The size distribution of inactivated and autolyzed yeasts was measured by flow cytometry following the protocol described by Lambrecht et al. (2018). Briefly, approximately 200 mg of spray-dried yeasts were dissolved and vortexed in 1 mL of phosphate buffer saline (PBS). Large debris was removed by centrifugation at 300 ×*g* for 5 min at 4 °C. The supernatant was transferred into a new tube and centrifuged at 21000 ×*g* for 10 min at 4 °C. The supernatant was discarded, and the

pellet was dissolved in 1 mL 2% formaldehyde in PBS. The sample was incubated at room temperature for 30 min. After incubation, the sample was centrifuged at 2100 ×*g*, for 5 min at 4 °C and the pellet was re-suspended in 1 mL PBS. For the staining, samples were incubated with SYBR Green (Thermo Fisher Scientific, San Jose, CA, USA) diluted 1:10000 overnight at 4 °C in the dark. After washing twice with PBS, stained yeast was analyzed using a MoFlo Asterios EQ (Beckman-Coulter, Brea, California, USA). Data acquisition was performed with the Summit version 4.3 software (Beckman-Coulter, Brea, California, USA), and analysis was performed using Kaluza software version 2.1 (Beckman Coulter, Brea, California, USA).

2.4.2. Nitrogen solubility test

The N solubility was measured in duplicate according to a previously described method (Teuling et al., 2019). Approximately 200 mg of spray-dried yeast samples were suspended in 4 mL potassium phosphate buffer (pH 8.0, 50 mM, Sigma Aldrich). The suspension was incubated in a shaking water bath at 25 °C for 30 min, and subsequently centrifuged at 15000 ×*g* for 10 min at 20 °C. After centrifugation, the N contents of the supernatant and the starting sample were analyzed using the CHNS Elemental Analyzer method.

2.4.3. In vitro protein digestibility test

In vitro protein digestibility was determined in triplicate according to the method described in Hansen et al. (2021). Approximately 1 g spray-dried yeast sample was dispersed in 9.6 mL of pepsin solution (Pepsin 416.7 U mL^{-1} in 0.084 mM HCl, 35 mM NaCl, pH 2.0, Sigma Aldrich). The sample was thereafter incubated in a shaking water bath at 37 °C for 6 h. Subsequently, 675 μL of 1 M NaOH was added to inactivate the pepsin activity, and pH was adjusted to 7.8 by adding approximately 30 mL of 10 mM PBS (pH 7.8, Sigma Aldrich). Thereafter, the samples were incubated for 1 h at 37 °C, after which 0.6 mL of the intestinal enzyme cocktail (Trypsin 2100 U mL^{-1} and Chymotrypsin 100 U mL^{-1} in 10 mM phosphate buffer, pH 7.8, Sigma-Aldrich) was added. After 18 h of incubation, the samples were boiled immediately to inactivate the enzyme cocktail. The digested samples were centrifuged at 20000 ×*g* for 20 min. The pellets were further analyzed for crude protein (N × 6.25) using Kjeldahl method and *in vitro* protein digestibility was calculated according to the equation expressed in Tibbetts et al. (2016).

Free AA in the digested samples were determined using a modified TNBS assay method (Adler-Nissen, 1979). Briefly, 35 μL of supernatant collected from each sample was added in triplicates into a 96-well plate (Maxisorp Thermo Fisher Scientific), followed by addition of 70 μL of pre-heated 0.1% TNBS-solution and 70 μL of 10 mM PBS (pH = 7.8). The TNBS-solution (Sigma-Aldrich) and PBS were pre-heated to 60 °C for 30 min before adding into the 96-well plate. Thereafter, the plate was incubated at 60 °C for 1 h in a heating cabinet with constant mixing. After incubation, the reaction was stopped with 70 μL of 1 M hydrochloric acid and absorbance was measured at 320 nm using a Spectramax microplate reader (Molecular Devices). The free AA (mmol AA released/g of crude protein weighed) was determined from a standard curve generated with DL-alanine.

2.5. Statistical analysis

All statistical analyses were conducted using the SPSS statistical software package version 27 (IBM Institute, Armonk, NY, USA). Data on growth performance, ADCs of nutrients in the diets, digesta viscosity and digesta DM were analyzed using the one-way analysis of variance (ANOVA). In addition, data on ADCs of nutrients in the yeast were analyzed using a 2-way ANOVA by testing for the effects of yeast species, DSP, and their interaction. In both cases, significant mean differences ($P < 0.05$) were detected using the Tukey comparison test. Linear relationships between ADCs of protein from yeasts and viscosities of yeasts, diets and digesta, as well as digesta DM were evaluated using linear regression analysis. Also, linear relationship between ADCs of protein from yeasts and N solubility, *in vitro* protein digestibility, free AA, and cell wall thickness of yeasts reported in Agboola et al. (2021)) were evaluated through the linear regression model. Significant relationships were considered at $P < 0.05$.

3. Results and discussion

3.1. Chemical composition and amino acid profile of yeasts

Limited information on nutritional composition of *C. jadinii*, *B. adeninivorans* and *W. anomalus* yeasts exists in literature (Agboola et al., 2020). In the present experiment, the crude protein content ranged from 37 to 53% on DM basis in the yeast species (Table 2). These values correspond with values obtained earlier for the same three yeast species (Lapeña et al., 2020a; Lapeña et al., 2020b). Lapeña et al. (2020b) reported that fermentation media (organic *vs.* inorganic) and mode of fermentation (batch, fed-batch and continuous) were important factors influencing the crude protein content of yeast. The content of non-protein nitrogen (NPN) in yeasts is quite high. About 40–44% of the crude protein in *C. jadinii* was NPN, while NPN made up 30% in *B. adeninivorans* and 28–30% in *W. anomalus* (Table 2). These values were higher than what were reported by Lapeña et al. (2020a), which ranged from 14 to 20% of crude protein for *C. jadinii, B. adeninivorans*

Table 2
Nutritional composition of inactivated and autolyzed yeasts included in the diets[1].

	ICJ	ACJ	IBA	ABA	IWA	AWA
Macro-nutrients (g/kg DM)						
Dry matter, g/kg[2]	940.3	924.1	952.5	942.8	949.3	936.5
Crude protein[2]	455.6	475.8	388.8	374.2	528.4	527.9
Crude lipids[2]	59.8	62.3	85.5	85.0	87.8	90.6
Ash[2]	77.7	81.4	60.5	62.6	33.4	32.3
Gross energy (MJ/kg DM)	20.2	20.5	22.1	21.8	22.8	22.7
Essential amino acids (g/kg DM)[3]						
Arginine	16.8	13.0	11.8	4.0	24.3	20.4
Histidine	7.5	7.7	7.6	7.5	12.0	11.8
Isoleucine	14.5	15.6	13.3	14.2	21.7	21.3
Leucine	21.4	21.9	20.7	21.0	31.5	31.4
Lysine	22.8	24.1	22.3	23.5	31.7	31.9
Methionine	3.9	3.7	4.7	4.6	7.0	6.9
Phenylalanine	13.4	13.2	12.9	11.5	19.4	17.5
Threonine	15.3	15.5	15.0	8.1	20.0	18.7
Valine	15.6	16.5	15.3	16.8	19.1	19.0
Non-essential amino acids (g/kg DM)[3]						
Alanine	17.6	19.0	16.3	20.2	24.2	25.4
Aspartic acid	27.1	27.4	25.6	25.6	40.3	39.6
Glycine	13.4	13.8	13.9	14.3	20.9	20.8
Glutamic acid	44.2	37.7	39.0	39.5	54.7	54.6
Cysteine	3.3	3.1	2.4	2.2	3.6	3.3
Tyrosine	10.2	8.7	14.7	14.0	14.0	12.9
Proline	11.4	12.2	15.7	16.2	18.2	18.2
Serine	13.7	14.0	13.2	8.7	19.4	18.6
Sum amino acids[4]	272.2	267.0	264.0	251.6	381.9	372.2
Non-protein nitrogen[5]	183.4	208.9	124.8	122.6	146.5	155.7
Minerals (g/kg DM)						
Sulphur	7.7	7.8	6.8	6.9	7.7	7.7
Phosphorus	17.6	18.3	10.7	10.8	6.1	5.8
Calcium	6.8	7.0	2.1	2.3	1.0	1.1
Zinc	0.08	0.09	0.09	0.09	0.03	0.03
Magnesium	1.3	1.4	1.0	1.0	0.7	0.7
Potassium	13.6	14.3	9.5	9.5	7.9	8.1
Iron	0.13	0.13	0.09	0.09	0.08	0.09

[1] ICJ – inactivated *Cyberlindnera jadinii*; ACJ – autolyzed *C. jadinii*, IBA – inactivated *Blastobotrys adeninivorans*; ABA – autolyzed *B. adeninivorans*; IWA – inactivated *Wickerhamomyces anomalus*; AWA – autolyzed *W. anomalus*.
[2] Already presented in Agboola et al. (2021).
[3] Determined using water corrected molecular weights.
[4] Sum amino acids = essential + non-essential amino acids.
[5] Non-protein nitrogen = crude protein – sum amino acids.

and *W. anomalus*.

The discrepancy in NPN content might be explained by the fact that the amino acid compositions of yeasts were not expressed in the same way in these two studies. In the hydrolysis process before chromatographic determination, 1 molecule of water is added to the amino acids for each cleaved peptide bond (can vary a bit, depending on the AA composition of the protein). In Lapeña et al. (2020a), the amino acids were expressed as g/kg DM – but these values were calculated using standard molecular weights for each AA. However, in the present study, AA compositions were expressed using water-corrected (dehydrated) molecular weights for the different AA (and then expressed as g/kg DM). This approach is often used in nutrition-related studies and give a more correct amount of each AA in the proteins, indicating that the AA composition of yeasts presented in Lapeña et al. (2020a) may have been overestimated, thereby underestimating the NPN content. Furthermore, in contrast to the current study, the yeasts in Lapeña et al. (2020a) were harvested after 24 h batch cultivation, when the cells had stop growing and had entered the stationary phase. Nucleic acid content of yeasts is dependent on their growth rate, as yeast in stationary phase tends to have low concentration of nucleic acids compared to those in exponential growth phase (Halasz and Lasztity, 1991). Studies have shown that the NPN in yeasts are mostly in the form of nucleic acids (Halasz and Lasztity, 1991; Lapeña et al., 2020a). Although the nucleic acid analysis of yeast was not conducted in this study, we expect that the content is higher than earlier reported by Lapeña et al. (2020a) because the yeasts were harvested when the cells were still in the exponential growth phase. Compared to the conventional ingredients, yeasts contain high nucleic acid content which can hinder their use in fish feeds (Nasseri et al., 2011; Sharif et al., 2021). It is worthy to mention that the urolytic pathway of Atlantic salmon is well regulated to cope with high levels of nucleic acids (Andersen et al., 2006). Thus, salmonids are able to metabolize high level of dietary nucleic acids without adverse effects, as demonstrated in previous studies (Rumsey et al., 1992; Rumsey et al., 1991a).

A well-balanced AA profile is imperative for considering a novel ingredient (*e.g.* yeast) as potential protein resource for fish feeds. Our data showed that yeasts have similar AA profile (Table 2) to conventional fishmeal and soybean meal (presented in Supplementary Table S2), except for the sulphur-containing AAs, methionine, and cysteine. Compared to fishmeal, methionine is the most limiting AA in yeasts (Agboola et al., 2020; Lapeña et al., 2020b; Mahnken et al., 1980; Øverland et al., 2013) which limits their use as major protein ingredients in fish feeds (Oliva-Teles and Gonçalves, 2001). Yeasts are particularly rich ($>$ 20 g/kg DM) in leucine, lysine, aspartic acid, and glutamic acid (Table 2). There was no major effect of autolysis on AA contents of yeast species under consideration, except for arginine and glutamic acid (Table 2). The effect of autolysis on arginine and glutamic acid differed among the three yeast species. Autolysis reduced the arginine content of the three yeasts, but the effect was more pronounced in *B. adeninivorans* (Table 2). The reduction in arginine contents of yeasts can be explained by the increased content of ornithine in autolyzed yeasts (Supplementary Table S3 & Fig. S1a-c). After autolysis, ornithine contents (g/kg DM) of *C. jadinii*, *B. adeninivorans* and *W. anomalus* increased from 0.9 to 2.1, 2.6 to 7.6, and 2.3 to 4.7; respectively (Table S3). There are several routes to ornithine from arginine, and these can vary between microbial candidates. It is well known that *S. cerevisiae* in the presence of arginase converts arginine to ornithine, and then to putrescine (Qin et al., 2015). However, the exact route for production of ornithine from arginine during autolysis in the present yeasts remains unknown. Similarly, glutamic acid content reduced (44 to 38 g/kg DM) in *C. jadinii* after the autolysis process, but it was unaffected in the other two yeasts (Table 2). This could be attributed to the increased content of γ-aminobutyric acid (GABA) in autolyzed *C. jadinii* (Supplementary Table S3 & Fig. S1a). The GABA content of *C. jadinii* increased from 4.5 to 8.6 g/kg DM due to autolysis, but it remained unchanged in the other two yeasts (Supplementary Table S3). GABA is a metabolite that can be produced from glutamic acid (Majumdar et al., 2016). It is worthy to mention that the GABA content of *W. anomalus* (11.5 g/kg DM) was quite high compared to the other two yeasts. Taurine content of the three yeasts ranged from 2.8 to 3.5 g/kg DM and was not affected by the autolysis process (Supplementary Table S3).

The content of P, Ca, and K in the three yeasts followed similar trends in which *C. jadinii* had numerically highest contents, followed by *B. adeninivorans,* while *W. anomalus* had the lowest values (Table 2). The content of Zn and Fe were present in trace amounts in the three yeasts (Table 2). This trend was similar to values reported in previous paper for the same three yeast species (Lapeña et al., 2020a). There was no effect of autolysis on mineral contents of the three yeast species.

3.2. Fish growth performance

Fish survival was more than 99% for all the dietary treatments with no noticeable abnormal behaviour observed during the experimental period. Fish fed REF diet doubled their body weight during the experimental period, but not fish fed the test diets. As expected, fish fed reference diet showed better FCR and SGR compared to fish fed yeast-based diets (Table 3). Among the yeast-based diets, fish fed autolyzed *W. anomalus* yeast had the highest SGR and lowest FCR, while fish fed inactivated *B. adeninivorans* and autolyzed *B. adeninivorans* had the lowest growth performance (Table 3); probably as a result of the lower protein levels in these diets (Supplementary Table S1).

3.3. Apparent digestibility coefficients of nutrients on yeast level

The ADCs of macro-nutrients and AAs were significantly affected by the dietary treatments (Supplementary Table S4). The ADCs of protein on diet level ranged from 82 to 89%, with the highest values in fish fed REF diet, whereas the ADCs of total sum AA ranged from 84 to 91%. These indicate that the dietary crude protein and sum AA were moderately to highly digestible.

The ADCs of DM and crude protein on yeast level are shown in Table 4a. There were no significant interactions ($P > 0.05$) between yeast species and DSP on ADCs of DM and crude protein. The ADCs of protein in inactivated yeasts ranged from 63 to 72%, with highest value recorded for *B. adeninivorans.* The protein digestibility of inactivated yeasts as seen in the current study is in line with values presented for intact yeasts in sea bass (Oliva-Teles and Gonçalves, 2001), rainbow trout (Cheng et al., 2004; Hauptman et al., 2014), Atlantic salmon (Hansen et al., 2021; Sharma, 2018; Øverland et al., 2013) and Artic charr (Langeland et al., 2016). In contrast, previous studies have documented higher protein digestibility in different intact yeast species. Øverland et al. (2013) observed protein digestibility of 81–87% for both intact *C. jadinii* and *Kluyveromyces marxianus* in Atlantic salmon. Likewise, higher protein digestibility (76% and 91%) were observed for intact *S. cerevisiae* in rainbow trout (Vidakovic et al., 2020) and European perch (Langeland et al., 2016), respectively. The variability in protein digestibility of yeasts across studies could be attributed to different strains, and the difference in fermentation and drying conditions used during the yeast production.

The effect of autolysis on ADCs of protein differed among the three yeasts. Autolysis increased the ADCs of protein in *C. jadinii* and *W. anomalus* by 12% and 9%, respectively, while no effect was observed for *B. adeninivorans* (Table 4a). The effect of autolysis on protein digestibility observed in the current study is minimal compared to recent study of Hansen et al. (2021), despite similar autolysis conditions. Hansen et al. (2021) observed 60% increase in protein digestibility of *S. cerevisiae* after 16 h of autolysis compared to inactivated yeast (from 56 to 89%). These discrepancies suggested that the effect of autolysis on protein digestibility appears to be dependent on the yeast species. However, in the study of Hansen et al. (2021), the autolysis was performed on fresh yeasts paste, while in the current study the yeasts were stored frozen (−20 °C) for 3–8 months prior to the autolysis process.

Table 3
Growth performance and feed intake juvenile Atlantic salmon fed reference diet and test diets with 30% of the different inactivated and autolyzed yeasts[1].

	REF	ICJ	ACJ	IBA	ABA	IWA	AWA	SEM[2]	*P-values*[3]
Initial weight (g/fish)	45.7	45.3	45.0	46.1	45.5	45.5	45.8	0.13	0.50
Final weight (g/fish)	93.0[a]	85.1[c]	85.9[c]	81.5[d]	81.0[d]	86.2[bc]	88.8[b]	0.87	< 0.001
Specific growth rate (%/d)	1.69[a]	1.50[c]	1.54[bc]	1.36[d]	1.37[d]	1.52[c]	1.58[b]	0.02	< 0.001
DM FI (g/fish/d)[4]	0.75[a]	0.72[b]	0.72[b]	0.70[cd]	0.68[d]	0.71[bc]	0.72[b]	0.01	< 0.001
Feed conversion ratio	0.67[a]	0.76[d]	0.74[c]	0.83[e]	0.81[e]	0.73[c]	0.70[b]	0.01	< 0.001

[1] REF – reference diet. ICJ, ACJ, IBA, ABA, IWA and AWA diets contain 70% reference diet and 30% each of yeast biomass, respectively. ICJ – inactivated *Cyberlindnera jadinii*; ACJ – autolyzed *C. jadinii*, IBA – inactivated *Blastobotrys adeninivorans*; ABA – autolyzed *B. adeninivorans*; IWA – inactivated *Wickerhamomyces anomalus*; AWA – autolyzed *W. anomalus*.
[2] Standard error of mean.
[3] Means in the same row but with different superscript ([a-e]) denote significant ($P < 0.05$) difference among the treatments, which was detected using Tukey comparison test.. $n = 3$ replicate tanks per treatment.
[4] Dry matter feed intake.

Table 4a
Apparent digestibility coefficients (%; ADC) of dry matter, crude protein, and essential amino acids on ingredient level in juvenile Atlantic salmon fed reference diet and test diets with 30% of the different inactivated and autolyzed yeasts[1].

Yeast species[2]	DSP[3]	DM	CP	Arg	His	Ile	Leu	Lys	Met	Phe	Thr	Val
Means for interaction effects												
C. jadinii	Inactivated	40.8	63.3	72.2[c]	64.3	58.6[d]	59.2[d]	66.5[d]	46.9[c]	58.0[b]	42.6[c]	60.8[d]
	Autolyzed	39.4	70.7	75.3[bc]	72.9	70.5[c]	71.7[c]	76.8[bc]	58.8[c]	69.2[a]	50.0[bc]	70.7[c]
B. adeninivorans	Inactivated	38.9	71.5	81.0[ab]	75.1	77.7[ab]	80.5[ab]	79.2[b]	81.4[ab]	71.9[a]	65.0[a]	76.4[b]
	Autolyzed	43.7	72.6	73.1[c]	78.3	83.7[a]	85.6[a]	84.4[a]	84.9[a]	75.4[a]	46.2[c]	82.8[a]
W. anomalus	Inactivated	38.8	65.9	81.4[ab]	72.4	72.4[bc]	73.4[c]	71.8[c]	73.6[ab]	72.3[a]	60.3[ab]	69.9[c]
	Autolyzed	52.2	72.0	82.5[a]	75.6	75.8[bc]	77.1[bc]	81.1[ab]	72.1[b]	73.0[a]	60.3[ab]	72.9[bc]
Means for main effects of yeast species												
C. jadinii		40.1	67.0	73.8	68.6[b]	64.6	65.4	71.6	52.8	63.6	46.3	65.8
B. adeninivorans		41.3	72.1	77.0	76.7[a]	80.7	83.1	81.8	83.2	73.6	55.6	79.6
W. anomalus		45.5	68.9	81.9	74.0[a]	74.1	72.3	76.5	72.8	72.7	60.3	71.4
Means for main effects of DSP												
	Inactivated	39.5	66.9	78.2	70.6	69.6	71.0	72.5	67.3	67.4	56.0	69.0
	Autolyzed	45.1	71.7	77.0	75.6	76.7	78.1	80.8	71.9	72.5	54.1	75.5
SEM[4]		1.5	1.1	1.1	1.2	1.9	2.1	1.5	3.3	1.5	2.2	1.7
P-values[5]												
Yeast species		0.244	0.075	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
DSP		0.051	0.011	0.297	0.002	<0.001	<0.001	<0.001	0.045	0.004	0.081	<0.001
Yeast species × DSP		0.055	0.27	0.004	0.174	0.019	0.014	0.069	0.062	0.031	0.001	0.031

[1] n = 3 for the interaction effect, $n = 6$ for main effects of yeast species and $n = 9$ for main effects of DSP. DM, CP, Arg, His, Ile, Leu, Lys, Met, Phe, Thr and Val denote dry matter, crude protein, arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, and valine, respectively.
[2] Yeast species: *C. jadinii* – *Cyberlindnera jadinii*; *B. adeninivorans* – *Blastobotrys adeninivorans*; and *W. anomalus* – *Wickerhamomyces anomalus*.
[3] DSP – Down-stream processing: inactivation and autolysis of each yeast.
[4] Standard error of mean.
[5] Means in the same column but with different superscript ([a-d]) denote significant ($P < 0.05$) difference among the treatments, which was detected using Tukey comparison test.

Thus, it is possible that the long freezing step led to partial inactivation of the endogenous enzymes in the yeasts, and thus reduced the efficiency of autolysis.

Apart from autolysis, other methods such as cell wall extraction (Langeland et al., 2016; Rumsey et al., 1991b) and mechanical disruption, *e.g.* with a microfluidizer (Hansen et al., 2021; Rumsey et al., 1991b), have been used to improve the protein digestibility of yeast. After cell wall extraction, protein digestibility of *S. cerevisiae* increased from 71 to 96% in Arctic charr and Eurasian perch (Langeland et al., 2016). In this study, *S. cerevisiae* was autolyzed followed by cell wall removal by centrifugation. To our knowledge, only one study has documented the protein digestibility of *W. anomalus* in fish. Protein digestibility (86–90%) of diets containing a mixture of *W. anomalus* and *S. cerevisiae* in a 70:30 ration were reported in rainbow trout (Vidakovic et al., 2020). However, no data on protein digestibility of *W. anomalus* or *B. adeninivorans* on ingredient level in fish is reported in the literature.

For ingredients (such as yeasts) with high and batch-batch variation in NPN content, it is important to evaluate the digestibility of total and specific amino acids in addition to crude protein. For this reason, the ADCs of AAs on yeast level are presented in Table 4a and 4b. There were significant interactions ($P < 0.05$) between yeast species and DSP for ADCs of arginine, isoleucine, leucine, phenylalanine, threonine, valine, and serine. Similarly, lysine ($P = 0.069$), methionine ($P = 0.062$) and proline ($P = 0.057$) show tendency for interaction between yeast species and DSP. This indicate that ADCs of these AAs are dependent on the type of yeast and the DSP used after harvesting. There were no interactions ($P > 0.05$) between yeast species and DSP on digestibility of histidine, alanine, aspartic acid, glycine, glutamic acid, cysteine, tyrosine, and sum AAs (Tables 4a, b).

In general, the digestibility of sum AA was in line with the digestibility of crude protein, however, the numerically differences were larger and statistical different between yeast species in comparison with ADC of crude protein. In the present study, the ADCs of sum AA for inactivated yeasts were 57%, 73% and 68% in *C. jadinii*, *B. adeninivorans*, and *W. anomalus,* respectively. Øverland et al. (2013) reported higher values for *C. jadinii,* but similar values for *K. marxianus* and *S. cerevisiae* yeast species. To compare digestibility coefficients across different studies, it is very important to take into consideration how the digestibility trial have been done and most important, the fecal collection method used (Shomorin et al., 2019). Different feces

Table 4b
Apparent digestibility coefficients (%; ADC) of non-essential amino acids on ingredient level in juvenile Atlantic salmon fed reference diet and test diets with 30% of the different inactivated and autolyzed yeasts[1].

Yeast species[2]	DSP[3]	Ala	Asp	Gly	Glu	Cys	Tyr	Pro	Ser	Sum AA	NPN
Means for interaction effects											
C. jadinii	Inactivated	59.9	54.0	38.5	60.9	46.5	43.8	45.3^{c}	51.3^{b}	56.6	73.8^{ab}
	Autolyzed	70.0	65.4	48.1	63.3	36.6	59.2	58.5^{b}	60.6^{ab}	65.4	77.8^{a}
B. adeninivorans	Inactivated	74.9	73.6	73.2	78.4	35.7	30.7	80.2^{a}	70.7^{a}	72.7	69.8^{ab}
	Autolyzed	83.1	76.5	81.5	81.9	41.6	35.3	83.4^{a}	62.5^{a}	76.6	62.9^{b}
W. anomalus	Inactivated	71.3	63.5	51.8	69.2	24.8	59.2	58.6^{b}	62.3^{a}	67.5	61.7^{b}
	Autolyzed	75.1	67.3	56.3	72.7	29.4	61.7	62.5^{b}	65.1^{a}	71.0	74.7^{ab}
Means for main effects of yeast species											
C. jadinii		65.0^{c}	59.7^{c}	43.3^{c}	62.0^{c}	41.6^{a}	51.5^{a}	51.9	56.0	61.0^{c}	75.8
B. adeninivorans		79.0^{a}	75.0^{a}	77.4^{a}	80.2^{a}	38.7^{a}	33.0^{b}	81.8	66.6	74.7^{a}	66.4
W. anomalus		73.2^{b}	65.4^{b}	54.1^{b}	70.9^{b}	27.1^{b}	60.5^{a}	60.5	63.7	69.3^{b}	68.2
Means for main effects of DSP											
	Inactivated	68.7	63.7	54.5	69.5	35.7	44.6	61.4	61.4	65.6	68.4
	Autolyzed	76.1	69.7	62.0	72.6	35.8	52.1	68.1	62.8	71.0	71.8
SEM[4]		1.8	1.9	3.7	1.9	2.3	3.3	3.3	1.6	1.7	1.8
P-values[5]											
Yeast species		<0.001	<0.001	<0.001	<0.001	0.016	<0.001	<0.001	0.001	<0.001	0.020
DSP		<0.001	0.005	0.005	0.019	0.966	0.038	0.002	0.480	0.002	0.190
Yeast species × DSP		0.151	0.318	0.651	0.918	0.157	0.244	0.057	0.006	0.275	0.020

[1] n = 3 for the interaction effect, n = 6 for main effects of yeast species and n = 9 for main effects of DSP. Ala, Asp, Gly, Glu, Cys, Tyr, Pro, Ser, Sum AA and NPN denote alanine, aspartic acid, glycine, glutamic acid, cysteine, tyrosine, proline, serine, sum of amino acids and non-protein nitrogen, respectively.

[2] Yeast species: *C. jadinii* – *Cyberlindnera jadinii*; *B. adeninivorans* – *Blastobotrys adeninivorans*; and *W. anomalus* – *Wickerhamomyces anomalus*.

[3] DSP – Down-stream processing: inactivation and autolysis of each yeast.

[4] Standard error of mean.

[5] Means in the same column but with different superscript ([a-d]) denote significant ($P < 0.05$) difference among the treatments, which was detected using Tukey comparison test.

collection methods could influence the digestibility estimation of an ingredient. In the present study, the ADCs of lysine in inactivated yeasts ranged from 67 to 81%, with the lowest value recorded for *C. jadinii.* These values were similar compared to ADC of lysine reported on diet basis for intact *S. cerevisiae* in rainbow trout (Vidakovic et al., 2020). In contrast, the ADCs of lysine reported for yeasts in the current study were lower compared to values reported for inactivated *S. cerevisiae* (85–90%) in Artic charr and European perch where they use settling column to collect the feces (Langeland et al., 2016). The settling column used by Langeland et al. (2016) may have overestimated the ADC of nutrient in yeasts compared to the manually stripping method used in the current study, which may also underestimate the ADCs of nutrient in the three yeasts.

The ADCs of methionine in inactivated yeasts ranged from 47 to 81%, with the lowest values reported for *C. jadinii* and the highest for *B. adeninivorans.* The ADCs of cysteine were low (27–47%) for the three yeasts species. The digestibility of methionine and cysteine observed in the current study was in agreement with previous results obtained for *C. jadinii, Kluyveromyces marxianus* and *S. Cerevisiae* yeast species in Atlantic salmon (Øverland et al., 2013). The low ADCs of sulphur-containing methionine (in *C. jadinii)* and cysteine may be attributed to conformational changes that occur in protein due to thermal treatment used during spray drying. A previous study has demonstrated that the content and digestibility of AAs in spray-dried fish hydrolysate reduced when the temperature of the spray-drier was raised from 150 °C at 180 °C (Abdul-Hamid et al., 2002). This conformational changes in protein may be linked to the formation of disulfide cross-linkage that impaired the bioavailability and digestibility of protein (Clemente et al., 2000; Salazar-Villanea et al., 2016). Opstvedt et al. (1984) showed that heat-induced disulfide cross-linkage reduced the digestibility of methionine and cysteine from fish protein in rainbow trout. However, knowledge on effects of processing (heating, freezing, *etc.*) on protein quality of yeasts is scarce in literature and should be of consideration in future studies. Another reason for the low ADCs of cysteine might be associated with endogenous production of cysteine. Cysteine is a non-essential AA that can be synthesized from methionine in fish (Wilson, 2003). Endogenous synthesis/losses of nutrient were unaccounted for in the current calculations, thus the ADCs of cysteine in yeast may be underestimated in the current study.

The impact of autolysis on AA digestibility varied among yeast species (Tables 4a,b). Autolysis increased the digestibility of sum AA by 15.6%, 5.4%, and 5.2% in *C. jadinii*, *B. adeninivorans*, and *W. anomalus,* respectively. Similarly, autolysis improved lysine digestibility by 15%, 7% and 13% in *C. jadinii, B. adeninivorans* and *W. anomalus*, respectively. The ADC of methionine was improved by 26% and 4% for *C. jadinii* and *B. adeninivorans,* respectively, while slightly reduced (by 2%) in *W. anomalus*. Generally, the effect of autolysis on AA digestibility seemed to be more apparent in yeast with initial low digestibility values (*i.e.* inactivated yeasts). The exact reason for this phenomenon remains unclear. Additionally, despite having the lowest sum AAs (25–26% of DM), *B. adeninivorans* had the highest AA digestibility among the three yeasts. This is an indication that aside from protein content, the AA digestibility is an important factor to consider when selecting a novel ingredient for fish feeds.

Dietary fecal excretion which is the percentage of ingested minerals (Table 5) that are excreted through feces were expressed as 100 – ADC of each mineral (Kraugerud et al., 2007; Storebakken et al., 2001; Weththasinghe et al., 2021). Dietary fecal excretion was expressed as such since it is impossible to distinguish between the ingested mineral and mineral uptake (through the gills or skin) from the culture water. Therefore, it is more ideal to present this as mineral excretion rather than ADC, which is mostly used for nutrient digested after ingesting a given feed. Fecal excretions of minerals considered in this study, except Zn were affected ($P < 0.05$) by the dietary treatments. Fecal excretion of P in Atlantic salmon fed the experimental diets ranged from 44 to 70%, with the highest excretion observed for fish fed the ACJ diet. Compared to inactivated yeasts, autolysis increased (by 9–27%) fecal excretion of P in fish fed ACJ and ABA diets, whereas it declined (by 10%) in fish fed AWA diet. The P excretion in the current study is higher than previous values (27–39%) reported for *C. jadinii* produced from three different substrates (Sharma, 2018). Fecal excretion of Ca in fish fed the experimental diets were about 100% and even greater than 100% (103–104%) in fish fed REF and ACJ diets. Similar values of Ca excretion were observed for various microalgal sources in both Nile tilapia and African

Table 5
Fecal excretion of minerals (%) in juvenile Atlantic salmon fed reference diet and test diets with different inactivated and autolyzed yeasts[1].

	REF	ICJ	ACJ	IBA	ABA	IWA	AWA	SEM[2]	*P-values*[3]
Sulphur	42.2[b]	54.9[a]	55.0[a]	53.8[a]	53.9[a]	54.7[a]	49.8[ab]	1.1	0.001
Phosphorus	63.4[ab]	55.1[abc]	70.2[a]	49.4[bc]	53.7[bc]	49.3[bc]	44.6[c]	2.1	0.001
Calcium	104.0[a]	98.5[ab]	102.5[a]	99.7[ab]	93.4[ab]	95.0[ab]	81.1[b]	2.1	0.039
Zinc	40.5	61.0	57.7	60.0	39.9	68.5	47.1	3.4	0.138
Magnesium	38.9[ab]	46.1[ab]	48.1[a]	43.0[ab]	44.2[ab]	34.2[b]	36.2[ab]	1.3	0.01
Potassium	5.0[b]	5.3[b]	5.0[b]	6.5[ab]	7.2[a]	5.9[ab]	6.3[ab]	0.2	0.009
Iron	90.7[a]	86.4[ab]	93.0[a]	87.4[ab]	83.1[ab]	54.0[c]	72.0[b]	3.0	< 0.001

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[1] REF – reference diet. ICJ, ACJ, IBA, ABA, IWA and AWA diets contain 70% reference diet and 30% each yeast biomass, respectively. ICJ – inactivated *Cyberlindnera jadinii*; ACJ – autolyzed *C. jadinii*, IBA – inactivated *Blastobotrys adeninivorans*; ABA – autolyzed *B. adeninivorans*; IWA – inactivated *Wickerhamomyces anomalus*; AWA – autolyzed *W. anomalus*. % fecal excretion = 100 – ADC (%) of each mineral.
[2] Standard error of mean.
[3] Means in the same row but with different superscript ([a-e]) denote significant ($P < 0.05$) difference among the treatments, which was detected using Tukey comparison test.

catfish (Teuling et al., 2017; Teuling et al., 2019). Fecal excretion of Zn ranged from 40 to 69% for all the dietary treatments, with the lowest excretion observed for fish fed ABA diets. Sharma et al. (2018) observed similar level of Zn excretion in *C. jadinii* fermented with brown seaweed and woody hydrolysates. Autolysis reduced fecal excretion of Zn in fish fed diets containing autolyzed yeasts (ACJ/ABA/AWA), compared to inactivated yeasts (ICJ/IBA/IWA).

Data on fecal excretion of feedstuff in fish is usually confounded by the ability of fish to utilize additional minerals from the rearing water. Fecal excretion of Ca in fish fed REF and ACJ diets were higher than 100%, implying that excretion of Ca in the feces was greater than the level supplied through the diets. This was expected because fish compensate for their mineral needs by absorbing additional minerals from rearing water. Therefore, the excess minerals in the feces might come from gill and skin uptake, which was not accounted for in our digestibility calculations. The inconsistency observation on the effect of autolysis on fecal excretion of minerals in yeast-based diets is an indication that the down-stream processing has varying effects on bioavailability of minerals in these yeasts. Autolysis reduced the bioavailability of P and Fe, whereas that of Zn increases. The low bioavailability of minerals in autolyzed yeasts could be associated with liberation of constituents that are able to bind and prevents the availability of minerals during the autolysis process. However, the low availability of minerals could be overcome through exogenous supplementation of mineral sources when microbial ingredients are used as fish feed resources. It is also important to state that the composition, content, and bioavailability of minerals in yeasts depend on the minerals in the fermentation media. This is the strategy behind the commercial production of selenium enriched yeast, a commercially produced specialty yeast known for its highly bioavailable form of Se (Esmaeili et al., 2012; Suhajda et al., 2000).

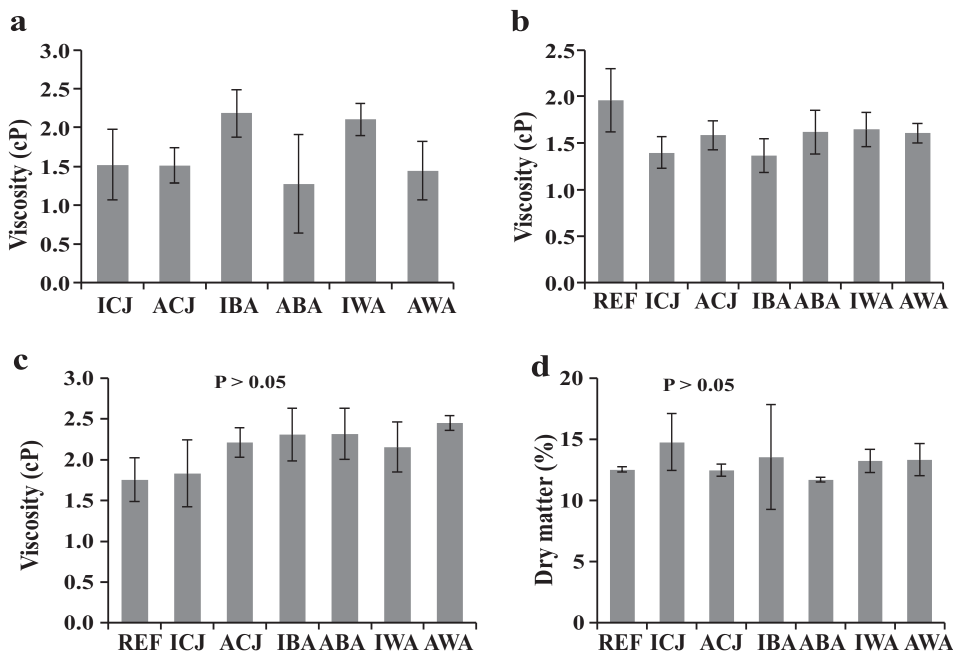

Fig. 1. Viscosity (centipoise; cP) of yeasts (a), diets (b) and digesta (c). REF is reference diet, Inactivated (ICJ) *vs* Autolyzed *Cyberlindnera jadinii* (ACJ); Inactivated (IBA) *vs* Autolyzed *Blastobotrys adeninivorans*; and Inactivated (IWA) *vs* Autolyzed *Wickerhamomyces anomalus* (AWA). The digesta dry matter (d) was expressed in percentage (%).

3.4. Viscosity of yeast, diets and digesta

The viscosity of yeasts, diets, and digesta were measured to understand the impact of viscosity on nutrient digestibility of yeasts (Fig. 1). Like cereal grains such as barley and oats, yeast contains β-glucan, which is known to influence digesta viscosity and consequently nutrient digestibility in animals (O'Neill et al., 2014). ICJ, ACJ ABA and AWA yeasts had similar viscosity values ranging from 1.3–1.5 cP (Fig. 1a). The viscosity of IBA and IWA yeasts (2.1–2.2 cP) were similar, but numerically higher than the remaining yeast products (Fig. 1a). The viscosity values of yeasts are comparable with values recorded for wheat (2.7 cP), but lower than in barley (8.7 cP) and oats (6.9 cP) (Svihus et al., 2000). The difference in viscosity of yeast and barley may be explained by the configuration of β-glucan in these two ingredients. Cereal-derived β-glucans contain β-1,3 and β-1,4 glycosidic linkages, whereas yeast-derived β-glucans contain a mixture of β-1,3 and β-1,6 glycosidic linkages (Kaur et al., 2020; Manners et al., 1973; Nakashima et al., 2018). Yeast derived β-glucans are quite rigid (incorporated into the cell wall) and insoluble in water, unlike β-glucans in barley (Nakashima et al., 2018). Although this study did not examine the direct relationship between the viscosity and solubility of β-glucans. Other factors such as number of glycosidic linkages; their 3-dimensional interactions with each other; and their individual molecular weights that influences the solubility of β-glucans may contribute to the varying viscosity of β-glucans in different sources.

There were no significant differences ($P > 0.05$) in digesta viscosity (1.8–2.5 cP) and digesta DM (12–15%) among all the dietary treatments (Fig. 1c,d). These values are similar compared to values recorded for digesta viscosity in Atlantic salmon fed different types of soybean (Refstie et al., 1999). Leenhouwers et al. (2006) also reported similar level of digesta viscosity (2 cP) in African catfish fed fishmeal-based reference diet. However, addition of 4–8% guar gum to the reference diet elevated digesta viscosity to 66–110 cP (Leenhouwers et al., 2006). Furthermore, 40% inclusion of rye caused high digesta viscosity (about 10 cP) in Nile tilapia (Leenhouwers et al., 2007). The differences in digesta viscosity of fish across studies, can be attributed to the amounts of soluble non-starch polysaccharides present in these ingredients. Additionally, yeast exhibits plastic behaviour when added to water. Prior to feed pelleting, Hansen et al. (2021) through visual examination observed an increased plasticity of mash in yeast-based diets compared to fishmeal-based diets. Similar plastifying effect of yeasts on feed mash was observed in the current study. However, the effects of yeast plasticity on digesta viscosity were not investigated in the current study.

3.5. Effects of down-stream processing on cell wall integrity

Impacts of DSP on cell wall integrity of yeast were measured through flow cytometry, N solubility test, *in vitro* protein digestibility and free AA tests (Fig. 2). The flow cytometry was used to determine the size distribution of inactivated and autolyzed yeast cells. The graphs (Fig. 2a-c) showed that autolysis reduced size distribution of the three yeasts; indicating cell shrinkage in autolyzed yeasts compared to inactivated yeasts. However, the effect was more pronounced in *B. adeninivorans and W. anomalus,* than in *C. jadinii* which was inconsistent with our protein digestibility results. The reduction in yeast size after the autolysis process is similar with findings of Hansen et al. (2021) in differently processed *S. cerevisiae.* The effect of DSP on size distribution of yeast cells was consistent with their micrographs (using both scanning- and transmission electron microscopes) documented in our previous study (Agboola et al., 2021). There, we have shown that the inactivated yeast

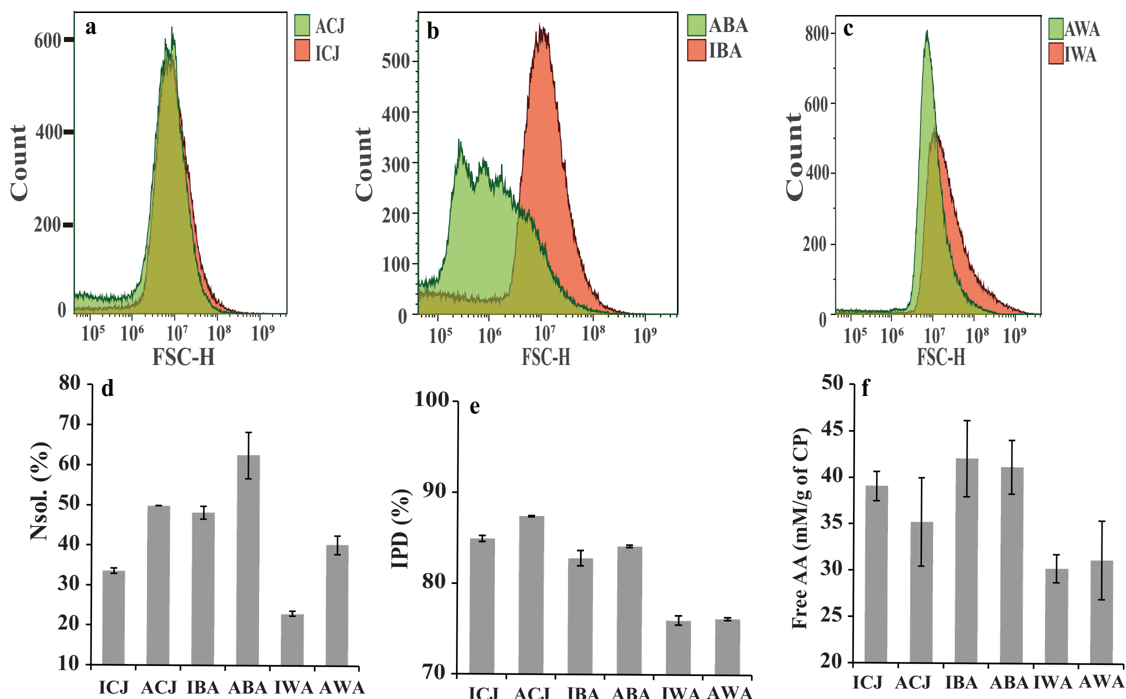

Fig. 2. Effects of processing on cell wall integrity of yeasts. Impact of processing on size distribution (a-c), nitrogen solubility (d) *in vitro* protein digestibility and release of free AA (e-f) of yeasts. Inactivated (ICJ) *vs* Autolyzed *Cyberlindnera jadinii* (ACJ); Inactivated (IBA) *vs* Autolyzed *Blastobotrys adeninivorans* (ABA); and Inactivated (IWA) *vs* Autolyzed *Wickerhamomyces anomalus* (AWA). Sample staining in a-c was done with SYBR green. The light scattered by cells in the flow cytometry was measured by forward scatter (FSC-H), which is used for discrimination of cell by size (a-c). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

cells possessed thicker cell walls with well-organized intracellular layers, whereas the autolyzed yeast cells appeared shrivelled with thinner and distorted intracellular layers (Agboola et al., 2021).

The N solubility can be used to determine the extent of protein release from the cell wall (Teuling et al., 2019). The N solubility ranged from 23 to 48% in inactivated yeasts (Fig. 2d). These are higher than N solubility (11%) of inactivated *S. cerevisiae* (Hansen et al., 2021). Autolysis increased N solubility of yeasts by 49%, 30%, and 75% for *C. jadinii*, *B. adeninivorans,* and *W. anomalus,* respectively. Previous studies have demonstrated that protein solubility of *S. cerevisiae* increased after the autolysis process (Hansen et al., 2021; Takalloo et al., 2020). The impact of autolysis on N solubility of yeasts in the current study was higher than the values observed for *S. cerevisiae* (Hansen et al., 2021), despite similar autolysis conditions. The observed discrepancy may be partly related to difference in yeast species or the buffer used during the solubility tests. Potassium phosphate buffer was used for solubilizing the yeast in the current study, whereas Hansen et al. (2021) used deionized water. Also, in the current study, the yeast pastes were stored frozen for a longer period before being thawed and then autolyzed/dried. In general, freezing/thawing is a method to increase yield when extracting various molecules from cells. This could be an additional reason for a general higher protein solubility in this experiment than in Hansen et al. (2021). Similar to our results, Tibbetts et al. (2016) showed high protein solubility values (64–84%) in different microalgae solubilized in potassium hydroxide buffer. Additionally, Teuling et al. (2019) have demonstrated that N solubility of microalgae can be improved with different cell wall disruption methods.

In vitro digestibility of protein differs among the three inactivated yeasts (Fig. 2e). Inactivated *C. jadinii* had the highest *in vitro* protein digestibility (84%) while the lowest value (76%) was recorded for inactivated *W. anomalus.* The effect of autolysis on *in vitro* protein digestibility was inconsistent among the three yeasts. Autolysis increased *in vitro* protein digestibility in *C. jadinii* and *B. adeninivorans,* but remained unchanged for *W. anomalus* (Fig. 2e). These observations were inconsistent with the ADCs of protein, suggesting that the *in vitro* protein digestibility method used in the current study may not exactly mimic protein digestibility of yeast in fish. The content of free AA (mmol AA released/g of crude protein weighed) varied among the three yeasts and was unaffected by the autolysis. Free AA contents in the three yeasts were 39 in *C. jadinii,* 42 in *B. adeninivorans and* 30 in *W. anomalus* (Fig. 2f).

3.6. Relationships between ADCs of protein in yeasts and viscosity, digesta dry matter, nitrogen solubility and in vitro protein digestibility measurements

The ADCs of protein in yeasts were positively correlated with digesta viscosity ($P < 0.001$, $r^2 = 0.5$) (Fig. 3a). The increased protein ADCs in yeast with increasing digesta viscosity of fish was unexpected. Studies have shown that increased viscosity negatively affects nutrient digestibility of ingredients due to reduced interaction of nutrients with the intestinal brush border enzymes (Leenhouwers et al., 2006; Leenhouwers et al., 2007; Storebakken, 1985). However, the trend observed in the current study may be explained by the transit time of the intestinal content. A longer transit time implies increased accessibility of intestinal contents to proteolytic enzymes, and thus could explain the increased protein digestibility of yeasts with increased digesta viscosity observed in the current study. The ADCs of protein in yeasts correlate negatively ($P < 0.01$, $r^2 = 0.3$) with digesta DM (Fig. 3b), indicating that an increase in protein ADC caused a decrease in digesta DM, which is according to our expectation. The more digestible an ingredient is, the less DM is expected to be voided as fecal material.

The ADCs of protein in yeasts were positively correlated with N solubility ($P < 0.001$, $r^2 = 0.4$) (Fig. 3c). The increased protein ADCs in yeast with increasing N solubility of yeasts was in line with our expectations. The increased N solubility of yeast could indicate an increase in

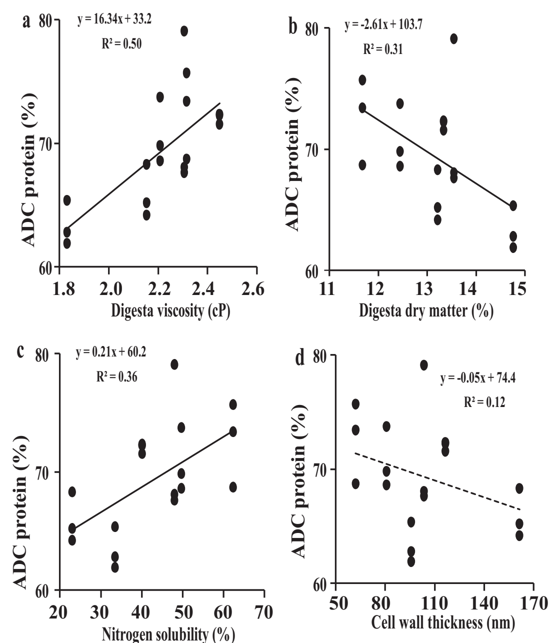

Fig. 3. Linear relationships between the apparent digestibility coefficients (% ADC) of protein from yeasts in juvenile Atlantic salmon and (a) digesta viscosity; (b) digesta dry matter (DM); (c) nitrogen solubility of yeasts; and (d) cell wall thickness of yeasts. Solid and dotted lines denote significant ($P < 0.05$) and non-significant ($P > 0.05$) relationships, respectively. Cell wall thickness of yeasts was presented in Agboola et al. (2021).

cell wall porosity, which consequently leads to higher release of protein to proteolytic enzymes present in the fish gut. Similar positive relationships between N solubility and protein digestibility of microbial ingredients have been reported for various fish species (Agboola et al., 2019; Hansen et al., 2021; Teuling et al., 2019). It is worthy to mention that the r^2 values of the relationships are quite low (0.12–0.5 in Fig. 3) and this could be explained by the limited data points and high variability in the raw data used for the models. Although the graphs (in Fig. 3) are beginning to show some trends, the statistical power of these relationships remain low (*e.g.*, r^2 values of <0.6), thus we recommend that further work is required to validate/strengthen these apparent trends. There were no significant linear relationships between ADCs of protein in yeasts and yeast viscosity, diet viscosity, *in vitro* protein digestibility and free AA content of yeasts ($r^2 < 0.01$) (Figures not shown).

Previously, we have shown that *C. jadinii* (96 nm) had the lowest cell wall thickness among the three yeasts, followed by *B. adeninivorans* (104 nm) and *W. anomalus* (160 nm) (Agboola et al., 2021). Autolysis reduced the cell wall thickness of *C. jadinii* by 16%, *B. adeninivorans* by 40% and *W. anomalus* by 28% (Agboola et al., 2021). These cell wall thickness showed low but positive correlation with ADCs of protein in yeasts (Fig. 3d). This is contrary to our expectations, as we expected the cell wall thickness to be an impediment towards protein digestibility of yeasts in fish. Nevertheless, our results from N solubility indicate that the porosity of the yeast cell wall may have a dominant effect on cell wall thickness in determining nutrient digestibility of yeasts in fish.

4. Conclusions

The present study showed that nutrient digestibility differed among the *C. jadinii*, *B. adeninivorans* and *W. anomalus* yeast species in Atlantic salmon. Autolysis increased protein digestibility of *C. jadinii* and

W. anomalus, but not *B. adeninivorans*. Nutrient digestibility of yeast was dependent on digesta viscosity, but not the viscosity of yeast and diets. Furthermore, cell wall porosity as demonstrated by increased N solubility, had a larger impact on nutrient digestibility of yeasts than the cell wall thickness. The results of the study showed that nutrient digestibility of yeasts in Atlantic salmon depends on the type of yeasts and the downstream processing used after harvesting the yeast. The particular *in vitro* digestibility method used in the current study did not adequately reflect the protein digestibility of yeasts in Atlantic salmon.

Declaration of Competing Interest

The authors declared no competing interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2021.737312.

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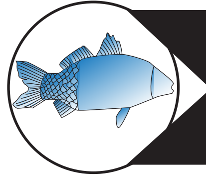

Paper V

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Article

Effects of Yeast Species and Processing on Intestinal Health and Transcriptomic Profiles of Atlantic Salmon (*Salmo salar*) Fed Soybean Meal-Based Diets in Seawater

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Abstract: The objective of the current study was to examine the effects of yeasts on intestinal health and transcriptomic profiles from the distal intestine and spleen tissue of Atlantic salmon fed SBM-based diets in seawater. *Cyberlindnera jadinii* (CJ) and *Wickerhamomyces anomalus* (WA) yeasts were heat-inactivated with spray-drying (ICJ and IWA) or autolyzed at 50 °C for 16 h (ACJ and AWA), followed by spray-drying. Six diets were formulated, one based on fishmeal (FM), a challenging diet with 30% soybean meal (SBM) and four other diets containing 30% SBM and 10% of each of the four yeast fractions (i.e., ICJ, ACJ, IWA and AWA). The inclusion of CJ yeasts reduced the loss of enterocyte supranuclear vacuolization and reduced the population of CD8α labeled cells present in the lamina propria of fish fed the SBM diet. The CJ yeasts controlled the inflammatory responses of fish fed SBM through up-regulation of pathways related to wound healing and taurine metabolism. The WA yeasts dampened the inflammatory profile of fish fed SBM through down-regulation of pathways related to toll-like receptor signaling, C-lectin receptor, cytokine receptor and signal transduction. This study suggests that the yeast species, *Cyberlindnera jadinii* and *Wickerhamomyces anomalus* are novel high-quality protein sources with health-beneficial effects in terms of reducing inflammation associated with feeding plant-based diets to Atlantic salmon.

Keywords: *Cyberlindnera jadinii*; *Wickerhamomyces anomalus*; intestinal health; SBMIE; transcriptomics; distal intestine; spleen; autolysis

1. Introduction

In recent decades, the composition of Atlantic salmon (*Salmo salar*) diets has changed towards the use of more plant ingredients [1] due to limited availability and increased market prices of fishmeal (FM) [2]. Currently, commercial salmon diets contain about 75% plant-derived ingredients [1]. Soybean meal (SBM) is an attractive plant ingredient due to its availability and high protein content as well as its low production cost [3]. It has already been described that SBM contains anti-nutritional factors (ANFs), especially saponin, which can induce inflammation in the distal intestine (DI) of Atlantic salmon, a condition commonly referred to as SBM-induced enteritis (SBMIE) [4,5]. Dietary inclusion of SBM induced both local and systemic responses in Atlantic salmon [6,7]. Studies have shown up-regulation of genes associated with increased gut permeability in Atlantic

salmon fed SBM-based diets [6,8], which may lead to translocation of opportunistic bacteria to the underlying mucosa [9,10]. Thus, the inclusion of SBM in salmon diets can have adverse effects on growth performance and fish health [11]. For these reasons, soy-protein concentrate, which is a refined soy product with low levels of ANFs, is currently used in commercial salmon diets. However, a recent study has shown that DI inflammation is still being observed in commercial salmon production in Norway [12].

Yeasts are gaining increasing interest as alternative ingredients for salmonids [12–14]. Yeasts are not only high-quality protein ingredients but also contain bioactive components that have the potential to mitigate SBMIE in Atlantic salmon [15–17]. The yeast cell wall contains bioactive components, including β-glucan, mannan and chitin, which has immune-modulating properties that reduce inflammation caused by SBM [15]. However, the bioactivity of these cell wall components depends on the yeast species, the fermentation conditions during yeast production and the downstream processing used before incorporating them into a salmon diet [13,18]. The inclusion of yeast in salmon diets could be a nutritional strategy to improve intestinal health and develop robust fish when feeding them with plant-based diets. Therefore, the objective of this study was to investigate the effect of yeasts on intestinal health and transcriptomic profiles of DI and spleen tissues from Atlantic salmon fed SBM-based diet in seawater. The applied yeasts, *Cyberlindnera jadinii* (CJ) and *Wickerhamomyces anomalus* (WA), were produced from wood sugars using in-house bioreactors. These yeasts were selected based on their functional effects reported in our previous study [15].

2. Results

2.1. Yeast Compositions

Yeast contained between 42 and 47% crude protein (Table 1). Autolysis increased the crude lipid content by 96% (from 2.9 to 5.7% DM) and 46% (from 2.8 to 4.1% DM) for CJ and WA, respectively (Table 1). Ash, total phosphorus, and gross energy contents of the two yeast species were similar and unaffected by the autolytic process (Table 1). The β-glucan, mannan and chitin content (% DM) of the inactivated yeast species were in the ranges of 15-16%, 8-11% and 0.3-0.5%. In both CJ and WA, autolysis reduced the content of β-glucan and mannan by 21-33% and 8-24%, respectively (Table 1).

Table 1. Composition of spray-dried yeasts with and without the autolysis treatment. All values are presented in % DM, except gross energy, which is presented in MJ/kg DM.

	Cyberlindnera jadinii		*Wickerhamomyces anomalus*	
	Inactivated	**Autolyzed**	**Inactivated**	**Autolyzed**
DM[1] (%)	96.3 ± 0.03	93.1 ± 0.04	96.1 ± 0.02	96.1 ± 0.06
Nutrients (% DM)[2]				
Crude protein	46.5 ± 0.47	47.4 ± 0.01	43.0 ± 0.04	42.1 ± 0.26
Crude lipids	2.9 ± 0.18	5.7 ± 0.17	2.8 ± 0.06	4.1 ± 0.02
Ash	5.7 ± 0.00	5.9 ± 0.01	5.5 ± 0.00	5.5 ± 0.00
Total phosphorus	0.6 ± 0.02	0.6 ± 0.01	0.5 ± 0.01	0.4 ± 0.02
Gross energy (MJ/kg DM)	21.8 ± 0.01	22.32 ± 0.02	21.1 ± 0.01	21.5 ± 0.01
Cell wall polysaccharides (% DM)[3]				
β-glucan	16.4 ± 3.19	11.1 ± 0.84	15.0 ± 1.41	11.8 ± 0.73
Mannan	7.9 ± 2.16	6.0 ± 0.66	11.3 ± 0.95	10.4 ± 0.67
Chitin	0.3 ± 0.07	0.2 ± 0.02	0.5 ± 0.05	0.4 ± 0.08

[1] DM—dry matter; [2] Crude protein, crude lipids, ash, total phosphorus, and gross energy contents of yeasts are mean values ± SD from duplicate analyses; [3] β-glucan, mannan and chitin contents of yeasts are mean values ± SD from triplicate analyses.

The amino acid content in the yeasts was reduced after autolysis (by 2.4 and 5.9% in CJ and WA, respectively), which is also reflected by the increase in non-protein nitrogen content of the yeasts by 13 and 10% in CJ and WA, respectively (Table S1).

2.2. Fish Performance and Nutrient Digestibility

There was no significant difference (P > 0.05) in biomass gain, specific growth rate (SGR), feed intake and feed conversion ratio (FCR) among the dietary treatments (Table S2). During the first week of the experiment, feed intake was low for all dietary groups (Figure S1). No fish mortality was observed throughout the experimental period. The apparent digestibility coefficient (ADC) of crude protein was significantly (P < 0.05) higher in fish fed the FM diet compared with the other dietary treatments (Table S2). Conversely, fish fed the IWA diet had significantly lower (P < 0.05) digestibility of crude lipids compared with the other treatments (Table S2).

2.3. Histopathological Changes in Fish

Mild to moderate inflammatory changes were observed in the DI mucosa of fish fed the experimental diets (Figure S2). The observed changes were characterized by a marked to total loss in enterocyte vacuolization, a mild to moderate decrease in mucosal fold height, and a mild infiltration of the submucosa and lamina propria by inflammatory cells (Figure S2). Fish fed the FM diet showed normal and healthy morphology (Figures 1a-d). Mild to moderate inflammatory changes were observed in fish fed the SBM diet and were not statistically different (P > 0.05) from fish fed ICJ, ACJ, IWA, and AWA diets (Figures 1a-c). Considering histological changes due to loss of supranuclear vacuolization, fish fed ICJ and ACJ diets were significantly different (P < 0.05) from fish fed SBM with marked changes (Figure 1d).

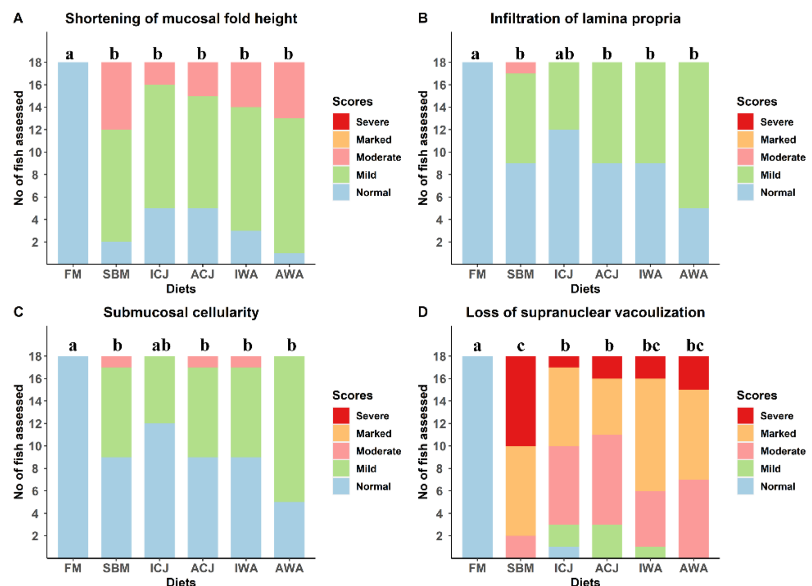

Figure 1. Histopathological changes in the distal intestine of Atlantic salmon smolts fed FM- or SBM-based diets with yeasts in seawater. The semi-quantitative scoring was obtained by measuring changes in four morphological parameters: (**A**) shortening of mucosal fold height; (**B**) infiltration of lamina propria; (**C**) submucosal cellularity; and (**D**) loss of supranuclear vacuolization. Each parameter was given a score of "0" representing normal morphology; "1" mild; "2" moderate; "3" marked; and "4" severe enteritis. Groups with different letters (a-c) above the bar charts are significantly different (P < 0.05). The diets were: FM—fishmeal-based; SBM—soybean meal-based; 4 experimental diets containing 300 g/kg SBM and 100 g/kg of ICJ—inactivated *Cyberlindnera jadinii*; ACJ—autolyzed *C. jadinii*; IWA—inactivated *Wickerhamomyces anomalus*; AWA—autolyzed *W. anomalus* diets.

2.4. Changes in T-lymphocyte Population

Both CD3ε- and CD8α-labeled cells were observed in all dietary groups with their expression more pronounced in the epithelium than the lamina propria (Figure S3). In general, there was a higher abundance of CD3ε positive lymphocytes compared with CD8α positive lymphocytes in the DI of fish fed all the experimental diets, (Figure S3). No statistical difference was observed for the area of epithelium occupied by CD3ε and CD8α positive cells among the diets (data not shown). Similarly, there was no significant difference ($P > 0.05$) for the area of lamina propria occupied by CD3ε positive cells among the diets (Figure 2). The area of lamina propria occupied by CD8α positive cells was significantly higher ($P < 0.05$) in fish fed the SBM diet, compared with the other diets (Figure 2). The simple fold length was significantly higher in fish fed the FM diet, compared with fish fed SBM, ICJ, ACJ, IWA and AWA diets (Figure 2).

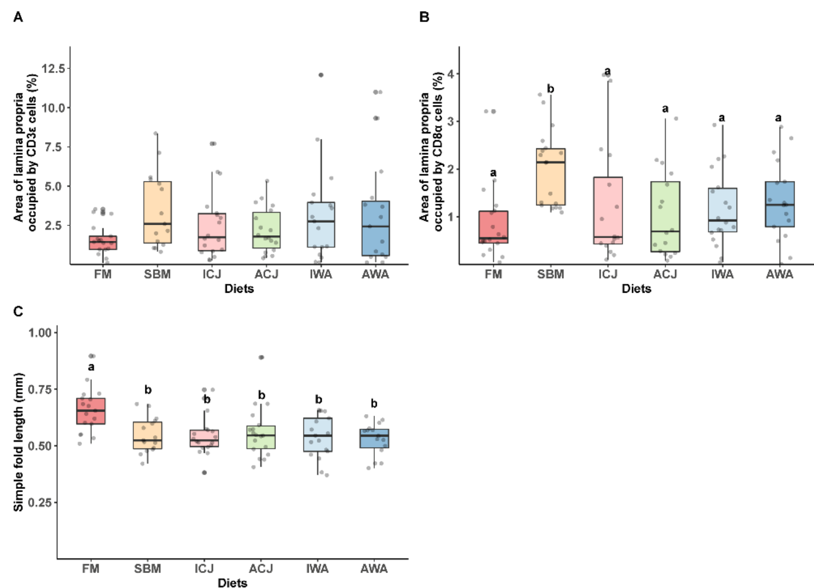

Figure 2. Area of lamina propria occupied by (**A**) CD3ε and (**B**) CD8α T-cells, and (**C**) simple fold length of the distal intestine of Atlantic salmon smolts fed FM- or SBM-based diets with yeasts in seawater. Groups with different letters (a-c) above the boxplots are significantly different ($P < 0.05$). The diets were: FM—fishmeal-based; SBM—soybean meal-based; 4 experimental diets containing 300 g/kg SBM and 100 g/kg of ICJ—inactivated *Cyberlindnera jadinii*; ACJ—autolyzed *C. jadinii*; IWA—inactivated *Wickerhamomyces anomalus*; AWA—autolyzed *W. anomalus* diets.

2.5. Transcriptomics

Higher differentially expressed genes (DEGs) were found between diet comparisons in DI tissue compared with spleen tissue (Table 2). In the DI, the comparison between fish fed SBM and FM diets showed 173 down-regulated and 143 up-regulated genes. A lower number of DEGs occurred when fish fed ICJ (71 down-regulated, 54 up-regulated) and ACJ (33 down-regulated, 31 up-regulated) diets were compared with fish fed the FM diet. The highest number of DEGs were observed in fish fed AWA (2685 down-regulated, 2714 up-regulated) and IWA (1299 down-regulated, 1036 up-regulated) diets, compared with those fed the FM diet. A list of significant DEGs between diet comparisons along with the name of each gene is attached in Table S3.

Table 2. Significant differentially expressed genes (DEGs) per diet-comparison[1].

Diet Comparison	Down-Regulated	Up-Regulated
Distal intestine		
SBM∣FM	173	143
ICJ∣FM	71	54
ACJ∣FM	33	31
IWA∣FM	1299	1036
AWA∣FM	2685	2714
Spleen		
SBM∣FM	6	20
ICJ∣FM	15	11
ACJ∣FM	5	12
IWA∣FM	7	6
AWA∣FM	12	19

[1] The diets were: FM—fishmeal-based; SBM—soybean meal-based; 4 other diets containing 300 g/kg SBM and 100 g/kg of ICJ—inactivated *Cyberlindnera jadinii*; ACJ—autolyzed *C. jadinii*; IWA—inactivated *Wickerhamomyces anomalus*; AWA—autolyzed *W. anomalus* diets.

2.6. Gene Ontology

For DI tissue, the Gene Ontology (GO) analysis when comparing fish fed SBM with those fed the FM diet, showed that the up-regulated terms in SBM were mainly related to transport-channel activity, lysosome, and tight junction function, while the down-regulated GO terms were related to metabolic pathways (SBM∣FM, Figure 3).

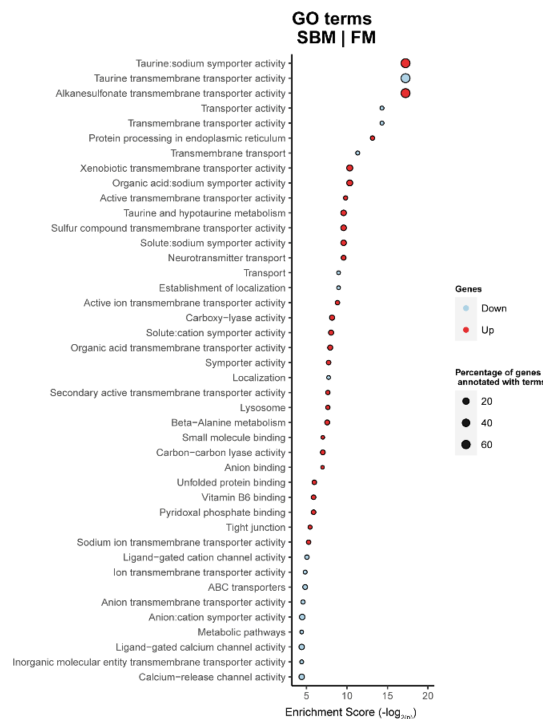

Figure 3. Significantly enriched gene ontology (GO) terms (minGSSize = 3) in the distal intestine of Atlantic salmon smolts fed SBM-based diet compared with fish fed the FM-based diet. The list is ordered by decreasing Enrichment Score (-log$_2$(P)). Up, up-regulated (in red); Down, down-regulated (in light blue). The diets were: FM—fishmeal-based and SBM—soybean meal-based diets.

The comparison between fish fed ICJ and FM revealed up-regulated GO terms relating to metabolic process, wound healing, vitamin B6 binding, taurine and hypotaurine metabolism, while the down-regulated GO terms were related to transport activity and biosynthetic processes (ICJ|FM, Figure 4A). Similar up-regulated and down-regulated GO terms were observed when fish fed ACJ were compared with those fed FM (ACJ|FM, Figure 4B).

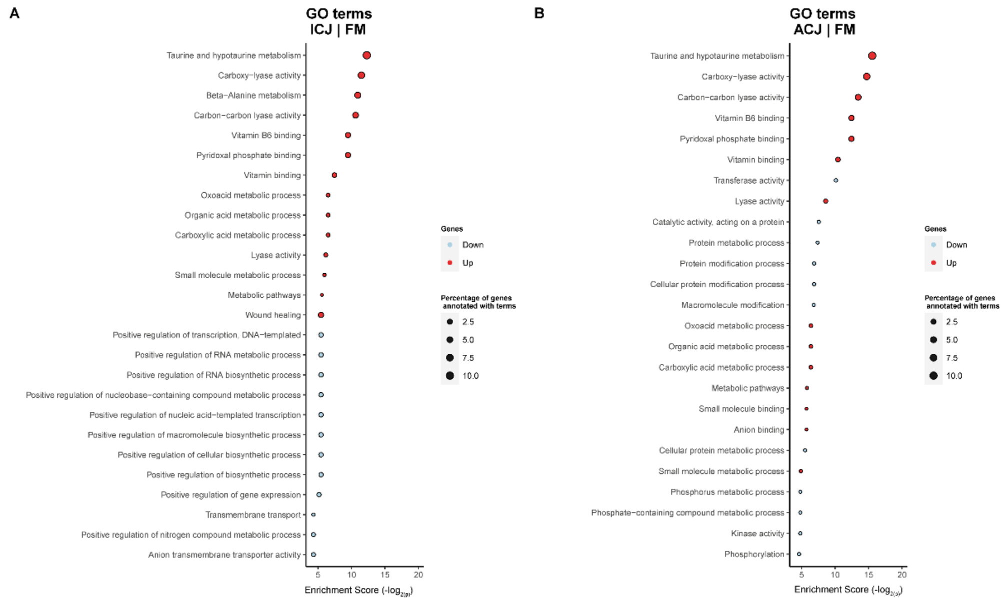

Figure 4. Significantly enriched gene ontology (GO) terms (minGSSize = 3) in the distal intestine of Atlantic salmon smolts fed ICJ (A) or ACJ (B) diets compared with fish fed the FM diet. The list is ordered by decreasing Enrichment Score (-log$_2$(P)). Up, up-regulated (in red); Down, down-regulated (in light blue). The diets were: FM—fishmeal-based; and diet containing 300 g/kg SBM with 100 g/kg of ICJ—inactivated *Cyberlindnera jadinii* and ACJ—autolyzed *C. jadinii* diets.

In addition, when comparing fish fed IWA and AWA diets with those fed the FM diet, the results showed up-regulated terms related to energy metabolism (Figures 5A and S4A), while the down-regulated GO terms were related to the immune response pathway and oxidation–reduction process (Figures 5B and S4B). There was no differentially significant GO term between diet comparisons for the spleen tissue.

Figure 5. Significantly enriched gene ontology (GO) terms (minGSSize = 3) in the distal intestine of Atlantic salmon smolts fed the IWA diet compared with fish fed the FM diet. The list is ordered by decreasing Enrichment Score (-log$_2$(P)). A. Up, up-regulated (in red); B. Down, down-regulated (in light blue). The diets were: FM—fishmeal-based; and diets containing 300 g/kg SBM with 100 g/kg of IWA—inactivated *Wickerhamomyces anomalus* diet. The top 40 up-regulated and down-regulated genes are presented.

Figure 6 showed shared pathways of network analysis of GO terms when comparing fish fed SBM-based diets with those fed the FM diet. Fish fed SBM and AWA diets shared similar pathways associated with transporter activity. The shared pathways were up-regulated in SBM and down-regulated in AWA. Fish fed ICJ and ACJ diets shared similarities associated with up-regulation of the acid metabolic process, lyase activity and small molecule metabolic process. Furthermore, we observed that fish fed IWA contained down-regulation of pathways related to the toll-like receptor signaling pathway, C-lectin receptor pathway, cytokine receptor activity and signal transduction, and these are connected to other pathways that were down-regulated in fish fed the AWA diet. Furthermore, many pathways related to nucleotide and carbohydrate binding and hydrolase, phosphatase, and ATPase are up-regulated in fish fed both IWA and AWA diets.

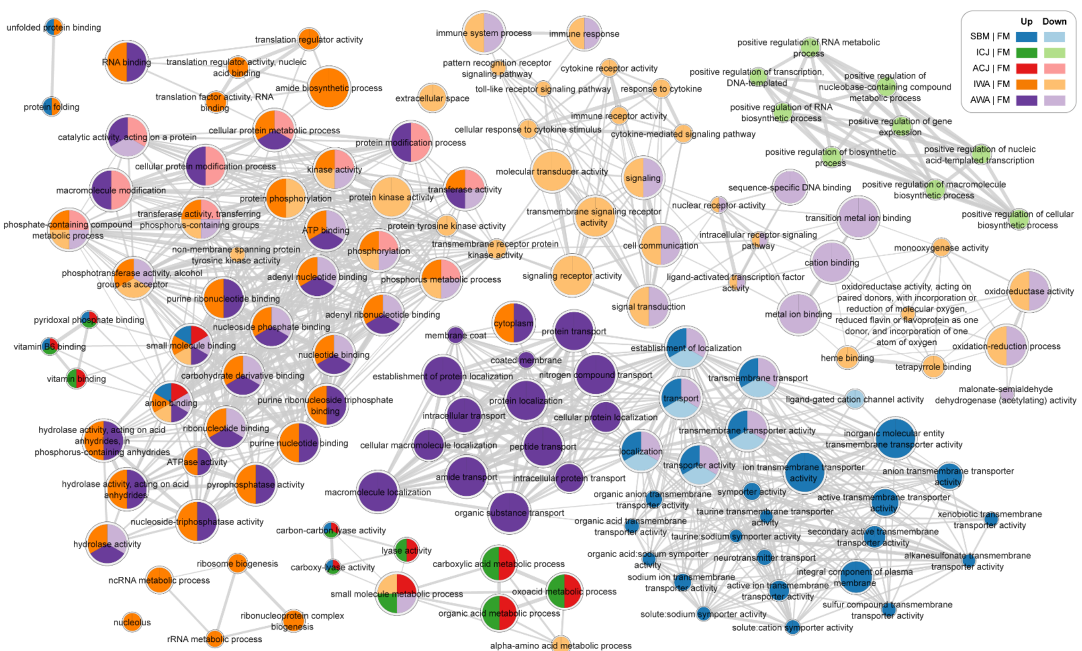

Figure 6. Network of significantly enriched GO terms between different diet comparisons. Each node is a GO term with size indicating the number of genes annotated with that term. Edges connect GO terms that are sufficiently similar to each other in terms of shared genes with edge thickness indicating the similarity coefficient (> 0.375). Node colors indicate diet comparisons in which the GO term was significantly enriched, according to the color legend. The diets were: FM—fishmeal-based; SBM—soybean meal-based; 4 experimental diets containing 300 g/kg SBM and 100 g/kg of ICJ—inactivated *Cyberlindnera jadinii*; ACJ—autolyzed *C. jadinii*; IWA—inactivated *Wickerhamomyces anomalus*; AWA—autolyzed *W. anomalus diets*. Cytoscape was used to visualize enriched GO terms for all diet comparisons in a network.

3. Discussion

The feed intake and growth data showed that the fish adjusted quickly to the seawater environment and obtained a reasonable feed intake two weeks post transfer. The length of the current study is too short to draw any conclusions on fish performance, but 10% dietary inclusion of the two yeast species did not compromise the growth of Atlantic salmon smolts. The reduced digestibility of crude protein in fish fed SBM-based diets reflected in numerically lower performance in fish fed these diets. In line with previous studies [4,5], fish fed the SBM diet developed classical symptoms of SBMIE, characterized by marked to severe loss in enterocyte vacuolization. The current results showed that the inclusion of ICJ and ACJ yeasts partially reduced the loss of supranuclear vacuolization in Atlantic salmon fed SBM-based diets. This is in accordance with previous studies, which demonstrated that CJ can be used to alleviate SBMIE in Atlantic salmon [15–17]. Based on the histological results, the inclusion of IWA and AWA yeasts in the diets did not prevent SBMIE, which contradicts the results of a recent work [15]. The WA used in the previous study [15], however, had a higher content of crude protein (19% more), mannan (38% more) and chitin (75% more) compared with the currently used batch. Conversely, the β-glucan content of the previous WA yeast was 31% lower than the present one. The disparity in the composition of bioactive components present in the yeast batch could account for their variation in preventing SBMIE in Atlantic salmon. The limited effect of WA on SBMIE in the present study could also be attributed to the difference in fish production stage, fish size or age, or to the severity of SBMIE. The previous study by

Agboola et al. [15] was conducted in freshwater with 5 g fish, which only displayed mild inflammatory changes when fed the SBM diet [15], suggesting that WA might be effective against mild inflammatory changes in Atlantic salmon.

Previous studies have demonstrated that T-cell mediated hypersensitivity is crucial to the development of SBMIE in Atlantic salmon [19–21]. Thus, CD3ε and CD8α positive cells are important biomarkers to evaluate inflammation caused by inclusion of SBM (or other plant ingredients) in salmon diets [17,19]. Furthermore, they are important biomarkers for studying the effect of functional diets on inflammatory responses of fish fed SBM [17]. In the present study, the higher abundance of both T-lymphocyte populations along the basal part of DI epithelium, compared with the lamina propria, was in accordance with a previous study [17]. Bakke-McKellep et al. [19] reported an increased presence of CD3ε-labeled cells in the lamina propria of fish presenting SBMIE, which is contrary to the observation in the present study. The discrepancy might be attributed to a number of factors such as variation in SBM used and increased tolerance of fish to SBM in recent years. Previous studies have shown that differences in the level of ANFs (especially saponin) in commercial sources of SBM can influence the degree of SBMIE in Atlantic salmon [22–24]. The increased tolerance of fish in recent years could be the result of breeding and genetic selection of fish for improved growth performance and adaptability to plant-based diets. Studies have shown increased growth performance and no detection of enteritis in strain of rainbow trout selected on a diet containing SBM, compared to non-selected strains [25,26]. However, similar studies in Atlantic salmon are scarce in scientific literature.

The area of lamina propria occupied by CD8α positive cells were higher in fish fed the SBM diet compared with those fed the FM diet, which is in line with previous studies [17,19]. However, there was a reduction in the population of CD8α cells in fish fed ICJ, ACJ, IWA and AWA diets compared with fish fed SBM, indicating an immunomodulatory effect of these yeasts when included in SBM-based diets. Reveco-Urzua et al. [17] reported a similar effect when 2.5% CJ was supplemented to a 20% SBM-based diet. Our results showed there was no effect of yeast supplementation on CD3ε cell population, and this could be explained by the large variability within the dietary group.

Our results at the transcriptional level revealed that fish fed SBM compared with FM, showed activation of transporters and channel activities, implying increased permeability in the DI of fish. In the current study, increased gut permeability is supported by up-regulation of solute carrier proteins, slc6a6 and SC6A6, in the DI of fish fed the SBM diet. This is similar to findings of previous studies that showed that SBM increases gut permeability through the up-regulation of genes associated with solute carriers and channel proteins [6,8,27]. The results also showed that fish fed SBM responded to inflammatory changes through up-regulation of genes associated with tight junction and lysosomal pathways. Tight junction proteins play important roles in intestinal fluid permeability in Atlantic salmon [28–31]. In this study, tight junction proteins such as aquaporin (aqp10b), claudin (cldn12) and nucleoporin (nup93) genes were up-regulated in response to feeding SBM. These have been previously reported in the intestine of Atlantic salmon fed SBM diets in response to increased gut permeability [8,28,32,33]. Lysosome is a vesicle that contains lysozyme, an anti-microbial protein responsible for pathogen degradation during innate immunity [34]. In this study, the up-regulation of the lysosomal pathway in fish fed SBM could be attributed to the translocation of opportunistic bacteria to the underlying mucosa due to increased intestinal permeability [9,10]. This result is in accordance with previous findings showing increased lysozyme production in the DI of Atlantic salmon fed SBM-based diet [6,35].

When comparing fish fed both CJ diets with FM, the results showed up-regulation of GO terms such as wound healing, as well as taurine and hypotaurine metabolism. Thus, it seems that the ICJ and ACJ yeasts were able to partially restore the integrity of the intestinal surface barrier following inflammatory damage caused by SBM. In response to the intestinal damage, genes participating in the wound healing process, such as vimentin

(VIME) and integrin protein (itgb3a), were activated in fish fed ICJ and ACJ diets. Vimentin (VIME) is known to interact with other structural proteins such as microtubules to maintain cellular integrity and provide resistance to cell damage [36]. Integrin protein (itgb3a) mediates the adhesive properties of intestinal epithelial cells and is needed to achieve mucosal wound closure [37]. Integrin interacts with the actin-binding protein, annexin A2, to facilitate movement of intestinal epithelial cells during wound resealing [37]. Our speculation is that the bioactive compounds in CJ support the wound resealing process, and this is partially responsible for its mode of action in counteracting SBMIE. Another possible mode of action of ICJ and ACJ may be connected to the reduction in oxidative stress through taurine and hypotaurine pathways. Taurine is categorized as a semi-essential keto acid that plays a key role in innate immunity and reduction in oxidative stress [38,39].

Fish fed IWA and AWA diets revealed alteration of genes associated with the immune responses compared with those fed the FM diet. Cytokines, such as tumor necrosis factor alpha (TNFα), interleukin-12 (IL-12), interferon gamma (IFNγ) and pattern recognition receptor (e.g., Toll-like receptor-7 (TLR7)) were down-regulated in fish fed IWA and AWA diets. This implies that these yeasts were able to control the inflammatory profile of SBM associated with M1 macrophages [40]. We speculate that the down-regulation of IL-12 reduced the inflammatory profile associated with SBM by suppressing the expression of TNFα and IFNγ. Previous studies have demonstrated increased expression TNFα and IFNγ in Atlantic salmon in response to dietary SBM [7,15]. Thus, the down-regulation of TNFα and IFNγ genes in the DI of fish fed IWA and AWA diets indicates the potential of these yeasts to regulate the inflammation caused by SBM. The suppression of genes associated with TNFα and IFNγ in fish fed IWA and AWA diets is supported by the down-regulation of genes associated with CD83 expression. In higher vertebrates, CD83 is expressed by mature dendritic cells, and its down-regulation can suppress T helper cells and, in the process, decrease the expression of TNFα and IFNγ cytokines [41,42]. Although the possible immunomodulatory potential of IWA and AWA yeasts was detected on a transcriptomic level, this effect was not clearly seen on the histological level.

Furthermore, the similar response of enriched pathways in fish fed the inactivated or autolyzed yeast diets (i.e., between ICJ vs. ACJ and IWA vs. AWA) suggests that processing by autolysis did not improve the beneficial health effect of the two yeast species in the current study, which is in contradiction with previous findings [7,15]. It is not clear what causes the discrepancy, but it might be related to the time lag between harvesting and autolysis of the yeast pastes in these experiments. The yeast pastes used by Agboola et al. [15] were stored for 5-6 months before the autolysis process. During this storage period, the yeasts might have undergone self-hydrolysis, which possibly contributes to their immunomodulatory effect in fish. On the contrary, yeasts used in the current study were autolyzed immediately after harvesting.

In the current study, the transcriptomic analysis of the spleen tissue revealed that the experimental diets did not induce systemic effects in fish. Previous studies on Atlantic salmon fed SBM-based diets have reported both similar effects [6] and contradicting effects [7] using head kidney and spleen, respectively. The reason for these differences is not clear, but it might be related to the sensitivity of immunological organs in the different life stages of fish. Similar to the current trial, Kiron et al.'s study [6] was conducted in seawater, whereas Morales-Lange et al.'s study [7] was conducted in freshwater. Thus, studies identifying possible factors influencing the systemic effect of fish fed SBM and SBM in combination with functional ingredients in both freshwater and seawater is warranted in the future. This might be key to our understanding of when to include functional ingredients in fish diets.

4. Materials and Methods

4.1. Yeast Production and Processing

In the present study, CJ and WA yeasts were cultured according to Lapeña et al. [43], using a growth medium containing a blend of enzymatic hydrolysates of pre-treated spruce wood (Picea abies) and chicken by-products. The yeast biomass was produced aerobically in a 42 L Techfors S bioreactor (Infors, Bottmingen, Switzerland) with 25 L working volume running as repeated batch fermentations. After harvesting, the yeasts were washed and re-suspended in 7 ºC deionized water in a 30 L reactor, equipped with a helical impeller (Einar, Belach Bioteknik, Skogås, Sweden). The washed yeasts were further centrifuged to obtain a paste with 32% and 41% dry matter content for CJ and WA, respectively. The processing of the yeast paste was performed according to the method described by Agboola et al. [15]. Briefly, the paste from each yeast biomass was mixed and divided into two homogenous parts. The first part of the yeast paste was inactivated by spray-drying using a SPX 150 MS (SPX Flow Technology, Søborg, Denmark). The other part of the paste was autolyzed at 50 ºC for 16 h in the 30 L Einar reactor, followed by spray-drying. The inlet and outlet temperatures of the spray-dryer were set at 180 and 80 ºC, respectively. Inactivated CJ (ICJ), autolyzed CJ (ACJ), inactivated WA (IWA), and autolyzed WA (AWA) were the four yeast ingredients used in this study.

4.2. Formulation and Production of Fish Feeds

A total of six experimental diets were used in the current trial. The diets were a fishmeal-based (FM) control diet, a 30% soybean meal-based (SBM) diet as a challenging positive control diet, and four experimental diets containing 30% SBM and 10% inclusion of the different processed yeasts (ICJ, ACJ, IWA and AWA), respectively. The formulation and analyzed nutritional compositions of the experimental diets are presented in Table 3. The diets contained a similar ratio of digestible protein to digestible energy and were formulated to meet the nutritional requirement of Atlantic salmon smolts [44,45]. The plant-based diets were supplemented with crystalline lysine and methionine to balance the essential amino acid profile to that of the FM control. For feed production, all dry ingredients were weighed and mixed in a Spiry 25 dough mixer (Moretti Forni, Mondolfo, Italy). Gelatin was firstly mixed in cold water, then heated up to 60 ºC in a microwave oven. The gelatin and fish oil were mixed with dry ingredients using the same mixer as described above. The mash was cold-pelleted using a P35A pasta extruder (Italgi, Carasco, Italy) and the resulting feed pellets were dried (to about 93% dry matter content) in small experimental dryers at about 60 ºC drying temperature. The feeds were stored at 4 ºC before and during the experimental period.

Table 3. Formulation and nutritional composition of the experimental diets*.

	FM	SBM	ICJ	ACJ	IWA	AWA
Diet formulation [a]						
Fish meal [b]	433.4	261.4	208.4	208.4	208.4	208.4
Soybean meal [c]	0	300	300	300	300	300
Yeast [n]	0	0	100	100	100	100
Wheat gluten [d]	170	136	111	111	111	111
Potato starch [e]	120	90	68	68	68	68
Cellulose	80	0	0	0	0	0
Fish oil [f]	130	130	130	130	130	130
Gelatin [g]	60	60	60	60	60	60
Monocalcium phosphate [h]	0	10	10	10	10	10
Premix [i]	5	5	5	5	5	5
L-lysine [j]	0	3	3	3	3	3
DL-Methionine [k]	0	3	3	3	3	3
Choline chloride [l]	1.5	1.5	1.5	1.5	1.5	1.5
Yttrium oxide [m]	0.1	0.1	0.1	0.1	0.1	0.1

Diet composition (analyzed values) [o]						
Dry matter (g/kg)	926	897	889	889	924	913
Crude protein	531	542	518	530	519	521
Starch	131	103	92	93	89	87
Ash	78	77	75	75	74	74
Carbon	509	510	503	518	513	511
Sulphur	6.0	6.3	6.2	6.0	6.1	6.0
Energy (MJ/kg DM)	23.3	23.1	23.3	23.3	23.1	23.1
DP:DE[p]	23.1	23.3	22.8	22.8	22.5	22.5

[a]Diet formulations are expressed in g/kg**;** [b]LT fishmeal, Norsildmel, Egersund, Norway; [c]Soybean meal, Denofa AS, Fredrikstad, Norway; [d]Wheat gluten, Amilina AB, Panevezys, Lithuania; [e]Lygel F 60, Lyckeby Culinar, Fjälkinge, Sweden; [f]NorSalmOil, Norsildmel, Egersund, Norway; [g]Rousselot 250 PS, Rousselot SAS, Courbevoie, France; [h]Monocalcium phosphate, Bolifor MCP-F, Yara, Oslo, Norway; [i]Premix fish, Norsk Mineralnæring AS, Hønefoss, Norway. Per kg feed ; Retinol 3150.0 IU, Cholecalciferol 1890.0 IU, α-tocopherol SD 250 mg, Menadione 12.6 mg, Thiamin 18.9 mg, Riboflavin 31.5 mg, d-Ca-Pantothenate 37.8 mg, Niacin 94.5 mg, Biotin 0.315 mg, Cyanocobalamin 0.025 mg, Folic acid 6.3 mg, Pyridoxine 37.8 mg, Ascorbate monophosphate 157.5 g, Cu: CuSulfate $5H_2O$ 6.3 mg, Zn: ZnSulfate 151.2 mg, Mn: Mn(II)Sulfate 18.9 mg, I: K-Iodide 3.78 mg, Ca 1.4 g; [j]L-Lysine CJ Biotech CO., Shenyang, China; [k]Rhodimet NP99, Adisseo ASA, Antony, France; [l]Choline chloride, 70% Vegetable, Indukern SA., Spain; [m]Y_2O_3. Metal Rare Earth Limited, Shenzhen, China**;** [n]ICJ—inactivated *Cyberlindnera jadinii*; ACJ—autolyzed *C. jadinii*; IWA—inactivated *Wickerhamomyces anomalus*; AWA—autolyzed *W. anomalus*; [o]Diet compositions are expressed in g/kg dry matter (DM) unless otherwise stated; [p]DP:DE = Digestible protein to digestible energy ratio. Calculated using internal digestibility values of various ingredients**;** *The diets were: FM—fishmeal-based; SBM—soybean meal-based; 4 other diets containing 300 g/kg SBM and 100 g/kg of ICJ, ACJ, IWA and AWA yeasts.

4.3. Fish Management and Feeding

A total of 450 vaccinated Atlantic salmon smolts with an average initial weight of 136 ± 0.25 g were sorted, batch weighed and transported in oxygenated plastic bags from the Center for sustainable Aquaculture at the Norwegian University of Life Sciences (Ås, Norway) to the research facility of Norwegian Institute of Water Resources (NIVA, Solbergstrand, Norway). The fish were randomly distributed into 18 fiber tanks (300 L) equipped with automatic feeders with 25 fish stocked into each tank. The six experimental diets were randomly allocated to all the tanks in triplicate. During the first week of the experiment, fish were fed 1% of their body weight, and feeding was subsequently increased based on feed consumption in each tank. Feeds were supplied 6 h a day using automatic feeders delivering feed every 12 min. Uneaten feed was collected after each feeding from the outlet water settling on a screen for each tank. Daily feed intake was calculated from the dry weight of the feed given and the dry weight of the recovered uneaten feed, corrected for feed recovery rate of each tank. Water salinity was gradually increased from 5 ppt at the start, until it reached full salinity (33 ppt) over the first 12 days of the experiment. Fish were kept under a 24 h light regime in a flow-through system with an average water temperature of 11.5 ºC and average oxygen saturation of 84%. The water flow was kept at an average of 5.5 L min-1 during the experimental period.

After 42 days of feeding, six fish were randomly selected from each tank, anesthetized with metacaine (MS-222, 50 mg L-1 water), and killed with a sharp blow to the head for tissue sampling. The body weight of individual selected fish was recorded and included in the total tank mean weight. After dissection, distal intestine (DI) and spleen tissue samples were collected from each selected fish. The DI is described as the segment from the increase in intestinal diameter and the appearance of transverse lumina folds to the anus. The DI was dissected longitudinally, the content was removed, and the tissue was carefully divided into two parts. One part was fixed in 10% phosphate-buffered formalin for 24 h before storage in 70% ethanol until further processing for both histological and immunohistochemistry analyses. The second part of the DI was cut into three pieces and immediately suspended in RNA-later and stored over night at 4 ºC, and later at -80

ºC until total RNA extraction. The spleen samples were treated in the same manner as the DI samples for total RNA extraction. The remaining fish per tank after tissue sampling were anesthetized, counted and group weighed for determination of fish growth performance. Furthermore, fecal samples were collected by stripping the remaining fish per tank for determination of nutrient digestibility. The fecal samples were stored at -20 ºC before freeze-drying.

4.4. Histological Examination

The DI tissue samples (18 samples per dietary group) were processed at the Veterinary Institute Laboratory in Oslo, Norway, according to standard techniques for histological assessment [4]. Briefly, formalin-fixed DI tissue samples were dehydrated in ethanol, equilibrated in xylene, and embedded in paraffin. Longitudinal sections of 3 μm thickness from each DI tissue sample were prepared and stained with hematoxylin and eosin. The sections were then blindly characterized under a light microscope with an emphasis on the morphological changes observed with SBMIE as previously described by Baeverfjord and Krogdahl [4]. The histological scores were obtained through a semi-quantitative scoring system, measuring changes in four morphological criteria: shortening of mucosal fold height, increase in width and cellularity of the submucosa and lamina propria, and loss of enterocyte supranuclear vacuolization [4]. Each criterion was given a score of 0–4, where 0 represented normal; 1 mild changes; 2 moderate changes; 3 marked changes; and 4 severe changes.

4.5. Detection of T-lymphocytes by Immunohistochemistry

The CD3ε and CD8α positive T-lymphocytes in the DI tissue of fish fed the experimental diets were detected using immunohistochemistry, following previously described protocol [17]. Briefly, for labeling of CD3ε and CD8α positive T-lymphocytes, primary monoclonal antibody (CD3ε mouse anti-trout) at 1:600 [46] and primary monoclonal antibody (CD8α mouse anti-salmon) at 1:50 [47] were used. The slides were incubated at room temperature for 1 h, followed by 30 min incubation with secondary antibody. Sections labeled for CD3ε were incubated with secondary antibody kit polymer-HRP anti-mouse (DAKO En Vision+ System-HRP, Dako, Glostrup, Denmark) while sections labeled for CD8α were incubated with biotinylated secondary anti-mouse IgG antibody (BA-9200, Vector laboratories, Burlingame, CA, USA). Peroxidase activity in the CD3ε and CD8α slides were detected with 3,3′ diaminobenzidine (DAKO En Vision+ System-HRP, Dako, Glostrup, Denmark) and 3-amino-9-ethylcarbazole (SK-4205, Vector laboratories, Burlingame, CA, USA); respectively. Sections for both T-lymphocytes were counterstained with hematoxylin for 10s and mounted using an Aquatex (Merck, Darmstadt, Germany). Slides incubated without the primary antibodies were included as negative controls for both T-lymphocytes. To estimate the area of lamina propria occupied by CD3ε and CD8α-labeled cells, QuPath digital pathology software (v0.2.3) [48] was used with some modifications to the previously described method of Reveco-Urzua et al. [17]. Fold length was measured from the stratum compactum to the tip of the simple fold using ImageJ software (v1.53c).

4.6. RNA Isolation, Library Preparation and RNA Sequencing

Total RNA was extracted from DI and spleen tissues collected from 36 fish (6 fish per dietary group) using Qiazol Lysis Reagent (Qiagen, Hilden, Germany) and chloroform following the protocol described by Toni et al. [49]. Thereafter, the RNA concentration was quantified using a NanoDrop TM 8000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). RNA integrity was determined using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). All samples showed high-quality integrity (RIN ≥ 8). Library preparation using TruSeq Stranded mRNA library prep (Illumina, San Diego, CA, USA) was performed at the Centre for Integrative Genetics (CIGENE, NMBU, Ås,

Norway). Libraries were pooled and RNA sequencing was performed using the Illumina NovaSeq S4 platform (150 bp paired-end reads) at the Norwegian Sequencing Center (UiO, Oslo, Norway).

4.7. Data Analysis of RNA Sequencing

RNA sequence data analysis was performed using the publicly available nf-core/RNA-seq pipeline version 3.3 implemented in Nextflow 21.04.0 [50]. In brief, raw reads were trimmed using Trim Galore and clean reads were thereafter aligned to Salmo salar genome Ssal_v3.1 (GenBank assembly accession: GCA_905237065.2) by STAR_RSEM. Gene-level assignment was performed using featureCounts (v1.4.6). Differentially expressed genes (DEGs) between diets were estimated using DESeqe2 (v1.22.1) and SARTools (v1.7.3) R packages. All genes with a $\log_2$ fold change > 2 or < -2 were designated up or down, respectively. Significant DEGs were determined when the adjusted p-value was < 0.05. To characterize DEGs, gene ontology (GO) enrichment using three categories (molecular function, biological process, and cellular components) was performed with the ShinyGO (v0.741) online tool, applying the False Discovery Rate (FDR) correction for multiple testing [51]. GO categories were selected (minGSSize = 3) and displayed as Enrichment Score ($-\log_2$(P). EnrichmentMap v3.3.3 [52] in Cytoscape v3.8.1 [53] was used to visualize enriched GO terms for all diet comparisons in a network. Two GO terms were connected if their similarity coefficient (mean of overlap and Jaccard coefficients) was greater than 0.375.

4.8. Composition of Yeasts and Fish Feeds

The nutritional compositions of yeasts and feeds were determined according to a previously described protocol by Agboola et al. [54]. The composition of yeast cell wall was estimated without prior cell wall isolation following the method described by Hansen et al. [55]. Briefly, the yeasts were hydrolyzed with sulphuric acid and the liberated sugar monomers (mannose, N-acetylglucosamine and glucose) were quantified by high-performance anion-exchange chromatography with pulsed amperometric detection [55], to determine the mannan, chitin and glucan content of the yeast cell wall.

4.9. Calculations and Statistical Analyses

The biomass gain, SGR and FCR were calculated based on the equations expressed in Agboola et al. [54]. In brief, the biomass gain was expressed as the difference between the average final weight and initial body weight of fish per tank. The FCR was expressed as the ratio between average feed intake per day and average biomass gain per day. The SGR was calculated as the logarithm differences between the average final and initial weight of fish divided by the experimental period. The ADCs of nutrients in the diets were determined based on the equation of Cho and Slinger [56].

Fish performance, nutrient digestibility, and area of lamina propria covered with T-lymphocytes were tested for treatment effects using one-way ANOVA. Significance difference ($p < 0.05$) among diets were detected using Tukey's HSD test. These analyses were performed using the SPSS statistical software package version 27 (IBM Institute, Armonk, NY, USA). Differences in histological scores for the various evaluated morphological characteristics of the DI tissue were analyzed for statistical significance using ordinal logistic regression in the R statistical package (version 3.6.2; 2019). Differences were examined based on odds ratios of the different feeding groups having different histology scores compared to the FM diet.

5. Conclusions

The results demonstrate that the inclusion of CJ yeast reduced the loss of supranuclear vacuolization and decreased population of CD8α positive cells in the DI of fish fed SBM-based diets. Inclusion of CJ and WA yeasts also induced transcriptomic changes

related to wound healing and immune response pathways in fish fed SBM-based diets. Processing by autolysis did not improve the beneficial health effect of CJ and WA yeasts. This study suggests that the yeast species *Cyberlindnera jadinii* and *Wickerhamomyces anomalus* are novel high-quality protein sources with health-beneficial effects in terms of reducing inflammation associated with feeding plant-based diets to Atlantic salmon.

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Institutional Review Board Statement: The experimental procedures were conducted according to the institutional and national guidelines under the applicable laws and regulations guiding experiments with live animals in Norway (regulated by the "Norwegian Animal Welfare Act" and "The Norwegian Regulation on Animal Experimentation" derived from the "Directive 2010/63/EU" on the protection of animals used for scientific purposes). The fish experiment was conducted at the Fish Laboratory of Norwegian Institute of Water Resources (NIVA, Solbergstrand, Norway), which is an experimental unit approved by the National Animal Research Authority, Norway (Permit No. 174).

Data Availability Statement: The obtained raw sequencing data were deposited in the Gene Expression Omnibus database (GEO-NCBI: GSE193239). Other raw data generated in this study are available from the corresponding authors, upon reasonable request.

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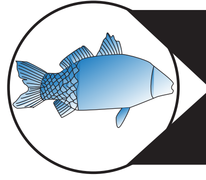

Paper VI

Effect of yeast species and processing on intestinal microbiota of Atlantic salmon (*Salmo salar*) fed soybean meal-based diets in seawater

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Background: Yeasts are gaining attention as alternative ingredients in aquafeeds. However, the impact of yeast inclusion on modulation of intestinal microbiota of fish fed plant-based ingredients is limited. Thus, the present study investigates the effects of yeast and processing on composition, diversity and predicted metabolic capacity of gut microbiota of Atlantic salmon smolt fed soybean meal (SBM)-based diet. Two yeasts, *Cyberlindnera jadinii* (CJ) and *Wickerhamomyces anomalus* (WA), were produced in-house and processed by direct heat-inactivation with spray-drying (ICJ and IWA) or autolyzed at 50 °C for 16 h, followed by spray-drying (ACJ and AWA). In a 42-day feeding experiment, fish were fed one of six diets: a fishmeal (FM)-based diet, a challenging diet with 30% SBM and four other diets containing 30% SBM and 10% of each of the four yeast products (i.e., ICJ, ACJ, IWA and AWA). Microbial profiling of digesta samples was conducted using 16S rRNA gene sequencing, and the predicted metabolic capacities of gut microbiota were determined using genome-scale metabolic models.

Results: The microbial composition and predicted metabolic capacity of gut microbiota differed between fish fed FM diet and those fed SBM diet. The digesta of fish fed SBM diet was dominated by members of lactic acid bacteria, which was similar to microbial composition in the digesta of fish fed the inactivated yeasts (ICJ and IWA diets). Inclusion of autolyzed yeasts (ACJ and AWA diets) reduced the richness and diversity of gut microbiota in fish. The gut microbiota of fish fed ACJ diet was dominated by the genus *Pediococcus* and showed a predicted increase in mucin O-glycan degradation compared with the other diets. The gut microbiota of fish fed AWA diet was highly dominated by the family *Bacillaceae*.

Conclusions: The present study showed that dietary inclusion of FM and SBM differentially modulate the composition and predicted metabolic capacity of gut microbiota of fish. The inclusion of inactivated yeasts did not alter the modulation caused by SBM-based diet. Fish fed ACJ diet increased relative abundance of *Pediococcus*, and mucin O-glycan degradation pathway compared with the other diets.

***Cyberlindnera jadinii* | *Wickerhamomyces anomalus* | gut microbiota | predicted metabolic capacity | SBMIE | microbial diversity | inactivated | autolysis**

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Background

Plant protein sources are increasingly being used in commercial aquafeeds (1, 2). Among the plant-based ingredients, the use of soybean meal (SBM) in diets of Atlantic salmon is restricted due to the presence of anti-nutritional factors (such as trypsin inhibitors, protease inhibitors and saponin) that compromise the growth performance, nutrient digestibility, and health of fish (3, 4). A number of studies (5–10) have reported that dietary inclusion of SBM induce inflammation in the distal intestine of Atlantic salmon; a condition widely known as SBM-induced enteritis (SBMIE), which is characterized by loss of enterocyte vacuolization, reduction in mucosal fold height, and infiltration of inflammatory cells in the lamina propria and epithelial submucosa. Considering these limitations, a refined soy-product known as soy-protein concentrate (SPC) with low level of anti-nutritional factors, is currently used in commercial salmon diets. The use of plant ingredients such as SPC in aquafeeds also raises ethical and environmental concerns as continuous use of SPC in aquafeeds may increase pressure on cultivable land, water and energy use, as well as decrease their availability for direct human consumption (11, 12). Therefore, there is an emerging need for sustainable novel ingredients for aquaculture.

Microbial ingredients such as yeasts are gaining attention as potential novel ingredient in aquaculture due to their ability to convert low-value by-products into high-value resources (13), high nutritional values (14–16), low environmental footprint (17) and functional effects in fish (18, 19). Studies have shown that dietary inclusion of yeasts could alleviate adverse effects of SBM in Atlantic salmon (18, 19), but little is known of their effects on intestinal microbiota of fish. The gut microbiota plays important roles in host physiological and metabolic processes, such as digestive function, growth performance, immune function, and health (20–22). A number of studies (23–26) have documented the effects of SBM inclusion on intestinal microbiota of Atlantic salmon. Identifying microbiota modulated by inclusion of yeasts in the diets may be crucial for improving nutrient utilization, growth performance, and health of Atlantic salmon fed plant-based diets. Therefore, the objective of the present study was to examine the effect of yeast species and processing on richness, diversity and predicted metabolic profile of gut microbiota of Atlantic salmon fed SBM-based diet in seawater. Two yeasts, *Cyberlindnera jadinii* (CJ) and *Wickerhamomyces anomalus* (WA) produced from wood sugars using in-house bioreactors, were used in the current study.

Methods

Yeasts, experimental diets, and fish feeding trial. The CJ and WA yeast biomass were produced in a 30 L bioreactor using a growth medium composed of a blend of enzymatic hydrolysates of pre-treated spruce wood (*Picea abies*) and chicken by-products as described by Lapeña et al. (13). After harvesting, the yeasts were processed following the protocol described by Agboola et al. (18). Briefly, the yeast biomass was washed, centrifuged and the resulting paste was divided into two equal parts. One part of the yeast paste was directly inactivated with a spray-dryer (SPX 150 MS, SPX Flow Technology, Denmark) set at 180 °C and 80 °C for inlet and outlet temperature, respectively. The other half of the yeast paste was autolyzed at 50 °C for 16 h in a stirred 30 L reactor (Einar, Belach Bioteknik, Sweden), followed by spray-drying using the same conditions as above. The resulting processed yeast products were: inactivated CJ (ICJ), autolyzed CJ (ACJ), inactivated WA (IWA), and autolyzed WA (AWA). The nutritional and cell wall compositions of the four yeast products are presented in Table S1.

Six experimental diets were formulated to meet or exceed (27, 28) the nutritional requirements of Atlantic salmon smolts; a fishmeal-based (FM) control diet, a challenging diet containing 30% soybean meal (SBM) and four diets containing 30% SBM with 10% inclusion of the different processed yeasts (ICJ, ACJ, IWA and AWA), respectively. Table 1 shows the ingredient and analyzed compositions of the six experimental diets. The diets were cold-pelleted using a P35A pasta extruder (Italgi, Carasco, Italy) and dried at 60 °C in small experimental driers. The production of the experimental diets is fully described in Agboola et al. (29).

A 42-day seawater feeding trial with Atlantic salmon smolts (initial body weight = 136 ± 0.25 g) was conducted at the research facility of the Norwegian Institute of Water Resources (NIVA, Solbergstrand, Norway). A total of 450 vaccinated salmon smolts were randomly allocated into 18 fiber tanks (300 L) and fed one of the six experimental diets (n = 3 tanks per diet) for 6 h per day using automatic feeders delivering feed every 12 minutes. The fish were reared under a 24 h light regime in a flow-through system with an average water temperature of 11.5 °C and average oxygen saturation of 84%. The water flow was kept at an average of 5.5 L min^{-1} during the experimental period. Water salinity was gradually increased from 5 ppt at the start, until it reached full salinity (33 ppt) during the first 12 days of the experiment.

Sample collection. At the end of the feeding trial, the average body weight of the fish was 179 ± 7.06 g. Six fish were randomly selected from each tank, anaesthetized with metacaine (MS-222, 50 mg L^{-1} water), and killed with a sharp blow to the head for digesta sampling. After dissection, the distal intestine was opened longitudinally and the digesta was carefully removed using sterile plastic spatulas. The digesta was placed in cryotubes, snap-frozen in liquid nitrogen and stored at -80 °C. To obtain sterile conditions, tools were cleaned and decontaminated using 70% ethanol and flaming between each fish. Additionally, feed and water samples were collected into sterile plastic containers and stored at -80 °C. Water samples were collected from both the source tank and the fish rearing tanks.

DNA extraction. Total DNA was extracted from 200 mg of digesta (18 samples per dietary group) and 100 mg of ground feed (3 replicates per diet) using QIAamp® Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany, Cat. No. 51604) following the manufacturer's specifications with some modifications as described elsewhere (30). In addition to the digesta and feed samples, total DNA was extracted from the water samples. 500 mL each of source water (2 samples) and rearing tank water (4 samples) were filtered through a MF-Millipore membrane filter with 0.22 µm pore size (Sigma-Aldrich, Cat. No GSWP04700) and total DNA was extracted using the same protocol described above. The rearing water (500 mL from each tank) samples were mixed, and four sub-samples (500 mL each) were taken and used for the DNA extraction. Total DNA was also extracted from blank filter paper used for the filtration of water samples. For quality control of the present workflow, a microbial community standard (mock), which consists of eight bacteria and two yeasts (ZymoBIOMICS™, Zymo Research, California, USA; Cat. No. D6300) was included for DNA extraction as positive control. In addition, a blank negative control was added to each batch of DNA extraction by omitting the input material. Total DNA were extracted from blank control, mock positive control and blank filter paper following the method used for digesta, feed and water samples. The DNA concentration of all the samples were measured in duplicates using Invitrogen™ Quant-iT™ Qubit™ dsDNA HS (High Sensitivity) assay kit (Thermo Fisher Scientific, California, USA, Cat. No. Q32854) with the Qubit 4 Fluorometer (Invitrogen™). The extracted DNA were stored at -20 °C until further analysis.

PCR amplification. The V3-V4 hypervariable regions of the bacterial 16S rRNA gene were amplified in a 25 µL reaction volume containing 2x KAPA HiFi HotStart Ready Mix (12.5 µL) (Roche Sequencing Solutions, Mat. No. 7958935001), DNA template (5 µL), and 1.33 µM primers (3.75 µL of each primer). The primers used for the amplicon PCR are 341F (5'-CCT ACG GGN GGC WGC AG-3') and 785R (5'-GAC TAC HVG GGT ATC TAA TCC-3'). The amplification was set at initial denaturation of 95 °C for 3 min; 25 cycles of denaturation at 95 °C for 30 s; annealing at 55 °C for 30 s; extension at 72 °C for 30 s; followed by a final extension at 72 °C for 5 min. After the amplification process, duplicate PCR products were pooled and purified using Agentcourt AMPure XP beads (Beckman Coulter, Indiana, USA, Cat. No. A63881), and the cleaned PCR products were examined by 1% agarose gel electrophoresis.

Library preparation and sequencing. The sequencing was carried out on a Miseq platform following the Illumina 16S metagenomic sequencing library preparation protocol (31). The cleaned PCR amplicons were multiplexed by dual indexing using the Nextera Index Kit v2 Set A (Illumina,

Table 1. Diet formulation and nutritional composition of the experimental diets*.

	FM	ICJ	ACJ	IWA	AWA	SBM
Diet formulation[a] (g/kg)						
Fishmeal[b]	433.4	208.4	208.4	208.4	208.4	261.4
Soybean meal[c]	0	300	300	300	300	300
Wheat gluten meal[d]	170	111	111	111	111	136
Potato starch[e]	120	68	68	68	68	90
Cellulose	80	0	0	0	0	0
Yeast[n]	0	100	100	100	100	0
Fish oil[f]	130	130	130	130	130	130
Gelatin[g]	60	60	60	60	60	60
Monocalcium phosphate[h]	0	10	10	10	10	10
Premix[i]	5	5	5	5	5	5
L-lysine[j]	0	3	3	3	3	3
DL-Methionine[k]	0	3	3	3	3	3
Chlorine chloride[l]	1.5	1.5	1.5	1.5	1.5	1.5
Yttrium[m]	0.1	0.1	0.1	0.1	0.1	0.1
Diet composition (analyzed values)[o] (g/kg)						
Dry matter	926.6	889.9	889.2	924.5	913.9	897.3
Crude protein	531.8	518.3	530.3	519.5	521.4	542.6
Starch	131.9	92.6	93.3	89.3	87.6	103.6
Ash	78.3	74.7	74.8	73.7	73.5	77.2
Carbon	509.1	502.5	517.8	513.1	511.0	509.7
Sulphur	6.0	6.2	6.0	6.1	6.0	6.3
Energy (MJ/kg DM)	23.3	23.3	23.3	23.1	23.1	23.1
DP:DE[p]	23.1	22.8	22.8	22.5	22.5	23.3

[a]Diet formulation are expressed in g/kg.
[b]LT fishmeal, Norsildmel, Egersund, Norway; [c]Soybean meal, Denofa AS, Fredrikstad, Norway; [d]Wheat gluten, Amilina AB, Panevezys, Lithuania; [e]Lygel F 60, Lyckeby Culinar, Fjälkinge, Sweden; [f]NorSalmOil, Norsildmel, Egersund, Norway; [g]Rousselot 250 PS, Rousselot SAS, Courbevoie, France; [h]Monocalcium phosphate, Bolifor MCP-F, Oslo, Norway Yara; [i]Premix fish, Norsk Mineralnæring AS, Hønefoss, Norway. Per kg feed ; Retinol 3150.0 IU, Cholecalciferol 1890.0 IU, α-tocopherol SD 250 mg, Menadione 12.6 mg, Thiamin 18.9 mg, Riboflavin 31.5 mg, d-Ca-Pantothenate 37.8 mg, Niacin 94.5 mg, Biotin 0.315 mg, Cyanocobalamin 0.025 mg, Folic acid 6.3 mg, Pyridoxine 37.8 mg, Ascorbate monophosphate 157.5 g, Cu: CuSulfate 5H2O 6.3 mg, Zn: ZnSulfate 151.2 mg, Mn: Mn(II)Sulfate 18.9 mg, I: K-Iodide 3.78 mg, Ca 1.4 g; [j]L-Lysine CJ Biotech CO., Shenyang, China; [k]Rhodimet NP99, Adisseo ASA, Antony, France; [l]Choline chloride, 70% Vegetable, Indukern SA., Spain; [m]Y_2O_3. Metal Rare Earth Limited, Shenzhen, China.
[n]ICJ – inactivated *Cyberlindnera jadinii*; ACJ – autolyzed *C. jadinii*; IWA – inactivated *Wickerhamomyces anomalus*; AWA – autolyzed *W. anomalus*.
[o]Diet composition are expressed in g/kg dry matter (DM) unless otherwise stated.
[p]DP:DE = Digestible protein to digestible energy ratio. Calculated using internal digestibility values of various ingredients.
*The diets are: FM – fishmeal-based; SBM – soybean meal-based; 4 other diets containing 300 g/kg SBM and 100 g/kg of ICJ, ACJ, IWA and AWA yeasts.

California, USA, Cat. No. FC-131-2001). The index PCR products were cleaned using the AMPure beads and quantified using the Invitrogen™ Quant-iT™ Qubit™ dsDNA BR (Broad range) assay kit (Thermo Fisher Scientific, California, USA, Cat. No. Q32853) with the Qubit 4 Fluorometer (Invitrogen™). To determine the library size representative, cleaned libraries were selected and analyzed using the Agilent DNA 1000 Kit (Agilent Technologies, California, USA, Cat. No. 067-1505). The libraries were diluted to 4 nM in 10mM Tris (pH 8.5) and pooled in an equal volume. The blank control samples with library concentrations lower than 4 nM were pooled directly without further dilution. The pooled library was denatured using 0.2 N NaOH. Due to low diversity of the amplicon library, 5% Illumina generated PhiX control (Illumina, San Diego, Waltham, MA, USA, Cat No: FC-110-3001) was spiked in by combining 570 µL amplicon library with 30 µL PhiX control. The library was then loaded at 8 pM and sequenced on the Miseq System (Illumina, San Diego, California, USA) using the Miseq Reagent Kit v3 (600-cycle) (Illumina; catalog no., MS-102-3003). The sequencing was done in two runs. To prevent potential batch effects between sequencing runs, the digesta and the feed samples were distributed between the runs with consideration that each dietary treatment and each experimental tank were equally represented. Also, water and control samples were evenly distributed between the two runs.

Sequence data processing. The sequence data were processed in R (version 4.0.5) (32). For each sequencing run, DADA2 was used to process the raw sequence data and generate amplicon sequence variants (ASVs) (33). Briefly, the demultiplexed pair-ended reads were trimmed off the primer sequences (first 17 bps of forward reads and first 21 bps of reverse reads), truncated at the position where the median Phred quality score crashed (forward reads at position 300 bp and reverse reads at position 230 bp for both runs) and filtered off low quality reads. After the trimming and filtering, a model of error rates was developed to remove error sequences. The forward and reverse reads were merged, and the ASV table for each run was constructed. The ASV table for each run were merged, and assigned with taxonomy using the reference database, SILVA version 138.1 (34, 35). A phyloseq object was constructed from the generated ASV table, the taxonomy table and the sample metadata using the phyloseq R package (version 1.34.0) (36). Taxa identified as chloroplasts or mitochondria were removed from the ASV table. The ASVs that had no phylum-level taxonomic assignments or appeared in less than three biological samples were conservatively filtered from the ASV table. The contaminating ASVs due to reagent contamination and cross contamination were identified and removed from ASV table as described elsewhere (37). The ASVs were then clustered using VSEARCH algorithm and subsequently curated with LULU (38). The post-clustering ASV table and representative sequences were used for the downstream data analysis. The core ASVs and alpha-diversity indices (observed ASVs, Pielou's evenness, Shannon's index and Faith's phylogenetic diversity (PD)) were computed according to Li et al. (37). Similarly, the beta-diversity indices (Jaccard distance, unweighted UniFrac distance, Aitchison distance and PhILR transformed Euclidean distance) were computed following Li et al. (37). The Jaccard distance and unweighted UniFrac distance were calculated by rarefying the ASV table into minimum sequence size i.e., 1,604 reads per sample (Fig. S1). Conversely, Aitchison distance and PhILR transformed Euclidean distance were computed using the unrarefied ASV table.

Metabolic reaction analysis of gut microbiota. The metabolic reaction analysis of gut microbiota was performed according to the method described by Yilmaz et al. (39). The ASVs for the digesta samples were mapped to metabolic reactions using an available collection of genome-scale metabolic models (GSMMs) of gut microbes (40). Only ASVs that could be mapped to family or lower taxonomic rank and to at least one GSMM were included in the reaction level analysis. For each sample, we calculated the normalized abundance of each reaction based on equation (1):

$$a_r(i) = \frac{\sum_{j=1}^{n} a_{\text{ASV}}(j)E(i,j)}{\sum_{j=1}^{n} a_{\text{ASV}}(j)} \qquad (1)$$

where $a_{\text{ASV}}(j)$ is the abundance of ASV j in the sample, n is the total number of ASVs, and E(i, j) is the expected probability (frequency of occurrences) of reaction i in the GSMMs

mapped to ASV *j*.

Statistical analysis. The statistical difference among the dietary groups for the microbial compositions at genus or lowest taxonomy ranks (top 15 most abundant taxa) were evaluated using Kruskal-Wallis test, followed by multiple comparison using Wilcox pair-wise comparison test. Similarly, the alpha-diversity measurements were evaluated using Kruskal-Wallis test and statistical differences among the dietary groups were detected using Wilcox pair-wise comparison test. The statistical difference among the dietary groups for the beta-diversity indices were computed using permutation multivariate analysis of variance (PERMANOVA) (41) with 999 permutations using the R package vegan 2.5.7 (42), followed by a pair-wise comparison. Principal coordinates analysis (PCoA) was used to visualize the beta-diversity indices. The homogeneity of multivariate dispersions among the dietary groups was computed by permutation test, PERMDISP (43), using the R package vegan (42) and visually assessed with boxplots. Significant differences with adjusted $p < 0.05$ among dietary groups were detected using the Benjamini-Hochberg procedure (44). For the metabolic reaction analysis, mean abundance of each reaction was tested using a two-sample *t*-test for each pair of diets. Multiple testing was corrected using the Benjamini-Hochberg procedure (44) and reactions with adjusted $p \leq 0.05$ were considered to be significantly different between diets. For each pair of diets, the enriched pathways among the significantly different reactions were computed using Fisher's exact test. The pathways with adjusted $p \leq 0.05$ based on Benjamini-Hochberg procedure were considered to be enriched. Additionally, principal component analysis (PCA) was performed separately on standardized ASVs (Fig. S2) and reaction abundances (*z*-scores) (Fig. S3).

Results

Characteristics of sequence data. After the sequence denoising, ASV filtering and clustering, a total number of 6.6 million reads were retained for the downstream data analysis. The median of reads per sample used for downstream analysis was 23,087, with the minimum and maximum values being 1,604 and 180,844, respectively. The reads for the downstream analysis generated a total of 906 unique ASVs, of which 76.4% were assigned at the genus level and 13.5% annotated at the species level.

Microbiota composition of mock and negative controls. All the eight bacterial species expected in the mock were successfully identified at genus level, with only *Staphylococcus aureus* being identified at species level (Fig. S4). The relative abundance of *S. aureus* was correctly estimated, whereas the abundance of *Salmonella, Pseudomonas* and *Escherichia-Shigella* were overestimated. Contrary, the relative abundance of *Listeria, Lactobacillus, Enterococcus* and *Bacillus* were underestimated. The average Pearson correlation coefficient (Pearson's *r*) between the expected and the observed taxonomic profile of the mock was 0.30, whereas the Pearson's *r* between the observed mock was 0.99. The dominant taxa identified as contaminants in the negative controls and the blank filter papers were *Actinobacteria* (47%), *Bacilli* (18%), and *Gammaproteobacteria* (15%) (Table S2).

Microbiota associated with feed and water. At phylum level, the feed-associated microbiota was dominated by *Firmicutes* and *Proteobacteria* (Fig. 1A). The ACJ (89%) and AWA (94%) feeds had higher abundance of *Firmicutes* compared with the remaining feeds (72-80%). On the other hand, the relative abundance of *Proteobacteria* was lower in ACJ (9%) and AWA (5.3%) feeds compared with the remaining diets (16-24%) (Fig. 1A). At genus or lowest taxonomic rank, the ACJ and AWA feeds were dominated by *Pediococcus* (62%) and *Bacillaceae* (68%), respectively (Fig. 1B). On the contrary, the microbiota composition in FM, ICJ, IWA and SBM feeds were dominated by *Lactobacillus* (21-25%), *Limosilactobacillus* (22-25%), *Photobacterium* (15-22%), *HT002 (10-11%)* and *Ligilactobacillus* (6.7-7.7%) (Fig. 1B).

The microbiota in the source water was dominated by phyla *Proteobacteria* (55%), *Actinobacteriota* (14%) and *SAR324 clade (Marine group B)* (14%), whereas the taxonomic compositions of the rearing tank water were dominated by phyla *Proteobacteria* (55%) and *Bacteroidota* (31%) (Fig. S5A). At the genus or lowest taxonomy level, *SUP05 cluster* (13%), *Candidatus Actinomarina* (10%) and *Clade II* (9%) dominated the microbiota in the source water (Fig. S5B). The microbiota in the rearing tank water were dominated by the taxa *Sulfitobacter* (11%), *Colwellia* (7%), *Hellea* (7%), *Lacinutrix* (5%) and *Maribacter* (5%) (Fig. S5B). *Bacillaceae* (0.01 - 0.2%) and *Pediococcus* (0.02 - 2%)were detected in both source water and tank water.

Digesta-associated microbiota. Regardless of the diets, the taxonomic compositions of the digesta samples at phylum level were dominated by *Firmicutes, Proteobacteria* and *Actinobacteriota* (Fig. 2A). Fish fed ACJ (97%), and AWA (97%) had higher abundance of *Firmicutes* compared with those fed the other diets (76-81%) (Fig. 2A). Conversely, fish fed ACJ (2.5%) and AWA (2.2%) diets had lower composition of *Proteobacteria* compared with fish fed the other diets (12-19%) (Fig. 2A). *Actinobacteriota* composition in the digesta of fish fed ACJ (0.2%) and AWA (0.4%) diets was lower compared with fish fed the remaining diets (3.3-4.1%) (Fig. 2A).

The taxonomic composition of digesta samples at the genus or lowest taxonomy rank was influenced by the dietary group (Figs. 2B & 3). Fish fed ACJ (92%) diet were significantly dominated by *Pediococcus* compared with the other diets (Figs. 2B & 3). Similarly, fish fed AWA (88%) diet were significantly dominated by *Bacillaceae* compared with fish fed the other diets (Figs. 2B & 3). *Lactobacillus* (12%) and *Limosilactobacillus* (21%) were significantly higher in fish fed FM compared with fish fed the other diets (Figs. 2B & 3). Fish fed ICJ, IWA and SBM diets (5.4-6.3%) had significantly higher abundant of *Enterococcus* compared with the other diets (Figs. 2B & 3). *Streptococcus, Peptostrep-*

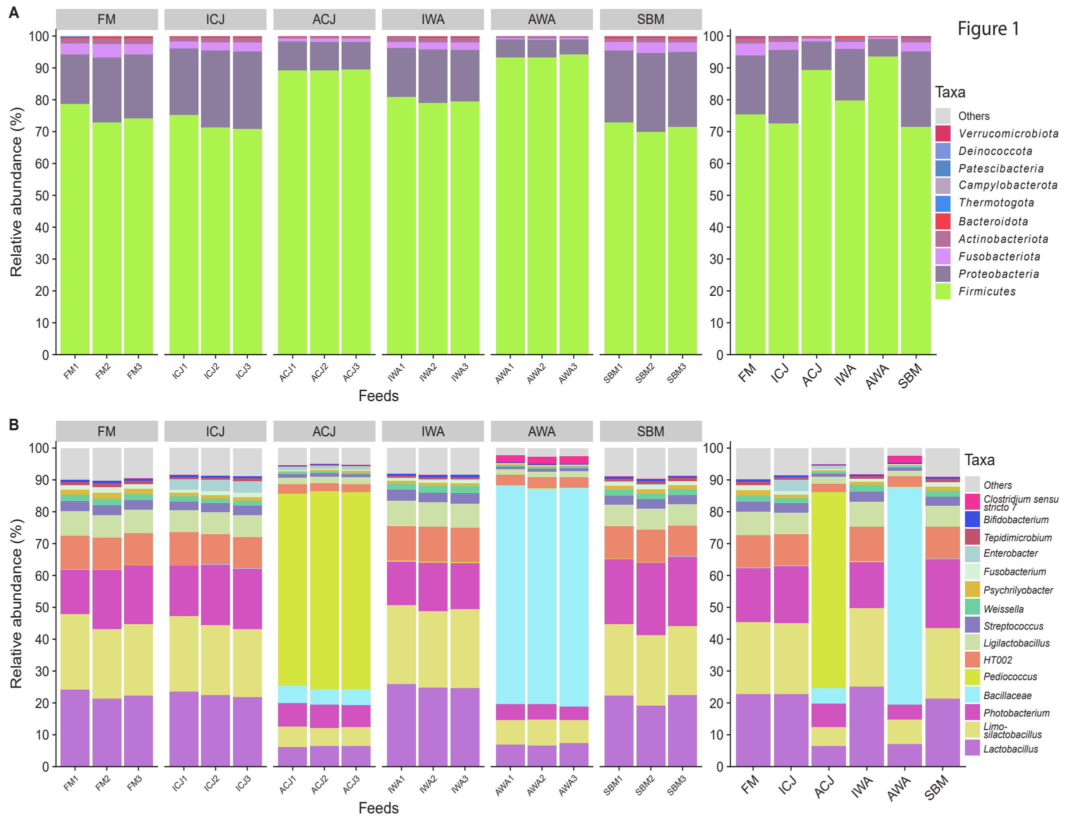

Fig. 1. Microbiota composition in the feed samples. Relative abundance of the top 10 most abundant taxa at phylum level (**A**) and top 15 most abundant taxa at genus or lowest taxonomic rank (**B**). The mean relative abundance of each taxon within the same diet is displayed on the right side. The samples are grouped by diets; FM – fishmeal-based; SBM – soybean meal-based; 4 experimental diets containing 300 g/kg SBM and 100 g/kg of ICJ – inactivated *Cyberlindnera jadinii*; ACJ – autolyzed *C. jadinii*; IWA – inactivated *Wickerhamomyces anomalus*; AWA – autolyzed *W. anomalus diets.*

387 *tococcus, HT002, RsaHf231, Weissella* and *Photobacterium* were significantly higher in fish fed FM, ICJ, IWA and SBM diets compared with fish fed ACJ and AWA diets (Figs. 2B & 3). 390

391 When comparing the ASVs of the gut, water and feed, the composition of the gut microbiota was similar to that of the feed, but different from the water microbiota (Fig. 4). The ASVs overlap between the gut and the feed was higher than between the gut and water. 395

396 **Core microbiota.** In total, 94 ASVs were identified as core microbiota (present in 80% of the digesta samples) in fish fed the experimental diets (Fig. S6A-B; Table S3). Fifteen ASVs classified as *Peptostreptococcus, Limosilactobacillus, Weissella, Ligilactobacillus, Streptococcus* and *Lachnospiraceae* were identified to be present in all the dietary groups. Fish fed FM and SBM diets shared 37 primary core ASVs, belonging to members of *Peptostreptococcus, Photobacterium, RsaHf231*, and lactic acid bacteria (LAB) including *Streptococcus, Lactobacillus, Limosilactobacillus, Weissella, Ligilactobacillus* and *HT002*. 406

407 **Alpha-diversity.** Based on the four indices, the microbial diversity of fish fed ACJ and AWA diets was significantly lower compared with fish fed the other diets (Fig. 5; Table S4). The observed ASVs and Faith's PD showed that fish fed FM diet had significantly higher microbial diversity compared with fish fed ICJ, IWA and SBM diets (Figs. 5A, D). Contrarily, based on Shannon's index, the microbial diversity of fish fed FM diet was significantly lower compared with those fed ICJ, IWA and SBM diets (Fig. 5C). Excluding fish fed ACJ and AWA diets, the microbial diversity was similar among the other diets based on Pielou's evenness (Fig. 5B). The microbial compositions of fish fed ICJ, IWA and SBM were similar based on the four alpha-diversity indices (Fig. 5). 419

420 **Beta-diversity.** The PCoA plots built on the four beta-diversity indices showed that the microbiota of fish fed FM diet were clearly distinct from the other diets (Fig. 6). Based on the four beta-diversity indices, the PCoA plots showed that microbiota of fish fed ICJ, IWA and SBM diets were similar, and clearly clustered from those fed FM, ACJ and AWA diets (Fig. 6A-D). The PCoA plots based on Jaccard distance, unweighted UniFrac distance and PhILR transformed Euclidean distances showed separation of microbiota in fish 428

Fig. 2. Microbiota composition in the digesta of fish fed the experimental diets. Relative abundance of the top 10 most abundant taxa at phylum level (**A**) and top 15 most abundant taxa at genus or lowest taxonomic rank (**B**). The mean relative abundance of each taxon within the same diet is displayed on the right side. The samples are grouped by diets; FM – fishmeal-based; SBM – soybean meal-based; 4 experimental diets containing 300 g/kg SBM and 100 g/kg of ICJ – inactivated *Cyberlindnera jadinii*; ACJ – autolyzed *C. jadinii*; IWA – inactivated *Wickerhamomyces anomalus*; AWA – autolyzed *W. anomalus diets.*

429 fed ACJ diet compared with fish fed AWA diet (Fig. 6A, B, D). On the contrary, the microbiota of fish fed ACJ diet were similar compared with fish fed AWA diet based on Aitchison distance matrix (Fig. 6C). The PERMANOVA tests showed that beta-diversity were significantly influenced by the dietary groups, and the results were in line with the PCoA plots (Table S5). Based on the four distance matrices, the microbiota of fish fed FM diet were significantly different from those fed the other diets. Also, the PERMANOVA tests showed similarity in the microbiota of fish fed ICJ, IWA and SBM diets, which were different from those fed ACJ and AWA diets. The statistical tests showed that the microbiota of fish fed ACJ diet were significantly different from fish fed AWA diet. The tests for homogeneity and multivariate dispersions are presented in Fig. SS7 and Table S6. The multivariate dispersions were significantly affected by the dietary groups based on the four distance matrices. 445

446 **Metabolic capacity of gut microbiota.** Fifty-eight percent (526) of the 906 ASVs identified in the current study could be mapped to at least one model from a published collection of GSMMs of gut microbiota. Thirty-seven percent (338), 19% (176) and 1.3% (12) of the ASVs were matched to family, genus, and species, respectively (Fig. S8A). The ASVs matched to family, genus, and species were mapped to an average of 16, 13 and 1 model(s), respectively (Fig. S8B). The models mapped to ASVs contained 4802 different reactions, half of which (55%) were present in all samples. Most samples (90%) contained more than 90% of the reactions, but the abundances of many reactions differed significantly between samples and diets. Furthermore, the variability in the data could be explained in a few components using PCA of reaction abundances rather than ASV abundances (Figs. S2 and S3). 461

462 By classifying the reactions into metabolic pathways, ten pathways were enriched in pairwise comparisons between the dietary groups (Fig. 7). The differences in mean abundance of enriched pathways for each pair of diets are presented in Fig. S9. The gut microbiota of fish fed FM diet showed predicted enrichment of metabolic pathways related to mucin O-glycan degradation, valerate metabolism and O-Glycan degradation, as well as lower enrichment of purine and pyrimidine catabolism pathways compared with fish fed ICJ and SBM diets (Figs. 7 & S9A, E). The gut microbiota of fish fed ACJ diets showed predicted enrichment of mucin O-glycan degradation pathway compared with fish fed ICJ, 473

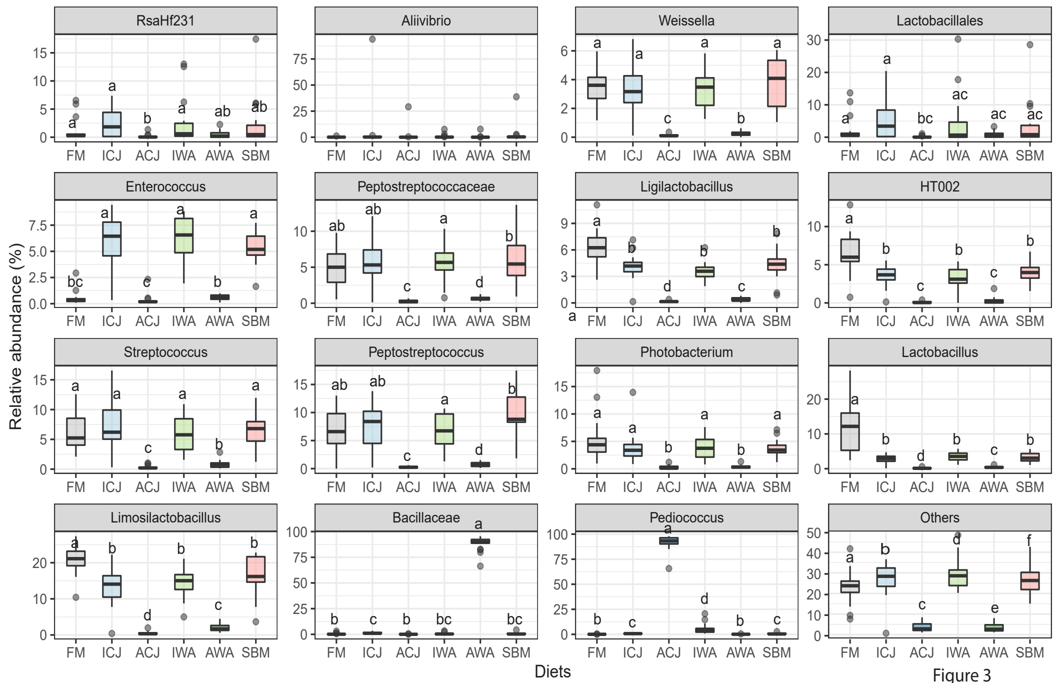

Fig. 3. Boxplots of relative abundance of the top 15 most abundant taxa (at genus or lowest taxonomic rank) in the digesta of fish fed the experimental diets. The samples are grouped by diets; FM – fishmeal-based; SBM – soybean meal-based; 4 experimental diets containing 300 g/kg SBM and 100 g/kg of ICJ – inactivated *Cyberlindnera jadinii*; ACJ – autolyzed *C. jadinii*; IWA – inactivated *Wickerhamomyces anomalus*; AWA – autolyzed *W. anomalus diets*. Different lower-case letters represent taxa with significantly different ($p < 0.05$) relative abundance among the diets.

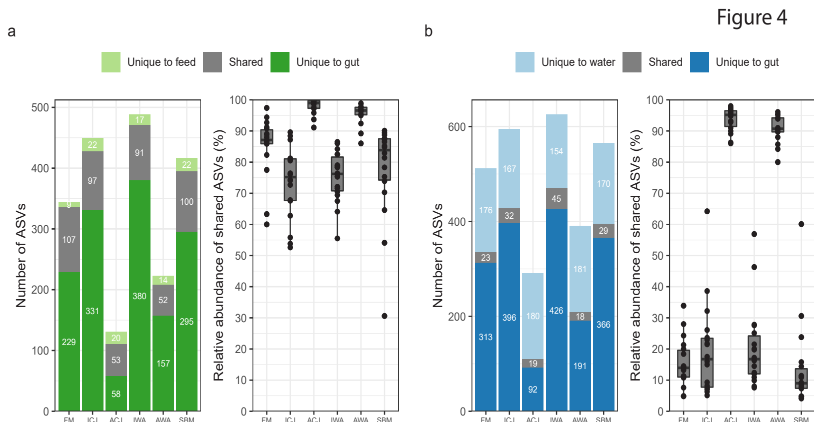

Fig. 4. The microbial overlap between the gut and feeds (a) and between the gut and the water (b). The number of shared amplicon sequence variants (ASVs) is shown in the left figure of each panel. The relative abundance of shared ASVs is shown in the right figure of each panel. The minimum relative abundance of ASVs to be considered as present in a sample was 0.05%. The samples are grouped by diets; FM – fishmeal-based; SBM – soybean meal-based; 4 experimental diets containing 300 g/kg SBM and 100 g/kg of ICJ – inactivated *Cyberlindnera jadinii*; ACJ – autolyzed *C. jadinii*; IWA – inactivated *Wickerhamomyces anomalus*; AWA – autolyzed *W. anomalus* diets.

IWA, AWA and SBM diets (Figs 7 & S9). The predicted enrichment of metabolic pathways was similar for fish fed FM and ACJ diets, except for glycerophospholipid pathway (enriched in fish fed FM) and nucleotide interconversion (enriched in fish fed ACJ) (Figs 7 & S9B).

Discussion

Core microbiota. In line with previous studies (45–47), Limosilactobacillus, Weissella, Ligilactobacillus and Streptococcus were annotated as core microbiota in the present study. *Limosilactobacillus*, *Weissella*, *Ligilactobacillus* and *Streptococcus* are commonly identified in the intestine of At-

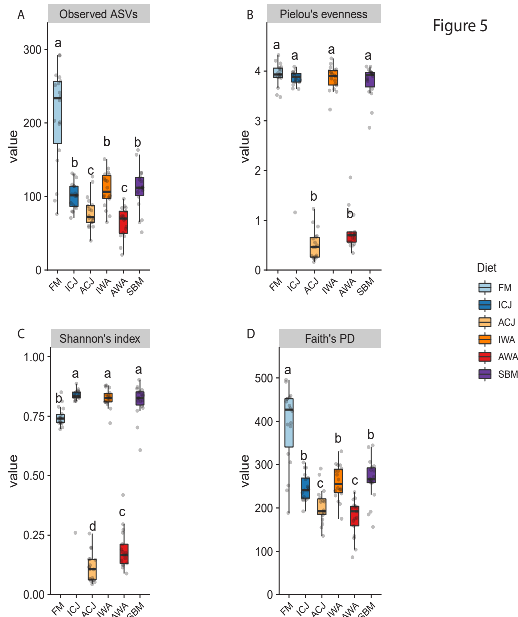

Fig. 5. Boxplots of alpha-diversity of gut microbiota of fish fed the experimental diets. The four alpha-diversity indices used are; (**A**) observed amplicon sequence variants (ASVs), (**B**) Pielou's evenness (**C**) Shannon's index and (**D**) Faith's phylogenetic diversity (PD). The samples are grouped by diets; FM – fishmeal-based; SBM – soybean meal-based; 4 experimental diets containing 300 g/kg SBM and 100 g/kg of ICJ – inactivated *Cyberlindnera jadinii*; ACJ – autolyzed *C. jadinii*; IWA – inactivated *Wickerhamomyces anomalus*; AWA – autolyzed *W. anomalus* diets. Indices with different lower-case letters are significantly different ($p < 0.05$) among the diets.

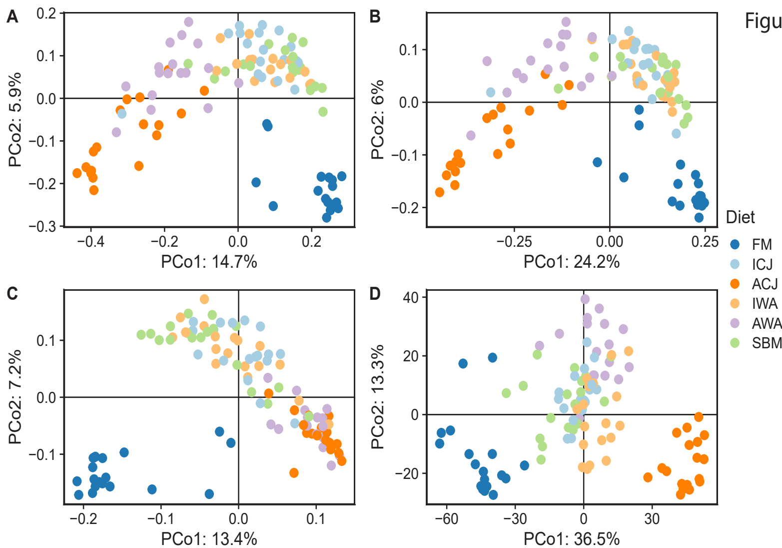

Fig. 6. Principal coordinates (PCo) analysis plots of beta-diversity of gut microbiota of fish fed the experimental diets. The four beta-diversity indices used are; (**A**) Jaccard distance, (**B**) Unweighted Unifrac distance (**C**) Aitchison distance and (**D**) PhILR transformed Euclidean distance. The samples are grouped by diets; FM – fishmeal-based; SBM – soybean meal-based; 4 experimental diets containing 300 g/kg SBM and 100 g/kg of ICJ – inactivated *Cyberlindnera jadinii*; ACJ – autolyzed *C. jadinii*; IWA – inactivated *Wickerhamomyces anomalus*; AWA – autolyzed *W. anomalus* diets.

lantic salmon reared in seawater (23, 37, 45). These taxa belong to the group of lactic acid bacteria (LAB), which are known to promote beneficial health effects in fish (48–50). The environmental factors (e.g., feeds) before seawater transfer possibly influenced the colonization of these microbiota in the fish gut. *Peptostreptococcus* and *Lachnospiraceae* were also identified as core taxa in the present study. These taxa have been found in the intestinal digesta of Atlantic salmon but are rarely identified as core microbiota (37, 45, 46). *Lachnospiraceae* are associated with produc-

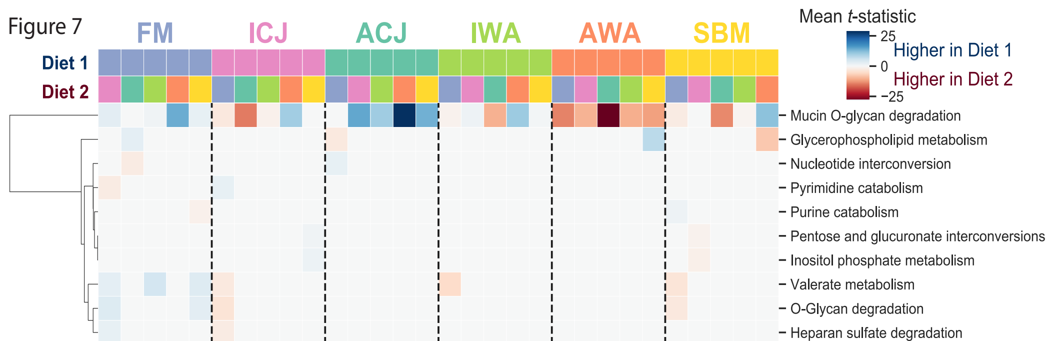

Fig. 7. Hierarchical clustering of the significantly enriched metabolic subsystems between each pair of dietary groups. Columns are diet pairs, rows are metabolic subsystem, and the color of each cell indicates whether the metabolic subsystem was enriched in diet 1 (blue) or diet 2 (red). The samples are grouped by diets; FM – fishmeal-based; SBM – soybean meal-based; 4 experimental diets containing 300 g/kg SBM and 100 g/kg of ICJ – inactivated *Cyberlindnera jadinii*; ACJ – autolyzed *C. jadinii*; IWA – inactivated *Wickerhamomyces anomalus*; AWA – autolyzed *W. anomalus* diets.

495 tion of short chain fatty acids (butyrate) (51), and has been reported to play a role in preventing inflammatory diseases in fish (52). It is noteworthy to state that, *Mycoplasma* which is commonly reported as core microbiota in the intestine of both wild and farmed Atlantic salmon (37, 47, 53–58), was not identified in the present study. It is unclear why *Mycoplasma* was not detected, but it might be linked to the differences in environmental factors during the early life stages of fish such as, live food, feeds, water temperature and salinity or simply lack of exposure to *Mycoplasma*. These factors are reported to influence the establishment of core microbiota in fish (48, 56–60). Also, a recent study has demonstrated that the establishment of *Mycoplasma* increased with time in seawater (58), implying that the experimental duration may be too short for its establishment in the gut of fish used in the current experiment. 510

511 **Soybean meal has a dominating effect on modulation of gut microbiota.** In accordance with previous findings in fish (23–26, 61–63), the present study observed differences between the gut microbiota of fish fed FM diet compared with those fed SBM diet. The microbial richness and diversity were higher in fish fed FM diet compared with fish fed SBM diet, which is in line with previous studies (23, 24). Most of the microbial taxa found in Atlantic salmon gut such as *Lactobacillus*, *Limosilactobacillus*, *Ligilactobacillus*, *HT002*, and *Vagococcus* were more abundant in fish fed FM diet compared with fish fed SBM diet. The current results showed that the microbiota of fish fed SBM were dominated by LAB such as *Lactobacillus*, *Limosilactobacillus*, *Ligilactobacillus*, *Weissella*, *Enterococcus* and *Streptococcus*, which is in accordance with previous findings (23–25). The high abundance of LAB in fish fed SBM-based diet has been attributed to the presence of soluble and insoluble oligosaccharides such as raffinose and stachyose, which can be used as substrates for metabolism and growth by the microbiota (23). Results from the present study published elsewhere (29) showed that fish fed SBM diet developed typical signs of SBMIE. As previously mentioned, LAB are generally considered as beneficial microbes promoting intestinal health and 533

534 growth of fish. Although members of LAB, such as some species of *Enterococcus* and *Streptococcus*, are considered pathogenic, it seems counterintuitive that LAB enrichment could be observed in fish that developed SBMIE. This observation challenges the general understanding that microbiota play a role in the development of SBMIE in fish. The relationship between increased relative abundance of LAB and development of SBMIE has been documented in previous studies (23, 24, 26). Reveco et al. (24) speculated that the increased relative abundance of LAB could be related to their capability to produce antimicrobial peptides (such as bacteriocins) against the certain bacteria in fish presenting SBMIE. Also, during the development of SBMIE, it is possible that the commensal bacteria (LAB) have less competition and more opportunity to proliferate. It remains unclear whether the increase in relative abundance of LAB is a cause or a consequence of the inflammatory response in fish presenting SBMIE. Further investigation is needed to clarify the role of intestinal microbiota in the development of SBMIE in Atlantic salmon fed plant-based diets. 553

554 The present study revealed that microbial richness and diversity were similar among fish fed ICJ, IWA and SBM diets. This implies that the inclusion of inactivated yeasts (CJ and WA) did not modulate the intestinal microbiota of fish fed SBM diet. This contradicts previous findings which showed that feeding diets containing *Saccharomyces cerevisiae* and WA yeasts modulated the intestinal microbiota in rainbow trout (64, 65). It is worthy of note that SBM was not used in the previous studies (64, 65). In line with our present results, dietary supplementation of mannan oligosaccharides (MOS) from yeasts did not modulate microbial diversity and richness of gilthead sea bream fed SBM-based diet (66). These contradicting results underscore the importance of ingredients used in diet formulation with respect to possible effects of yeast or its cell wall components on gut microbiota of fish (67). The cell wall polysaccharides of yeasts such as glucans and MOS can serve as substrates for microbial growth (68–70), and as a consequence modulates the intestinal microbiota in fish fed yeast-based diets (64, 65). However, our speculation is that 30% inclusion level of SBM possibly has a dominat- 573

ing effect in modulating gut microbiota when compared with 10% inclusion level of inactivated yeasts in the current study. Study on the effects of inactivated yeasts (CJ and WA) in Atlantic salmon fed SBM-free diets is recommended in the future. Despite the similarity in microbial composition of fish fed ICJ, IWA and SBM diets, the results of the present study reported elsewhere (29) showed that inclusion of inactivated yeasts (CJ and WA) dampened the inflammatory response in the distal intestine of fish fed SBM diet. Therefore, it can be hypothesized that the ameliorating effects of inactivated yeasts on SBMIE is related to their capability to stimulate immune responses rather than through modulation of intestinal microbiota in Atlantic salmon.

Autolyzed yeasts modulate gut microbiota of fish . The results of the present study revealed that the gut bacteria composition of fish fed ACJ and AWA diets were greatly affected by the diets when compared with the other groups. The ACJ and AWA diets promoted the dominance of genus *Pediococcus* and the family *Bacillaceae*, respectively. Such modulation consequently led to a decrease in richness and diversity of gut microbiota of fish fed ACJ and AWA diets compared with fish fed the remaining diets. A previous study reported that autolyzed *S. cerevisiae* reduced the microbial diversity of gilthead sea bream fed commercial-like diet (71).

The increased relative abundance of *Pediococcus* and *Bacillaceae* in Atlantic salmon fed the autolyzed yeasts may be explained by the autolytic conditions, feed-borne microbiota and/or feed composition. Based on BLAST analysis using the NCBI database, the *Pediococcus* ASV in our data set revealed sequence homologous to *Pediococcus acidilactici* and *P. claussenii*, whereas the *Bacillaceae* ASVs matched a wide range of members in the *Bacilli* microbial clade, including *Caldibacillus pasinlerensis*, *C. thermoamylovorans*, *Cerasibacillus terrae*, *C. quisquiliarum*, *Alkalihalobacillus gibsonii* and *A. lonarensis*. Optimum growth temperature for the genus *Pediococcus* (72) and the family *Bacillaceae* (73) ranged between 30 – 60 ºC. Thus, it is plausible that the growth of spores of these microbial taxa were selectively promoted during the autolytic process (at 50 °C for 16 h). Although thermal condition during the spray-drying was expected to inactivate the microbes in the yeast, dead or bacterial spores can still be profiled by the DNA sequencing methods. We could assert that the inclusion of autolyzed yeasts promotes the enrichment of a certain microbial taxon in the digesta of fish, but the effects seem to be yeast dependent. Therefore, the observed dominance of these microbial taxa in the gut of fish fed ACJ and AWA feeds probably reflects not only active microbes, but also dead microbes and spores transferred from the yeasts into the feeds. In future studies, analyzing the microbes in the yeast cream and the dried yeasts would further elucidate the extent to which the diet effects are attributable to the transfer of microbes from the yeasts to the diets. Techniques such as viability PCR and RNA sequencing (74), which are able to distinguish dead or active microbes, would provide useful information regarding the role of yeast- and feed-associated microbes in shaping the intestinal microbiota of fish fed yeast-based diets. Changes in cell wall polysaccharide of autolyzed yeasts may also partly contributed to the observed dominance of *Pediococcus* and *Bacillaceae* in fish fed ACJ and AWA diets. Previous studies have reported that the solubility (75) and biophysical properties (18, 76) of cell wall polysaccharides of yeasts are modified by the autolytic process. It is possible that the glucans and MOS in autolyzed yeasts are more available as substrates for the intestinal microbiota compared with intact yeasts. In the current study, it was impossible to distinguish whether the substrates for microbiota growth and metabolism were derived from SBM or from the yeast. Thus, the extent to which the modification of cell wall polysaccharides of yeasts contributed to the intestinal microbiota of fish could not be ascertained. This hypothesis can be tested by supplementing autolyzed yeasts to SBM-free diets and sequencing the intestinal microbiota of fish fed these diets.

It remains unclear whether the high abundance of a single taxon in fish fed ACJ or AWA diet was beneficial or caused dysbiosis in the host. The species *P. acidilactici* and *Bacillus subtilis* are among the most widely studied probiotic bacteria and have been reported to promote growth performance, nutrient digestion, disease resistance and intestinal health in farmed fish (20, 77–80). Based on this, it was expected that the high relative abundance of *Pediococcus* and *Bacillaceae* in fish fed the autolyzed yeasts would enhance the performance and intestinal health compared with fish fed the other diets. This was not the case, based on the results of fish performance and intestinal health presented in Agboola et al. (29). Fish performance was unaffected by the dietary treatments, and the inclusion of autolyzed yeasts in fish fed SBM did not alleviate SBMIE beyond the level observed for fish fed SBM with inactivated yeasts (29). Therefore, it is possible that the physiological response of fish to high relative abundance of both *Pediococcus* and *Bacillaceae* is limited by low feed intake and short experimental period used in the current study. Long-term experiments with *ad-libitum* fish feeding of diets containing autolyzed yeasts is recommended in the future. Also, it could simply be that the microbiota are dead and without probiotic effects in fish. The lack of difference in physiology of fish fed inactivated and autolyzed yeasts also supports the hypothesis that the dominance of a single taxon in the gut of fish fed ACJ and AWA is due to transfer of bacteria spores from the feeds to the fish gut. Thus, the reproducibility of microbiota modulated in fish fed yeast-based diets in the present study should be investigated in future studies. It is important to note that no mortality or noticeable signs of disease were recorded, suggesting that the high abundance of a single taxon in fish fed ACJ and AWA diets in the present study did not lead to dysbiosis.

Gut microbiota is driven by feed microbiota and less by water microbiota. Feed and rearing water are two environmental factors shaping the intestinal microbiota of fish (81–90). In agreement with previous studies in fish (81–86), there was high overlap between microbiota in the gut and the feeds. Still, it is unclear to what extent the carry-over microbes from the feeds influenced the intestinal microbiota. It would be interesting in the future to stain for live/dead

gut bacteria and then use fluorescence-activated cell sorting followed by 16S rRNA sequencing to identify the dead spores from the live bacteria. In the current study, microbial overlap between the intestine and the feeds was higher than the microbial overlap reported elsewhere (30, 86) in Atlantic salmon fed insect-based diets. The discrepancy can be attributed to the feed processing technology used in these studies. Contrary to the present study, feeds used in the previous studies (30, 86) were processed using extrusion technology. Extrusion is a hydrothermal process that is capable of inactivating microbes, thus, it is likely that the viability of feed microbes in this study was higher than the previous studies (30, 86). This may be responsible for the higher microbial overlap between the feeds and the intestine in the current study compared with earlier studies. However, it is reported that the feed processing (pre-conditioning *vs.* non-preconditioning) slightly influenced the gut microbiome of rainbow trout (91). Further investigation on the impact of extrusion treatment on intestinal microbiota of fish fed yeast-based diets in Atlantic salmon may be needed in the future. In accordance with previous studies (25, 30), water had a lower impact in shaping the intestinal microbiota of fish than the feeds. Microbial overlap between water and the intestine in the current study was higher than reported for Atlantic salmon reared in freshwater (25, 30, 86). In seawater, Atlantic salmon maintain osmoregulation by ingesting water to compensate for water loss to the hyperosmotic environment (92). Water drinking ability of salmon reared in seawater may facilitate uptake of microbes, and thus, be responsible for the higher microbial overlap between water and the intestine compared with previous studies in freshwater phase (25, 30, 86).

Metabolic capacity of gut microbiota. The gut microbiota plays a critical role in host physiology by supporting growth performance, nutrient digestion, metabolism and participating in immune system maturation and pathogen defense (93, 94). In the current study, a metagenome prediction tool was used to investigate the metabolic capacity of the gut microbiota of fish fed the experimental diets. The results revealed that the gut microbiota of fish fed ACJ diet were enriched in pathways related to mucin O-glycan degradation compared with fish fed the other diets. The gut microbiota of fish fed ACJ was dominated by *Pediococcus*, which has capability to adhere to intestinal mucus (95) and intestinal epithelial cells (96). The breakdown of mucin glycans by the gut microbiota generates a pool of microbial products that can be beneficial for host mucus production and for immune and metabolic responses (21, 22). This plays an important function in mucosal health, which is considered the first line of defense protecting the epithelial layer from pathogen invasion and other luminal compounds (21). Our results further showed that pathways related to valerate metabolism were enriched in fish fed FM diet compared with fish fed ICJ, IWA and SBM diets. Valerate is a scarcely studied short chain fatty acid that can be produced as an end product of microbial fermentation (97). The production of short chain fatty acids can act as link between the microbiota and the immune system by modulating the different aspects of intestinal epithelial cell (97, 98). It has been reported that valerate production can help to inhibit the growth of *Clostridioides difficile*, both *in vitro* and *in vivo* (99), a bacterium that has been implicated in the development of inflammatory bowel disease in humans (100). Although the role of valerate on fish physiology is not reported in literature, it is possible that increased valerate metabolism may be responsible for the normal intestinal health observed in fish fed FM diet in the current study (29).

Prediction tools are used to infer metabolic functions of gut microbiota produced through amplicon sequencing (101–103), but their validity is often questionable (103). The GSMMs used in the current study were based on human gut microbiota, and the predicted metabolic capacities may not exactly mimic that of fish gut microbiota. Additionally, only about half of the identified ASVs were matched to a known GSMM, thus limiting the ability of the analysis to represent the whole gut microbiota of fish used in the present study. Based on these shortcomings, the results of the predicted metabolic capacity of fish gut microbiota reported in this study should be interpreted with caution.

Conclusions

The present study showed that the richness and diversity of gut microbiota was lower in fish fed SBM compared with fish fed FM diet. The microbial composition and richness were similar among fish fed ICJ, IWA and SBM diets. Inclusion of autolyzed yeasts (ACJ and AWA) lowered the richness and diversity of gut microbiota in fish. Fish fed ACJ diet increased relative abundance of *Pediococcus*, and mucin O-glycan degradation pathway while fish fed AWA diet increased relative abundance of *Bacillaceae* compared with other diets. The results also suggest that the ameliorating effects of yeasts on SBMIE is related to their capability to stimulate immune cells rather than through modulation of intestinal microbiota in Atlantic salmon. Future research should focus on increasing our understanding of functional role of microbiota enhanced through inclusion of yeasts in fish diets.

Abbreviations

ASVs: amplicon sequence variants; CJ: *Cyberlindnera jadinii*; ICJ: inactivated CJ; ACJ: autolyzed CJ; WA: *Wickerhamomyces anomalus*; IWA: inactivated WA; AWA: autolyzed WA; BR: broad range; FM: fishmeal; GSMMs: genome-scale metabolic models; HS: high sensitivity; LAB: lactic acid bacteria; MOS: mannan oligosaccharides; PCA: principal component analysis; PCoA: principal coordinates analysis; PD: phylogenetic diversity; PERMANOVA: permutation multivariate analysis of variance; SBM: soybean meal; SBMIE: SBM-induced enteritis; SPC: soy protein concentrates.

Declarations

Ethics approval and consent to participate. The fish experiment was conducted at the research facility of Norwegian

Institute of Water Resources (NIVA, Solbergstrand, Norway), which is a research facility approved by Norwegian Animal Research Authority (permit no. 174). The experimental procedures were in accordance with the national guidelines for the care and use of animals (The Norwegian Animal Welfare Act and the Norwegian Regulation on Animal experimentation).

Consent for publication. Not applicable

Data and code availability

The raw 16S rRNA gene sequence data and metadata files are deposited at the NCBI SRA database under the BioProject PRJNA797563. Other data and the code for reproducing the results are available in the Github repository (https://github.com/Jeleel2020/Salmon_Yeasts_Microbiota).

Competing interests

The authors declared no competing interests

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Author's contributions

JOA, JØH, MØA, and MØ contributed to the conception, JOA, JØH, LTM and MØ designed the study. JOA, DDM, JØH, SDCR and DL involved in feed production, fish experiment and sampling. JOA and SDCR conducted the laboratory analysis. JOA and OØ performed the bioinformatics, statistical analyses, and data visualization. SJH, LTM and MØ acquired funding. JØH, MØA and MØ supervised the work. JOA wrote the first draft of the manuscript. All the authors read, revised, and approved the final version of the manuscript for publication.

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Supplementary Note 1: Supplemental tables

Table S1. Table S1. Composition of spray-dried yeasts with and without the autolysis treatment. All values are presented in % DM, except gross energy which is presented as MJ/kg DM.

	Cyberlindnera jadinii		*Wickerhamomyces anomalus*	
	Inactivated	Autolyzed	Inactivated	Autolyzed
DM (%)[1]	96.3 ± 0.03	93.1 ± 0.04	96.1 ± 0.02	96.1 ± 0.06
Nutrients (%DM)[2]				
Crude protein	46.5 ± 0.47	47.4 ± 0.01	43.0 ± 0.04	42.1 ± 0.26
Crude lipids	2.9 ± 0.18	5.7 ± 0.17	2.8 ± 0.06	4.1 ± 0.02
Ash	5.7 ± 0.00	5.9 ± 0.01	5.5 ± 0.00	5.5 ± 0.00
Total phosphorus	0.6 ± 0.02	0.6 ± 0.01	0.5 ± 0.01	0.4 ± 0.02
Gross energy (MJ/kg DM)	21.8 ± 0.01	22.32 ± 0.02	21.1 ± 0.01	21.5 ± 0.01
Cell wall polysaccharides (%DM)[3]				
β-glucan	16.4 ± 3.19	11.1 ± 0.84	15 ± 1.41	11.8 ± 0.73
Mannan	7.9 ± 2.16	6.0 ± 0.66	11.3 ± 0.95	10.4 ± 0.67
Chitin	0.3 ± 0.07	0.2 ± 0.02	0.5 ± 0.05	0.4 ± 0.08

[1]DM – dry matter.
[2]Crude protein, crude lipids, ash, total phosphorus, and gross energy contents of yeasts are mean values ± SD from duplicate analyses.
[3]β-glucan, mannan and chitin contents of yeasts are mean values ± SD from triplicate analyses.

Table S2. The dominant taxa identified as contaminants in the negative controls and the blank filter papers. (available as additional file 2_supplementary tables in the GitHub repository: https://github.com/Jeleel2020/Salmon_Yeasts_Microbiota/tree/main/Results

Table S3. The prevalence of core ASVs in the digesta of fish fed the experimental diets. (available as additional file 2_supplementary tables in the GitHub repository: https://github.com/Jeleel2020/Salmon_Yeasts_Microbiota/tree/main/Results

Table S4. Pair-wise comparisons of alpha-diversity indices of gut microbiota in Atlantic salmon smolts fed FM-based diet or SBM-based diet with yeasts.[1]

	Observed ASVs	Pielou's evenness	Shannon's index	Faith's PD
P-values[2]	<0.0001	<0.0001	<0.0001	<0.0001
Pair-wise comparisons[3]				
IWAvsAWA	<0.0001	<0.0001	<0.0001	<0.0001
IWAvsACJ	0.003	<0.0001	<0.0001	0.001
IWAvsSBM	0.81	1.000	0.9701	0.740
IWAvsICJ	0.77	1.000	0.9701	0.740
IWAvsFM	0.001	1.000	<0.0001	<0.0001
AWAvsACJ	0.62	0.052	0.007	0.580
AWAvsSBM	<0.0001	<0.0001	<0.0001	<0.0001
AWAvsICJ	<0.0001	<0.0001	<0.0001	<0.0001
AWAvsFM	<0.0001	<0.0001	<0.0001	<0.0001
ACJvsSBM	0.010	<0.0001	<0.0001	0.003
ACJvsICJ	0.010	<0.0001	<0.0001	0.002
ACJvsFM	<0.0001	<0.0001	<0.0001	<0.0001
SBMvsICJ	0.620	1.000	0.970	0.500
SBMvsFM	0.002	1.000	0.001	0.001
ICJvsFM	<0.0001	0.910	<0.0001	<0.0001

[1]The diets are: FM – fishmeal-based; SBM – soybean meal-based; 4 other diets containing 300 g/kg SBM and 100 g/kg of ICJ – inactivated *Cyberlindnera jadinii*; ACJ – autolyzed *C. jadinii*; IWA – inactivated *Wickerhamomyces anomalus*; AWA – autolyzed *W. anomalus* diets.
[2]P-values computed for diet effect with Kruskal-Wallis test.
[3]Wilcox pairwise comparison to identify differences between diets.

Table S5. PERMANOVA analysis for beta-diversity of gut microbiota in Atlantic salmon smolts fed FM-based diet or SBM-based diet with yeasts.[1]

	Jaccard dist.[2]	Unw. Unifrac dist.[2]	Aitchison dist.[3]	PhILR dist.[3]
P-values[4]	<0.001	<0.001	<0.001	<0.001
Pair-wise comparisons[3]				
IWAvsAWA	0.015	0.015	0.015	0.015
IWAvsACJ	0.015	0.015	0.015	0.015
IWAvsSBM	0.600	0.27	0.075	0.015
IWAvsICJ	1.000	1.000	1.000	0.345
IWAvsFM	0.015	0.015	0.015	0.015
AWAvsACJ	0.015	0.015	0.015	0.015
AWAvsSBM	0.015	0.015	0.015	0.015
AWAvsICJ	0.015	0.015	0.015	0.015
AWAvsFM	0.015	0.015	0.015	0.015
ACJvsSBM	0.015	0.015	0.015	0.015
ACJvsICJ	0.015	0.015	0.015	0.015
ACJvsFM	0.015	0.015	0.015	0.015
SBMvsICJ	0.600	1.000	0.195	1.000
SBMvsFM	0.015	0.015	0.015	0.015
ICJvsFM	0.015	0.015	0.015	0.015

[1]The diets are: FM – fishmeal-based; SBM – soybean meal-based; 4 other diets containing 300 g/kg SBM and 100 g/kg of ICJ – inactivated *Cyberlindnera jadinii*; ACJ – autolyzed *C. jadinii*; IWA – inactivated *Wickerhamomyces anomalus*; AWA – autolyzed *W. anomalus* diets. Jaccard dist. - Jaccard distance; Unw. Unifrac dist. - Unweighted Unifrac distance; Aitchison dist. - Robust Aitchison distance; and PhILR dist. - Phylogenetic isometric log-ratio (PhILR) transformed Euclidean distance
[2]Performed on phyloseq object rarefied to minimum read sequence in the sample.
[3]Performed on unrarefied phyloseq object.
[4]P-values of permutational multivariate analysis of variance (PERMANOVA) test for the four beta-diversity distances.
[5]PERMANOVA pairwise comparisons for the four beta-diversity distances.

Table S6. Test of homogeneity of multivariate dispersions among dietary groups.

	Jaccard dist.[2]	Unw. Unifrac dist.[2]	Aitchison dist.[3]	PhILR dist.[3]
P-values[4]	0.001	0.001	0.001	0.002
Pair-wise comparisons[3]				
IWAvsAWA	<0.001	0.023	<0.001	0.018
IWAvsACJ	<0.001	0.032	<0.001	0.824
IWAvsSBM	0.412	0.195	0.039	0.668
IWAvsICJ	0.84	0.90	0.109	0.648
IWAvsFM	0.051	0.009	0.041	0.150
AWAvsACJ	0.328	0.80	0.017	0.028
AWAvsSBM	0.001	0.002	0.001	0.002
AWAvsICJ	0.001	0.026	0.001	0.001
AWAvsFM	0.001	0.001	0.001	0.857
ACJvsSBM	0.007	0.002	0.001	0.522
ACJvsICJ	0.058	0.041	0.001	0.508
ACJvsFM	0.001	0.001	0.001	0.205
SBMvsICJ	0.418	0.24	0.471	0.950
SBMvsFM	0.333	0.214	0.842	0.085
ICJvsFM	0.086	0.013	0.402	0.083

[1]The diets are: FM – fishmeal-based; SBM – soybean meal-based; 4 other diets containing 300 g/kg SBM and 100 g/kg of ICJ – inactivated *Cyberlindnera jadinii*; ACJ – autolyzed *C. jadinii*; IWA – inactivated *Wickerhamomyces anomalus*; AWA – autolyzed *W. anomalus* diets. Jaccard dist. - Jaccard distance; Unw. Unifrac dist. - Unweighted Unifrac distance; Aitchison dist. - Robust Aitchison distance; and PhILR dist. - Phylogenetic isometric log-ratio (PhILR) transformed Euclidean distance
[2]Performed on phyloseq object rarefied to minimum read sequence in the sample.
[3]Performed on unrarefied phyloseq object.
[4]P-values of homogeneity of multivariate dispersions using PERMDISP test for the four beta-diversity distances.
[5]PERMDISP pairwise comparisons for the four beta-diversity distances.

Supplementary Note 2: Supplemental figures

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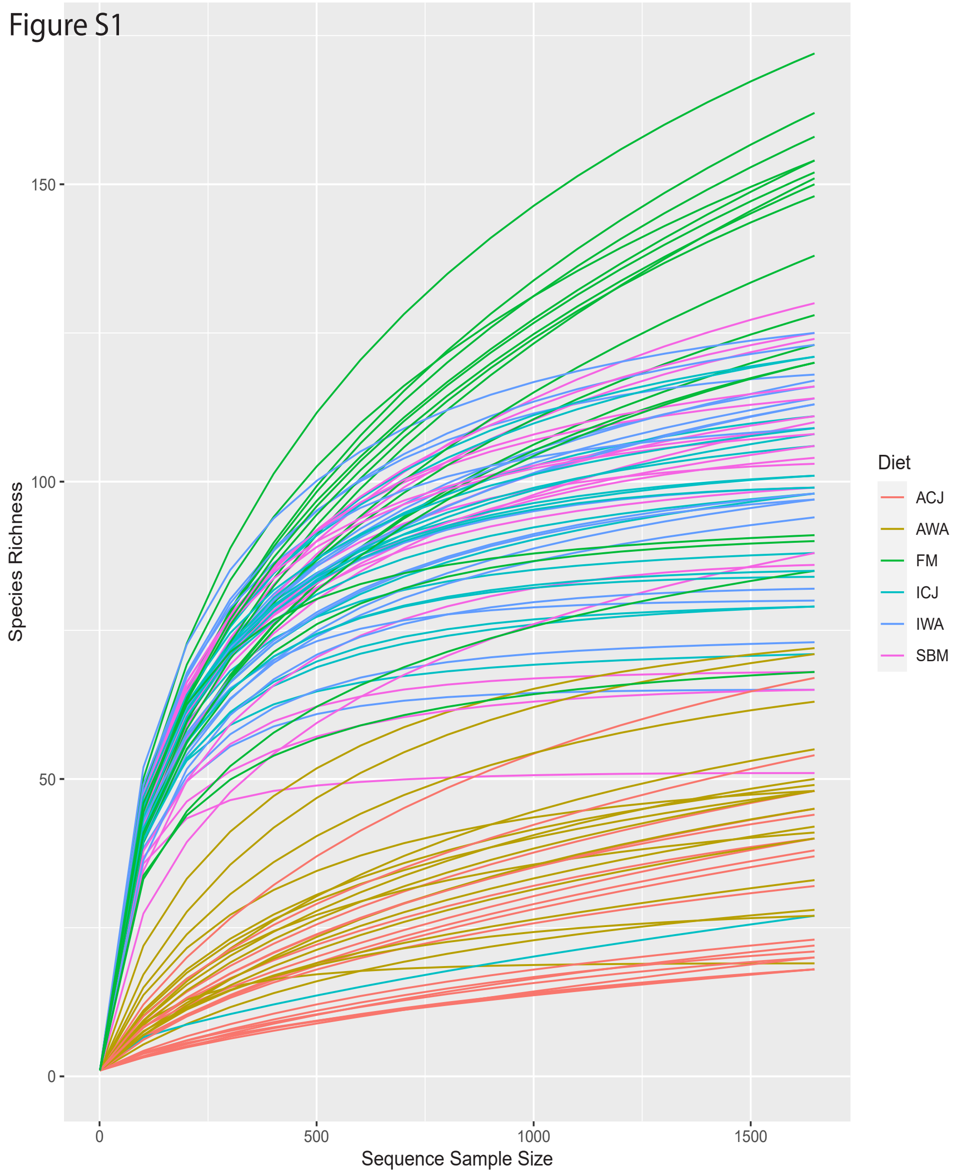


Fig. S1. Rarefaction curves showing subsampling of sample into minimum sample sequence (1,604 sequence per sample). The rarefied amplicon sequence variants table was used for computation of Jaccard and unweighted Unifrac beta-diversity distances. FM – fishmeal-based; SBM – soybean meal-based; 4 experimental diets containing 300 g/kg SBM and 100 g/kg of ICJ – inactivated *Cyberlindnera jadinii*; ACJ – autolyzed *C. jadinii*; IWA – inactivated *Wickerhamomyces anomalus*; AWA – autolyzed *W. anomalus* diets.

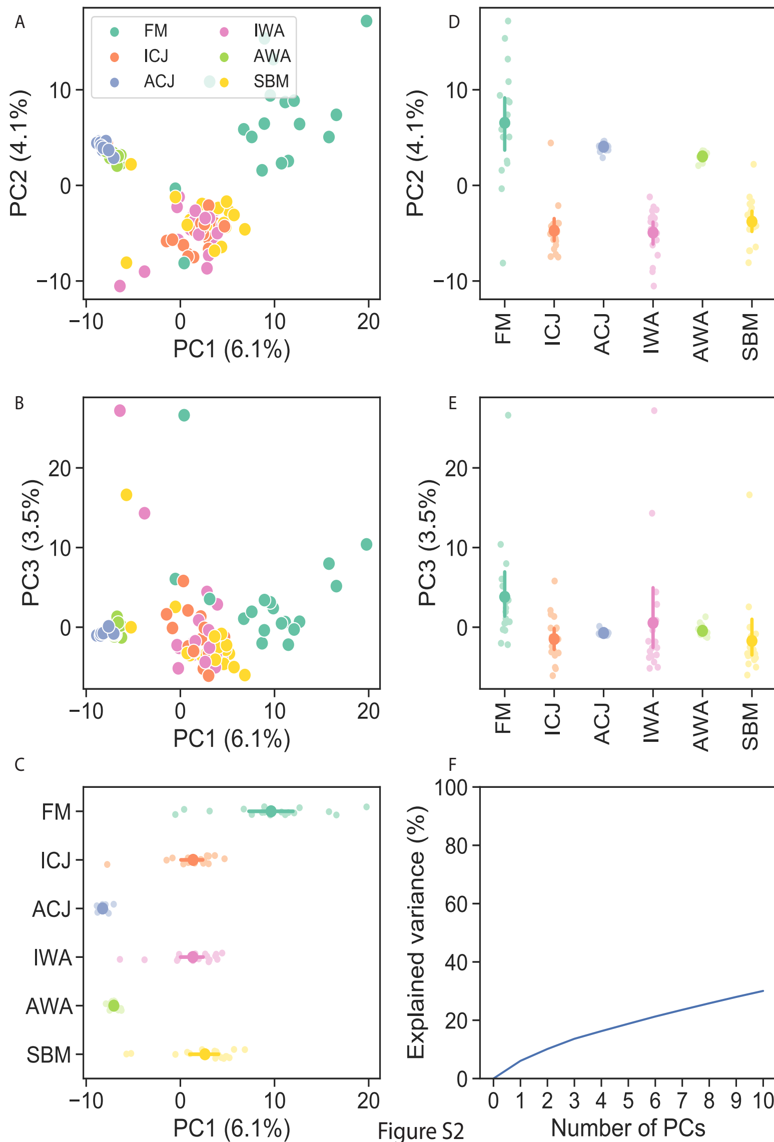


Fig. S2. Principal component (PC) analysis on standardized amplicon sequence variants (ASVs). Score plots for PC1 and PC2 (**A**) and PC1 and PC3 (**B**), mean scores with 95% confidence intervals for PC1 (**C**), PC2 (**d**), and PC3 (**E**), and percentage of variance explained by PCs (**F**).FM – fishmeal-based; SBM – soybean meal-based; 4 experimental diets containing 300 g/kg SBM and 100 g/kg of ICJ – inactivated *Cyberlindnera jadinii*; ACJ – autolyzed *C. jadinii*; IWA – inactivated *Wickerhamomyces anomalus*; AWA – autolyzed *W. anomalus* diets.

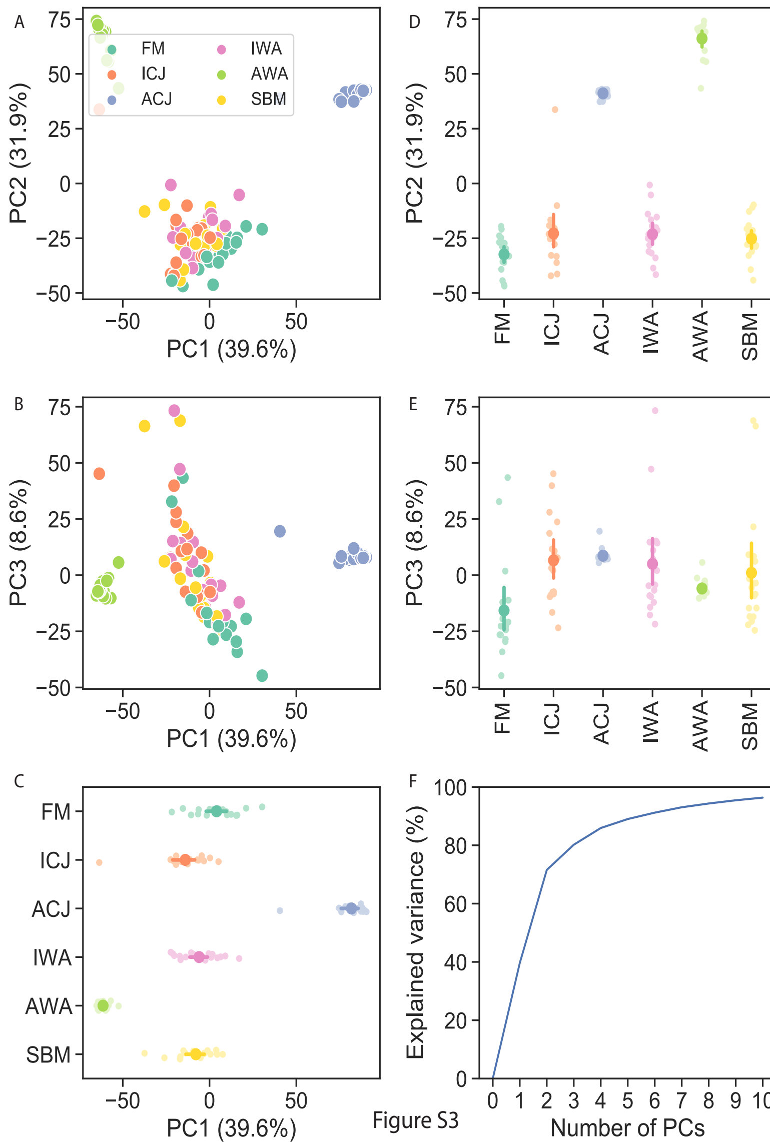


Fig. S3. Principal component (PC) analysis on standardized amplicon sequence variants (ASVs). Score plots for PC1 and PC2 (**A**) and PC1 and PC3 (**B**), mean scores with 95% confidence intervals for PC1 (**C**), PC2 (**d**), and PC3 (**E**), and percentage of variance explained by PCs (**F**).FM – fishmeal-based; SBM – soybean meal-based; 4 experimental diets containing 300 g/kg SBM and 100 g/kg of ICJ – inactivated *Cyberlindnera jadinii*; ACJ – autolyzed *C. jadinii*; IWA – inactivated *Wickerhamomyces anomalus*; AWA – autolyzed *W. anomalus* diets.

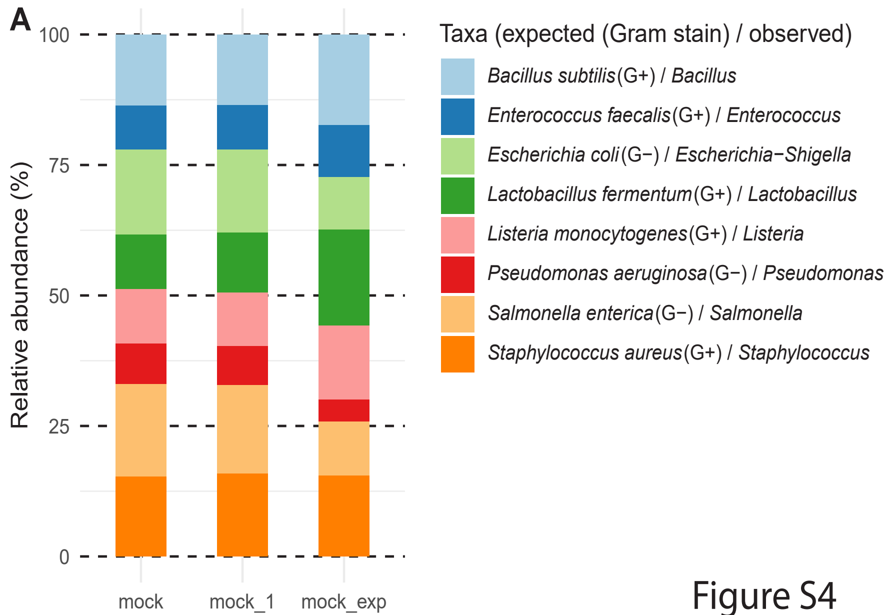


Fig. S4. Expected (mock_exp) and observed (mock and mock_1) taxonomic profiles of the mock microbial community standard).

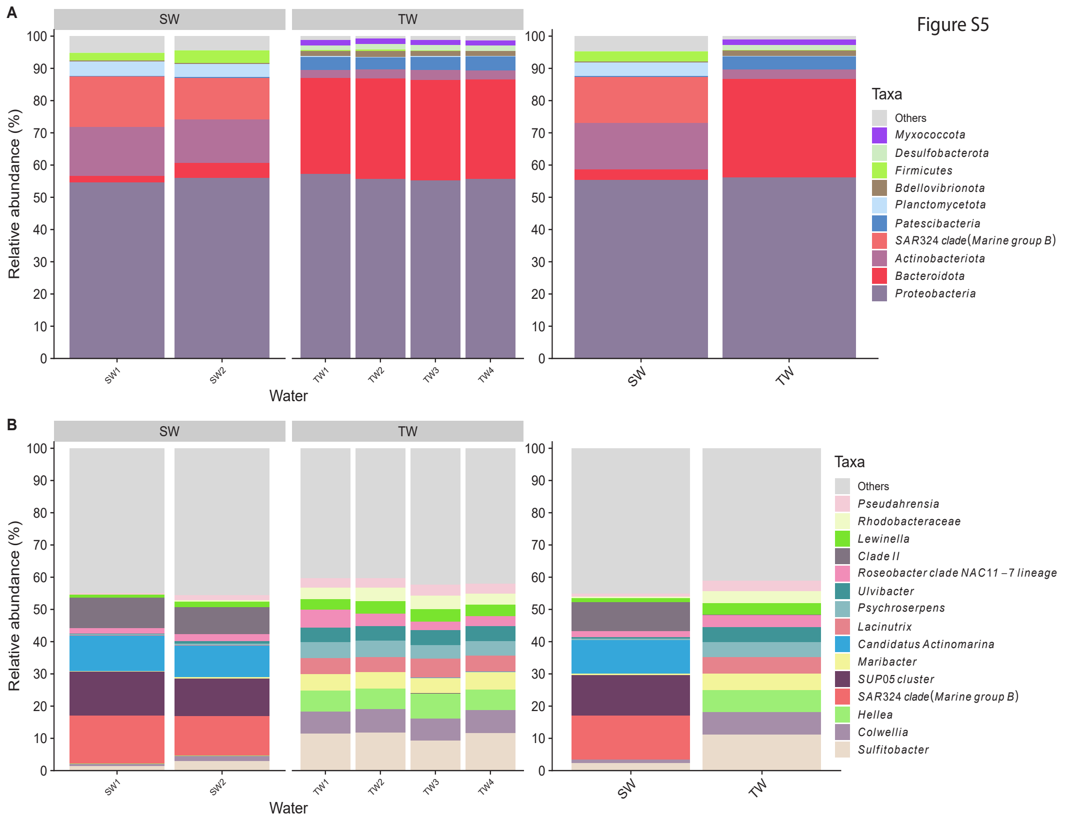


Fig. S5. Microbiota composition of water samples. Relative abundance of the top 10 most abundant taxa at phylum level (**A**) and top 15 most abundant taxa at genus or lowest taxonomic rank (**B**). The mean relative abundance of each taxon within the same water type is displayed on the right side. The samples are group water type; SW – water collected from the source tank (i.e., header tank) and TW – water collected from the fish rearing tanks. Water collected from the 18 fish tanks were mixed, four subsamples were taken and used for the microbiota analysis.

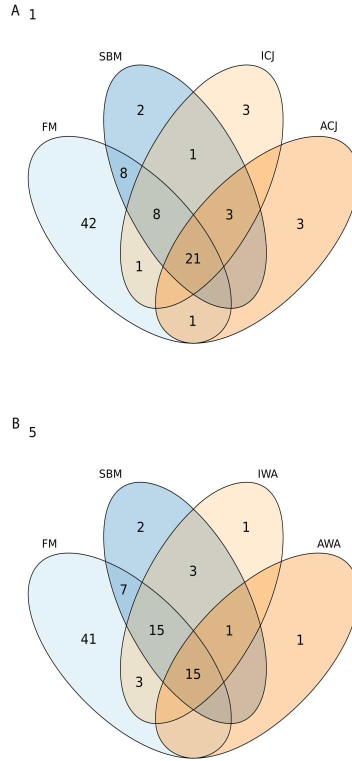


Fig. S6. Venn's diagram (A and B) showing the shared and the unique amplicon sequence variants (ASVs) in the digesta sample of fish fed the experimental diets. The ASVs were computed using a prevalence threshold of 80%. FM – fishmeal-based; SBM – soybean meal-based; 4 experimental diets containing 300 g/kg SBM and 100 g/kg of ICJ – inactivated *Cyberlindnera jadinii*; ACJ – autolyzed *C. jadinii*; IWA – inactivated *Wickerhamomyces anomalus*; AWA – autolyzed *W. anomalus* diets.

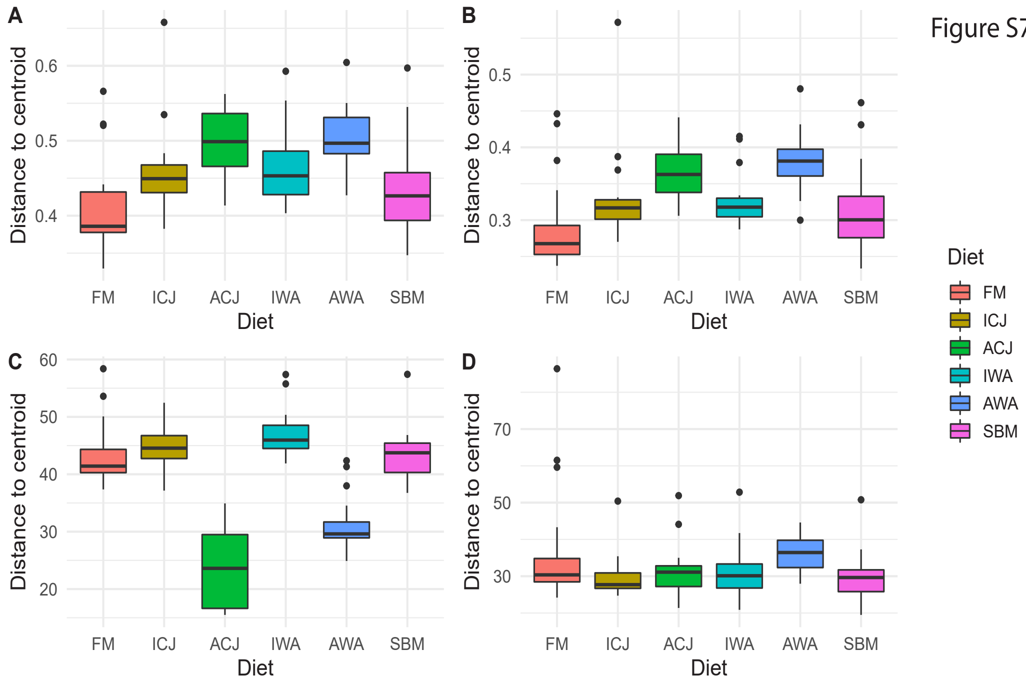


Fig. S7. Boxplots for homogeneity of multivariate dispersions (PERMDISP) in gut microbiota of fish fed experimental diets. The PERMDISP test was based on; (**A**) Jaccard distance, (**B**) Unweighted Unifrac distance (**C**) Aitchison distance and (**D**) PhILR transformed Euclidean distance. FM – fishmeal-based; SBM – soybean meal-based; 4 experimental diets containing 300 g/kg SBM and 100 g/kg of ICJ – inactivated *Cyberlindnera jadinii*; ACJ – autolyzed *C. jadinii*; IWA – inactivated *Wickerhamomyces anomalus*; AWA – autolyzed *W. anomalus* diets.

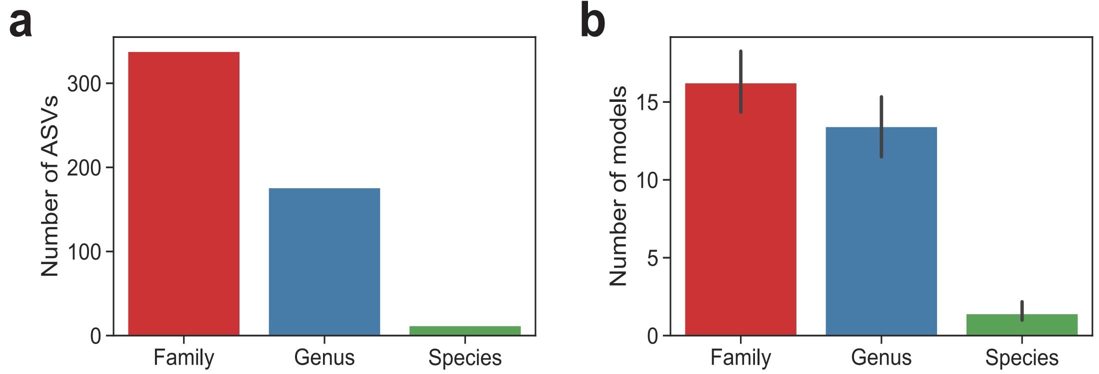


Fig. S8. Number of ASVs mapped to genome-scale metabolic models. Number of samples matched to models at different taxonomic levels (**A**) and the number of models mapped to each sample by taxonomic level (**B**).

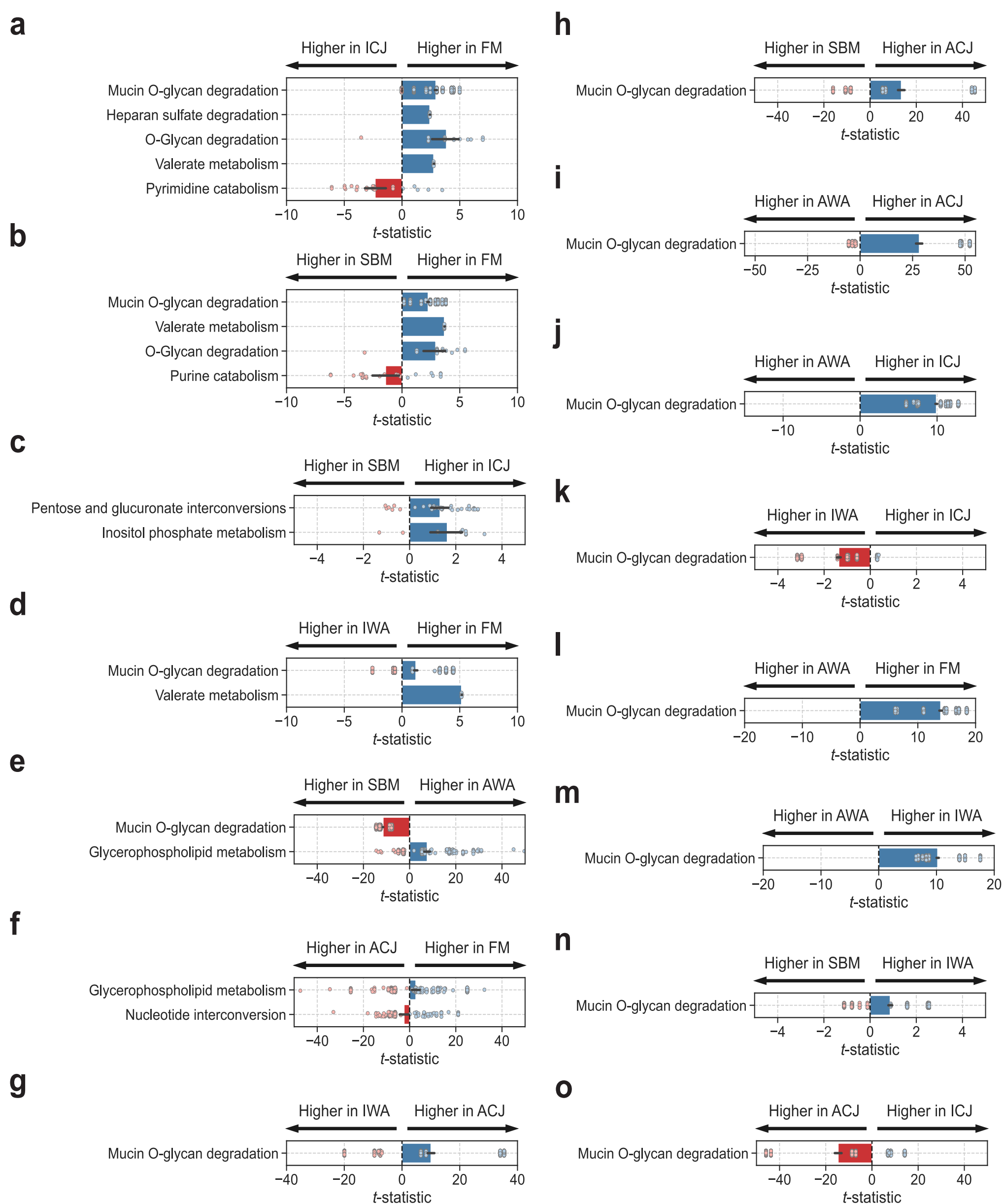
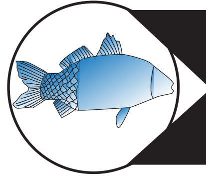


Fig. S9. The *t*-statistic tests comparing reaction abundances between each pair of diets. The *t*-statistic for each reaction is shown along with the mean across all reactions with 95% confidence interval for all significantly enriched subsystems. FM – fishmeal-based; SBM – soybean meal-based; 4 experimental diets containing 300 g/kg SBM and 100 g/kg of ICJ – inactivated *Cyberlindnera jadinii*; ACJ – autolyzed *C. jadinii*; IWA – inactivated *Wickerhamomyces anomalus*; AWA – autolyzed *W. anomalus* diets.



Paper VII

A meta-analysis to determine factors associated with the severity of enteritis in Atlantic salmon (*Salmo salar*) fed soybean meal-based diets

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22 **Abstract**

23 A meta-analytic approach was used to determine factors associated with the severity of enteritis
24 in distal intestine of Atlantic salmon fed soybean meal (SBM)-based diets. Dataset from 26
25 fish studies were extracted and standardized for use in the meta-analysis. After standardization,
26 the data were analyzed with ordinal logistic regression model by comparing the SBM
27 treatment(s) in each study with the neutral-reference treatment. The log-odds ratio of the
28 proportional odds model and its standard error were extracted and analyzed using the random
29 effects model to estimate the effect size of dietary SBM on enteritis using four semi-
30 quantitative histological variables: reduction in mucosal fold height; disappearance of
31 supranuclear vacuolization; inflammatory cell infiltration of lamina propria, and of submucosa.
32 Both univariate and multivariate meta-regression were used to identify study factors with
33 significant association to the severity of enteritis in Atlantic salmon. The results showed that
34 fish production phase, feed type, SBM inclusion level, year of study and water temperature are
35 significantly associated with the severity of enteritis in Atlantic salmon. Further meta-analysis
36 of sub datasets according to production phase, revealed that fish reared in seawater were more
37 prone to develop enteritis compared with fish reared in freshwater. The absence of positive
38 relationship between SBM inclusion level and the severity of enteritis was probably due to
39 difference in source, batch, processing, and level of anti-nutritional factors in the SBM used in
40 the different studies combined in the meta-analysis. Subgroup analysis based on year of study
41 revealed that the severity of enteritis in fish fed SBM-based diets has decreased over the years.
42 Additional results revealed that fish fed SBM-based diet at low water temperature showed
43 increased severity of enteritis, compared with fish raised in high water temperature. Linear and
44 quadratic regressions conducted to explore possible impact of enteritis on fish performance,
45 showed that the specific growth rate and thermal growth coefficient of fish decreased with
46 increased severity of enteritis. However, this relationship depends on the fish production phase

and the histological variables used for the regression analysis. The current study concluded that the severity of enteritis in Atlantic salmon fed SBM-based diets are significantly associated with fish production phase, feed type, SBM inclusion level, year of study and water temperature, but not the exposure time. Also, the study showed that increased severity of enteritis reduced specific growth rate and thermal growth coefficient of fish fed SBM-based diets.

Key words: Meta-regression, ordinal logistic regression, mucosal fold height, supranuclear vacuole, lamina propria, submucosal cellularity.

1.0 Introduction

Over the years, concerted efforts have been dedicated to finding alternative protein sources for use in formulated feeds for farmed salmon as fishmeal availability is limited and market prices have increased (Tacon, et al., 2011). In recent time, the dependency on marine ingredients has been reduced as more plant ingredients are used in salmon feeds (Ytrestøyl, et al., 2015; Aas, et al., 2019). Among several protein alternatives, soybean meal (SBM) is an attractive plant ingredient for fish feeds due to its high protein content, its availability and price competitiveness (Gatlin III, et al., 2007). A major reason for the low/no inclusion of SBM in Atlantic salmon feeds is that several studies have shown that its dietary inclusion can induce enteritis in the distal intestine of Atlantic salmon (Baeverfjord and Krogdahl, 1996; Marjara, et al., 2012; Urán, et al., 2008b; Urán, et al., 2009a; Van den Ingh, et al., 1996; Van den Ingh, et al., 1991). The cause of SBM-induced enteritis in Atlantic salmon is linked to various anti-nutritional factors (ANFs) present in SBM (Baeverfjord and Krogdahl, 1996; Bakke, et al., 2014; Krogdahl, et al., 2015; Krogdahl, et al., 2020; Refstie, et al., 1998). Alcohol-soluble components of SBM (especially saponins) have been implicated as the causative agents of SBM-induced enteritis, but there are indications that the severity might be amplified by presence of other ANFs (such as protease inhibitors, trypsin inhibitors and lectins) (Chikwati, et al., 2012; Knudsen, et al., 2007; Knudsen, et al., 2008; Van den Ingh, et al., 1996). Typical signs of SBM-induced enteritis, firstly described by Van Den Ingh and Krogdahl (1990), are reduction in mucosal fold height; disappearance of supranuclear vacuolization; thickening of both lamina propria and sub-epithelia mucosa with a severe infiltration of inflammatory cells (Baeverfjord and Krogdahl, 1996; Krogdahl, et al., 2003; Urán, et al., 2008b; Van den Ingh, et al., 1996). In several studies, SBM-induced enteritis has been assessed qualitatively by describing histological alterations of the distal intestine. However, Urán, et al. (2008b) realized that such method cannot be used to compare the impact of different study factors (e.g., variety

93 of SBM, SBM inclusion level, environmental factors) on the development of SBM-induced
94 enteritis between different fish studies. The authors, therefore, used a "semi-quantitative"
95 method, that quantifies the degree of enteritis by scoring system using five scales for different
96 parts of the distal intestine (Knudsen, et al., 2008; Urán, et al., 2008b). The scoring system was
97 based on an ordinal scale comprising of normal, mild, moderate, marked, and severe depending
98 on the degree of enteritis, where the lowest degree was regarded as normal, and the highest
99 scale was regarded as severe. Although, several studies have since adopted this scoring system
100 to assess the degree of SBM-induced enteritis in Atlantic salmon, to the best of our knowledge,
101 no study has quantitatively summarized the available information in a systematic literature
102 review.

103 Furthermore, several studies have investigated different ways to ameliorate SBM-
104 induced in fish. The most common approach is the use of single-cell ingredients (SCI) as
105 possible functional ingredients to counteract enteritis in fish (Agboola, et al., 2022; Agboola,
106 et al., 2021; Grammes, et al., 2013; Reveco-Urzua, et al., 2019; Romarheim, et al., 2013). The
107 SCI are a relatively broad class of resources including bacteria, yeast and microalgal derived
108 products or their combination and have potential to be used in aquafeeds (Glencross, et al.,
109 2020). The cell wall of SCI, particularly the yeasts and bacteria meal, contain bioactive
110 components such as β-glucan, mannan and chitin, which has immuno-modulating properties
111 that reduced inflammation caused by SBM in fish (Grammes, et al., 2013; Romarheim, et al.,
112 2013). Despite the number of available studies on the possible ameliorating effects of SCI on
113 SBM-induced enteritis in fish, no study has quantitatively evaluated this effect using a meta-
114 analytic approach.

115 Meta-analysis is a type of systematic review that can be used to draw important
116 conclusions from different, but related studies. Unlike narrative reviews, meta-analyses use
117 rigorous statistical procedure to provide objective and unbiased interpretation of findings from

118 multiple dataset (Higgins, et al., 2019; Sauvant, et al., 2008). Several studies have used meta-
119 analysis to provide quantitative evidence on different continuous variables (e.g., growth and
120 nutrient digestibility indices) (Hua and Bureau, 2012; Prabhu, et al., 2013; Sales and Glencross,
121 2011) in aquaculture, but there is no published meta-analysis on variables measured with
122 ordinal data, such as the effect of dietary SBM on enteritis in Atlantic salmon. Therefore, the
123 objective of the current study was to quantitatively evaluate factors associated with the severity
124 of enteritis in Atlantic salmon fed SBM-based diets. Additionally, in majority of studies on
125 development of enteritis in fish fed SBM, no clear effect on performance was shown, thus the
126 secondary objective of the present study was to evaluate the relationships between severity of
127 SBM-induced enteritis and growth performance of Atlantic salmon.

128

129 **2.0 Materials and methods**

130 ***2.1 Literature search strategy and study selection***

131 A literature search based on Oria, Web of Science, Scopus, and Google Scholar after duplicate
132 removal identified a total of 356 references related to the use of SBM using the following
133 search terms; “soybean meal”, “induced”, “enteritis”, “enteropathy”, “intestinal/gut health”,
134 “Atlantic salmon” and “*Salmo salar*”. The search strategy and literature selection process used
135 in the meta-analysis dataset is presented in Fig. 1. References were removed if the titles and
136 abstracts indicated use of a fish species other than Atlantic salmon, presented no histological
137 scores, were specifically related to subfractions of SBM (such as saponin and molasses),
138 focused on other plant protein sources (such as pea protein), presented only morphometric
139 scores of fish intestine or were review papers. This resulted in a list of 46 potentially relevant
140 articles after being assessed for the following eligibility criteria: (1) the trial was conducted
141 with Atlantic salmon; (2) the studies contained at least one SBM and a neutral-reference diet

142 (NRD); (3) random allocation of fish during the trial; (4) random selection of fish for
143 histological evaluation and the histology was conducted on the fish distal intestine (5) blind
144 evaluation of histological variables; and (6) the studies contained semi-quantitative scoring for
145 the histological variables. The NRD are fish feeds (without SBM) containing ingredients such
146 as fishmeal, soy-protein concentrate, wheat gluten meal among others which are known not to
147 induce enteritis in Atlantic salmon.

148 After literature review, the semi-quantitative scoring was presented as mean values or
149 as graphical representation of the actual fish number within each scoring category (i.e., normal
150 to severe enteritis). However, the histological scores were ordinal categorical variables and the
151 actual fish number in each scoring category were needed for this meta-analysis, thus the
152 original histological data were solicited from the authors of the relevant articles. These
153 solicitations yielded data for 16 peer-reviewed articles, which were included in the meta-
154 analysis database. The reference list of trials which failed to meet the inclusion criteria and/or
155 unavailability of raw dataset are presented in Table S1. Additionally, data from 10 unpublished
156 trials that met the inclusion criteria were also included in the meta-analysis, making a total of
157 26 experimental studies that were included in the final analysis. The description of studies
158 included in the meta-analysis database is presented in Table S2.

159 ***2.2 Data extraction and standardization***

160 An Excel spreadsheet was created for data extraction. Data were extracted for variables on
161 inclusion level (%) of SBM, SBM types, feed type (i.e., whether the feed contained SBM with
162 or without SCI), the scoring system used and fish production data. The fish production
163 variables collected were fish production phase, initial and final body weight of fish, water
164 parameters (temperature and salinity), and the exposure time. In addition, specific growth rate

165 (SGR) and thermal growth coefficient (TGC) were calculated from the extracted data using
166 equations (a) and (b), respectively.

167 $$SGR = \frac{\ln(FBW) - \ln(IBW)}{d} \times 100 \quad \text{(a)}$$

168 $$TGC = \frac{\sqrt[3]{FBW} - \sqrt[3]{IBW}}{t \times d} \times 1000 \quad \text{(b)}$$

169 Where FBW and IBW are final and initial body weight of fish; d is the exposure time in days;
170 and t is the average water temperature recorded during the experiment.

171 After reviewing the literature, different scoring system: 0-4, 0-2, 1-5, 0-10 and 1-10
172 generally denoting normal, mild, moderate, marked, and severe enteritis were used to evaluate
173 the degree of enteritis in Atlantic salmon. Regardless of the scoring system used, the data were
174 extracted based on the number of fish recorded for each score category (normal to severe
175 enteritis). Data from each study were extracted for the four histological variables normally used
176 to evaluate enteritis for estimation of effect size. The four histological variables observed to be
177 associated with inflammatory changes were: (1) reduction in mucosal fold height (MFH); (2)
178 disappearance of supranuclear vacuolization (SNV); (3) increases in cellularity of lamina
179 propria (LP); and (4) of submucosa (SC) due to infiltration by inflammatory cells.

180 The data in this study were transformed into trichotomous outcomes and analyzed using
181 ordinal logistic regression. In the newly created dataset, fish with normal score were grouped
182 into the normal category, fish with mild/moderate scores were grouped into moderate category
183 and fish with marked/severe scores were grouped into severe category for each study. These
184 new categories were assigned a score of 1 for normal, 2 for moderate and 3 for severe enteritis.
185 The data standardization used for the ordinal logistic regression analysis was done for both the
186 SBM and the NRD groups.

187 ***2.3 Statistical analysis***

188 Ordinal logistic regression was performed on the standardized trichotomous dataset using the
189 polr command from MASS package in R. The proportional odds logistic regression model was
190 performed by comparing the SBM treatment(s) with the NRD treatment in each study. The
191 NRD was used as reference point (log-odds ratio (LOR) = 0) in each study. From this
192 proportional odds regression model, the LOR and its standard error (Agresti, 2003; Higgins, et
193 al., 2019) for each SBM treatment were generated and extracted into a spreadsheet for the meta-
194 analysis. The meta-analysis was performed using the Comprehensive Meta-analysis (CMA)
195 software (version 3, Biostat Inc., USA). To determine factors associated with the severity of
196 enteritis, a meta-regression analysis was performed (using the Comprehensive Meta-analysis
197 v3) using the study factors in the database. Categorical variables including production phase,
198 and feed type, as well as continuous variables including SBM inclusion level, year of study,
199 water temperature, and exposure time were included in the meta-regression analysis. Meta-
200 regression was conducted by screening individual variables using univariate meta-regression.
201 Significant variables from the univariate meta-regression were subsequently combined in a
202 multivariate meta-regression analysis to determine how much of these variations could be
203 explained by the study factors combination. The meta-regression was conducted using a
204 random-effects model which has the underlying assumption that the distribution of effects
205 exists, resulting in heterogeneity among study results (Borenstein, et al., 2009; Lean, et al.,
206 2009). Heterogeneity across studies were assessed using the chi-squared (Q) test and the I^2
207 statistics (Borenstein, et al., 2009; Lean, et al., 2009). Significant variation across studies were
208 detected at $I^2 > 50\%$.

209 To control for variation across studies, the studies were stratified into different
210 production phase (seawater *vs.* freshwater). Further subgroup meta-analysis was conducted
211 with feed type (SBM with *vs.* without SCI) within each production phase to control for possible
212 effect of feed type on study variations. Additional subgroup meta-analysis was conducted using

213 study factors such as SBM inclusion level (< 20%, 20%, and > 20%), year of study (< 2014 *vs.*
214 ≥ 2014) and rearing water temperature (≤ 10 ºC *vs.* > 10 ºC) to evaluate the impact of these
215 factors on the heterogeneity observed across the studies in fish reared in seawater. There were
216 not enough datapoints to conduct similar analysis with SBM inclusion level, year of study and
217 water temperature in studies conducted in freshwater. The effect size for the analyzed variables
218 was determined as LOR for the meta-regression and odds ratio (OR) for the sub-group meta-
219 analysis at 95% level of confidence intervals. The significance of effect size was set at $P \leq$
220 0.05. The LOR and OR refers to the ratio of the probability that a particular event will occur to
221 the probability that it will not occur and can assume any number from zero to infinity. In this
222 study, publication bias was not examined due to presence of substantial heterogeneity with all
223 outcomes, which may lead to false-positive claims for publication bias (Ioannidis and
224 Trikalinos, 2007).

225 To determine the impact of enteritis on fish growth performance, linear and quadratic
226 regression analyses were performed between LOR and fish production parameters, such as
227 SGR, final weight and TGC of fish using the Excel 365 Software package (Microsoft Excel
228 version 2102, Microsoft Corp., Redmond, WA). Unlike random-effect model used for the
229 meta-regression analysis, the regression analyses between the fish production data and LOR
230 were non-weighted and assumed equal variance across studies.

231

232 **3.0 Results**

233 ***3.1 Study characteristics***

234 The characteristic of the studies included in the meta-analysis is presented in Table S2. Data
235 from a total of 26 fish experiments (16 peer-reviewed studies and 10 unpublished data),
236 representing 96 study datapoints were included in the meta-analysis. Fifty-eight percent of the

237 studies were conducted in seawater, while the remaining were conducted in freshwater. The
238 studies were conducted over a 19-year period (2001-2020) and included histological
239 assessment data from a total of 1486 fish. Dietary inclusion of SBM ranged from 5-46%, with
240 20% inclusion level most commonly used. A vast majority of the study (60%) used solvent
241 extracted SBM, whereas other SBM types such as HiPro SBM, full-fat SBM, genetically
242 modified (GM) SBM, biotechnologically-processed SBM and triple-null SBM were also used.

243 ***3.2 Factors associated with the severity of enteritis***

244 Consistent for the four histological variables, the results of the meta-regression analysis showed
245 that the severity of enteritis in Atlantic salmon fed SBM-based diet was significantly ($P < 0.05$)
246 associated with the fish production phase, feed type, SBM inclusion level, year of study and
247 water temperature, but not exposure time (Table 1). The R^2 values (0.19 – 0.50) of the
248 univariate analysis showed that water temperature had the highest effect on severity of enteritis
249 for all the four histological variables (Table 1). The multivariate meta-regression analysis
250 showed that ($P < 0.05$) production phase, feed type, SBM inclusion level, year of study and
251 water temperature in combination explained 44%, 24%, 70% and 54% of the variations
252 observed in enteritis associated with reduction in MFH, loss of SNV, infiltration of LP and SC,
253 respectively (Table S3).

254 ***3.3 Effect of fish production phase on severity of enteritis***

255 For the four histological analyses, the sub-group analysis showed that fish fed SBM-based diets
256 in seawater (ORs = 33 to 50) were more sensitive ($P < 0.05$) to develop enteritis, compared
257 with fish reared in freshwater (ORs = 4.3 to 18.7) (Table 2). Regardless of the production phase,
258 the results also showed that loss of SNV (ORs of 50 in seawater and 18.7 in freshwater) was
259 the most sensitive marker for evaluating SBM-induced enteritis, compared with reduction in
260 MFH, infiltration of LP and SC (Table 2). The significant of Q-statistics and I^2 values (33 –

80.5%) showed there was significant heterogeneity across studies on the effect of production phase on severity of enteritis in fish fed SBM-based diets in both seawater and freshwater (Table 2).

3.4 Effect of feed type on severity of enteritis

There was an inconsistent effect of dietary inclusion of SCI on severity of enteritis in Atlantic salmon fed SBM-based diets (Tables S4a & S4b). Based on the four histological variables, there were no significant effects ($P < 0.05$) of feeding SCI on severity of enteritis in fish fed SBM-based diets in seawater, except for SC (Table S4a). Based on SC, dietary inclusion of SCI reduced ($P < 0.05$) the severity of enteritis (from OR of 79 to 22) in fish fed SBM-based diets in seawater (Table S4a). Similarly, based on reduction in MFH and loss of SNV, inclusion of SCI decreased the severity of enteritis in fish fed SBM-based diets in freshwater (Table S4b). The values of 95% confidence intervals, Q-statistic and I^2 showed large variability among studies on the effect of feed type on severity of enteritis in fish fed SBM-based diets (Table S4a & S4b).

3.5 Effect of SBM inclusion level on severity of enteritis

Based on the four histological variables, there were no definite trends on the impact of SBM inclusion level on the severity of enteritis in fish fed SBM-based diets in seawater (Table S5). It was consistently shown that increasing the level of SBM ($< 20\%$, 20% or $> 20\%$) in the diets did not necessarily increase the severity of enteritis (Table S5). The 95% confidence intervals and I^2 statistic showed substantial variation across studies combined to evaluate the effect of SBM inclusion level on severity of enteritis in fish fed SBM-based diets in seawater.

3.6 Effect of year of study on severity of enteritis

Subgroup analysis based on year of study showed that the severity of enteritis was higher ($P < 0.05$) in fish fed SBM-based diets before 2014, compared with study conducted after 2014

285 (Table 3). These results were consistent for changes associated with reduction of MFH (ORs
286 of 54.3 before and 17.7 after 2014), infiltration of LP (ORs of 82.4 before and 10.2 after 2014)
287 and submucosal cellularity (ORs of 99.3 before and 9.9 after 2014), but not for the loss of SNV
288 (Table 3). There was no significant difference ($P > 0.05$) in severity of enteritis associated with
289 loss of SNV in studies conducted before (OR = 54.1) and after (OR = 43.7) 2014.

290 ***3.7 Effect of water temperature on severity of enteritis***

291 The results of the sub-group analysis revealed that the severity of enteritis in fish fed SBM-
292 based diets in seawater decreased ($P < 0.05$) with increasing water temperature (i.e., from < 10
293 ºC to > 10 ºC) (Table 4). These were consistent for changes determined for all four histological
294 variables (Table 4). By increasing water temperature from < 10 ºC to > 10 ºC, the ORs
295 decreased from 45.4 to 19.2, 54.8 to 33.0, 70.4 to 14.0 and 69.0 to 17.3 based on reduction in
296 MFH, loss of SNV, infiltration of LP and SC; respectively (Table 4).

297 ***3.8 Relationships between enteritis and fish growth performance***

298 Based on the four histological variables, there were both negative linear and quadratic
299 relationships ($P < 0.05$) in the full dataset between LOR and SGR of fish fed SBM-based diets
300 (Table 5). The SGR of fish decreased with increased severity of enteritis (higher LOR) in fish
301 fed SBM-based diets (Table 5). In the freshwater dataset, there were no linear relationships (P
302 > 0.05) between LOR and SGR of fish for all the histological variables, except for SC (Table
303 5). Conversely, there was negative quadratic relationships ($P < 0.05$) between LOR and SGR
304 of fish fed SBM-based diets based on reduction in MFH, loss of SNV, and SC. Based on loss
305 of SNV, there were both negative linear and quadratic relationships ($P < 0.05$) in the seawater
306 dataset between LOR and SGR of fish fed SBM-based diets.

307 None of the histological variables showed significant linear or quadratic relationships
308 ($P > 0.05$), neither in the full dataset nor the sub-dataset, between LOR and final weight of fish

309 fed SBM-based diets (Table S6). Similarly, based on the four histological variables, there were
310 no linear or quadratic relationships ($P > 0.05$) in the full dataset between LOR and TGC of fish
311 fed SBM-based diets (Table S7). However, in the freshwater dataset, there were negative
312 quadratic relationships ($P < 0.05$) between LOR and TGC of fish based on reduction in MFH,
313 loss of SNV and SC (Table S7). In the seawater dataset, there were no quadratic relationships
314 ($P > 0.05$) between LOR or TGC of fish for the histological variables, except for infiltration of
315 LP (Table S7).

316

317 **4.0 Discussion**

318 To our knowledge, the current study was the first to apply meta-analytic approach to semi-
319 quantitative histological (ordinal data) data in aquaculture. It was also the first study to
320 quantitatively determine various factors associated with the severity of enteritis in Atlantic
321 salmon fed SBM-based diets. In the current study, the four histological variables gave
322 consistent results based on meta-regression analysis. The results of the meta-regression
323 analysis consistently showed that the fish production phase, feed type, inclusion level of SBM,
324 year of study and water temperature were significantly associated with the severity of enteritis
325 in Atlantic salmon. The univariate meta-regression revealed a negative coefficient for SBM
326 inclusion levels, suggesting the higher the SBM level, the less the severity of enteritis. It is
327 likely that SBM inclusion level was confounded by other variables in the dataset. This could
328 be explained by the reduced sensitivity of fish to SBM-induced enteritis over the years. To
329 induce moderate to severe enteritis and uniformity in the sample group, the authors of the
330 studies in the meta-analysis intentionally increased the level of SBM in the diet from 20%
331 which was considered the most common dietary challenge before 2014 to 30 and 40%. Even
332 at these high inclusion levels only mild to moderate enteritis with large individual variation

333 among fish was observed e.g., Agboola, et al. (2021); Hansen, et al. (2019); Reveco-Urzua, et
334 al. (2019). The second reason for the observation may be linked to the variation in level of
335 ANFs present in different commercial sources of SBM used in the studies combined for the
336 meta-analysis. Different commercial sources of SBM contain different levels of ANFs
337 (especially saponin), which does not necessarily correlate with SBM inclusion level in the diets.
338 Thus, it is possible that the true effect of SBM inclusion level was masked by the level of ANFs
339 in the diets and thus responsible for the negative coefficient observed with the univariate meta-
340 regression. However, data on content of ANFs in the experimental diets used in the study
341 combined were lacking, and thus we could not use level of ANFs in the diets as a covariate for
342 the meta-regression analysis. The meta-regression analysis consistently showed that exposure
343 time (from 20 – 224 days) had no association with the severity of enteritis in Atlantic salmon.
344 This was in line with the results of previous studies where it had been documented that the
345 development of full enteritis in Atlantic salmon occurs after 5-7 days of feeding diet containing
346 20-33% SBM (Baeverfjord and Krogdahl, 1996; Marjara, et al., 2012; Urán, et al., 2008b;
347 Urán, et al., 2009b). Therefore, fish developed full enteritis after few days of feeding,
348 suggesting that there was no time related effect after 5-7 days of feeding SBM-based diet.

349 Sub-group analysis of studies based on production phase consistently showed for all the
350 histological variables, that fish reared in seawater were more prone to develop enteritis than
351 fish reared in freshwater. This finding was consistent with results of several studies conducted
352 over the years. Typical intestinal changes associated with SBM-induced enteritis have been
353 well documented in seawater-adapted Atlantic salmon (Baeverfjord and Krogdahl, 1996; Urán,
354 et al., 2008a; Urán, et al., 2008b; Van den Ingh, et al., 1991), but mild inflammatory responses
355 have been reported in fish reared in freshwater (Agboola, et al., 2021; Hansen, et al., 2019;
356 Sahlmann, et al., 2015; Sanden, et al., 2005). The enteritis has been linked to a T-cell-mediated
357 hypersensitivity in seawater-adapted salmon (Bakke-McKellep, et al., 2007; Marjara, et al.,

358 2012). However, in a study investigating the effect of SBM from start feeding on ontogeny of
359 the digestive system of Atlantic salmon, Sahlmann, et al. (2015) suggested that the Atlantic
360 salmon juveniles' under-developed adaptive immune system may not have been equipped to
361 provoke inflammatory responses, compared to post-smolt fish. This corroborated the findings
362 of an earlier study that showed increased cell proliferation, but no inflammatory response in
363 fish fed diet containing 12.5% SBM in freshwater (Sanden, et al., 2005). In this, meta-analysis,
364 the disparity in the maturation of the fish adaptive immune system, and their ability to mount
365 inflammatory responses, may be responsible for the variation in the severity of enteritis
366 between the seawater and freshwater phase. In the current study, loss of SNV showed the
367 highest sensitivity among the typical variables of SBM-induced enteritis. This supported the
368 results of previous findings which showed shrinkage of SNV (earliest noticeable signs after 3
369 days) in Atlantic salmon fed diets containing 20% SBM (Baeverfjord and Krogdahl, 1996;
370 Urán, et al., 2009b).

371 Other factors which may account for the observed disparity between the production
372 phases are differences in water salinity, fish size, fish age, fish developmental stage,
373 smoltification, drinking rate of fish and composition of the basal diets. During smoltification,
374 Atlantic salmon maintain osmoregulation by ingesting water to compensate for water lost to
375 the hyperosmotic environment. During this process, the osmoregulatory function of the gut
376 increases active transport of ions across the intestinal mucosa (Usher, et al., 1990). Active
377 transport of ions increases the gut permeability (Hu, et al., 2016), and possibly facilitates
378 transfer of enteritis-inducing components of SBM into the underlying mucosa (Knudsen, et al.,
379 2007; Knudsen, et al., 2008; Kortner, et al., 2012). Thereby, the inflammatory response of fish
380 fed SBM in seawater may be aggravated compared to the situation in freshwater. As possible
381 impacts of water salinity, fish size, fish age, and feed composition on severity of SBM-induced

382 enteritis were not tested in the current meta-analysis (due to lack of/insufficient number of
383 datapoints), further studies elucidating the role of these factors are warranted.

384 Further subset analysis based on feed type showed that there was inconsistency
385 regarding the ameliorating effect of single cell ingredient (SCI) on severity of enteritis between
386 the two production phases and among the four histological variables. Dietary inclusion of SCI
387 reduced the severity of enteritis as indicated by SC in fish raised in seawater, whereas MFH,
388 loss of SNV and infiltration of LP did not show similar relationship. In freshwater reared fish,
389 inclusion of SCI in fish diets reduced the severity of enteritis based on loss of SNV, but not on
390 the other histology markers. The large variability observed on the impact of SCI in reducing
391 the severity of enteritis could be linked to types of SCI, strain of SCI, bioactive components
392 present in the SCI, batch-to-batch variation in the composition of SCI, inclusion level of SCI
393 in the fish diet, and processing methods used after SCI harvest (Agboola, et al., 2021;
394 Grammes, et al., 2013; Hansen, et al., 2019). The SCI used in the studies included in this meta-
395 analysis were bacteria meal (Romarheim, et al., 2013), yeasts (Agboola, et al., 2021; Grammes,
396 et al., 2013; Hansen, et al., 2019; Reveco-Urzua, et al., 2019) and microalgae (Grammes, et al.,
397 2013). These differ in content and physio-chemical properties of functional components.
398 However, there are insufficient number of studies in this meta-analysis for further study
399 stratification to account for the differences among the SCI.

400 Additional subgroup analysis based on SBM inclusion level showed that increasing
401 dietary inclusion of SBM (from 8 to 46% in the diets) did not necessarily increase the severity
402 of enteritis. This observation was consistent for the four histological variables. This observation
403 is in contrast to the findings of previous studies, which reported dose-dependent increase in the
404 severity of enteritis in fish fed diet containing increasing level of SBM (Krogdahl, et al., 2003;
405 Urán, et al., 2009b). Several factors such as sources of SBM, batch-to-batch variation,
406 processing method used and level of ANFs remaining in the SBM products, could influence

407 the severity of enteritis in Atlantic salmon (Urán, et al., 2009a). The SBM of studies combined
408 in this meta-analysis differed based on the aforementioned factors. Therefore, the discrepancy
409 in our results and that of the previous studies (Krogdahl, et al., 2003; Urán, et al., 2009b),
410 implies that other factors than SBM inclusion level may have a dominating effect on severity
411 of enteritis. Thus, to study the impact of SBM inclusion level on severity of enteritis, source,
412 batch, processing, and level of ANFs in SBM must be considered. The findings of the current
413 meta-analysis showed that SBM-induced enteritis occur at dietary SBM inclusion level of <
414 20% (average SBM inclusion level of 14%), which correspond with the previous studies
415 (Krogdahl, et al., 2003; Urán, et al., 2009b).

416 Partitioning the study based on year of study showed that the severity of enteritis using
417 the four histological variables, except for loss of SNV consistently declined over the years. The
418 severity of enteritis was higher in fish fed SBM-based diets before year 2014, compared with
419 studies conducted after 2014. The reduction in severity of enteritis over the years could be
420 attributed to a number of factors such as increased tolerance of fish to SBM, the physico-
421 chemical changes in SBM (such as breeding to reduce/eliminate ANFs), as well as improved
422 feed formulation and processing in recent years. The tolerance of fish over the years could be
423 the result of breeding and genetic selection of fish for improved growth performance and
424 adaptability to plant-based diets. Previous studies have reported increased nutrient utilization,
425 improved growth rate, and no signs of enteritis in strain of rainbow trout selected on a diet
426 containing SBM, compared to non-selected strain (Abernathy, et al., 2017; Callet, et al., 2017;
427 Overturf, et al., 2013; Venold, et al., 2012). However, similar studies in Atlantic salmon are
428 scarce in scientific literature. Additionally, several factors such as genetics, breeding to
429 reduce/eliminate ANFs, cultivation conditions, harvesting, processing and storage, may have
430 considerable effect on nutritional, physical and chemical properties of SBM. These factors are
431 constantly changing and might have altered the potency of SBM to induce enteritis in Atlantic

432 salmon. In recent years, there has been substantial improvement in the area of fish feed
433 formulation and processing such as, the addition of premixes with claimed health beneficial
434 components that may indirectly influence the response of fish to dietary SBM in recent years.
435 Our results showed that there was no impact of year of study on severity of enteritis based on
436 loss of SNV, implying that this parameter could still be used as an important marker in future
437 studies to document the response of Atlantic salmon to dietary SBM and/or other plant
438 ingredients as well as to evaluate the impact of functional feed on enteritis. Also, it supported
439 earlier finding in this paper regarding the high sensitivity of this marker to enteritis, compared
440 with the other variables. This finding calls for investigation of the role of the SNV in the distal
441 intestine.

442 Subset analysis based on water temperature (≤ 10 ºC or > 10 ºC) showed that the
443 severity of enteritis decreased with increasing water temperature. This observation was similar
444 for all the histological variables. However, this was not expected, and it was contrary to the
445 findings of previous studies, which showed increased severity in enteritis with increasing water
446 temperature (Chikwati, et al., 2013; Urán, et al., 2008b). Temperature drive feed intake in fish,
447 thus, we expected that higher feed intake at higher water temperature would increase the
448 amount of enteritis-inducing components of SBM exposed to the intestinal mucosa, thereby
449 increasing the severity of enteritis. However, possible explanation for our findings could be
450 attributed to the short period of no/low feed intake (possibly starvation) experienced by fish
451 immediately after seawater transfer. Fish undergo various physiological changes after seawater
452 transfer, as a consequent they can go through a period of starvation or depressed feed intake
453 (Usher, et al., 1991). Fish reared at low water temperature are possibly more prone to this
454 starvation, which may trigger inflammatory responses that can be responsible for the increased
455 severity of enteritis observed in fish in the present meta-analysis. This position is supported by
456 the results of a previous trial, which demonstrated that fish can develop inflammatory response

457 that resembles typical SBM-induced enteritis after 7 days of starvation (Baeverfjord and
458 Krogdahl, 1996). Another possible reason is that water temperature was confounded by year
459 of study in this meta-analysis. Majority of the study with water temperature of < 10 ºC were
460 conducted before 2014, coinciding with the period of high sensitivity of fish to dietary SBM
461 (already shown in this study). Confounding can complicate interpretation of a meta-analysis
462 and can lead to misleading conclusions (Higgins, et al., 2019). For this reason, the impact of
463 water temperature on severity of enteritis reported in this study should be interpreted with
464 caution.

465 The results of the linear and quadratic regression revealed negative relationship
466 between LOR and fish performance indices such as SGR and TGC, indicating lower fish
467 performance with increased severity of enteritis. However, this observation depends on the fish
468 production phase and the histological variables considered for the regression analysis.
469 Compared with freshwater, there were no clear relationship between severity of enteritis and
470 growth performance of fish fed SBM-based diets in seawater. The discrepancy between the
471 production phases may be linked to low feed intake and short exposure time in seawater-
472 adapted fish. The feed intake and exposure time in seawater reared fish are possibly limiting
473 the apparent effect of enteritis on growth performance of fish.

474

475 **5.0 Conclusions**

476 Based on the findings of studies included in this meta-analysis, the severity of enteritis in
477 Atlantic salmon fed SBM-based diets was associated with fish production phase, feed type,
478 SBM inclusion level, year of study and water temperature, but not the exposure time. Further
479 regression analysis showed that increased severity of enteritis reduced SGR and TGC of fish

480 fed SBM-based diets. However, this effect was more apparent in fish reared in freshwater,
481 compared with seawater.

482

483 **6.0 Limitations**

484 The inclusion of unpublished data is a recurring debate when it comes to systemic review with
485 meta-analysis. Experts seem to be divided on whether unpublished data should be included in
486 a meta-analysis. A group of experts believed that studies derived from both published and
487 unpublished studies can be used in a meta-analysis (Higgins, et al., 2019; Kelley and Kelley,
488 2019; Liberati, et al., 2009). Their position is based on the principle that publication bias is a
489 major threat to the validity of systematic reviews, and obtaining and including data from
490 unpublished trials appears to be one obvious way of avoiding this problem (Kelley and Kelley,
491 2019; Lean, et al., 2009). There is convincing evidence that results that are statistically non-
492 significant and unfavourable to the experimental intervention are less likely to be published
493 than statistically significant results, and hence are less easily identified by the systemic review
494 (Higgins, et al., 2019). Van Driel, et al. (2009) concluded that the difficulty in retrieving
495 unpublished work could lead to selection bias. The group of authors that argued against the
496 inclusion of unpublished data in a meta-analysis based their position on the following reasons.
497 Firstly, the inclusion of unpublished data can itself introduce bias in that the unpublished
498 studies may be an unrepresentative subset of the unpublished studies in existence. However, it
499 has been stated that this bias is of less concern than the bias introduced by excluding all
500 unpublished studies, based on what is known about the impact of report bias (Higgins, et al.,
501 2019). Secondly, a major concern regarding the inclusion of unpublished studies is the
502 assumption that their methodological qualities are poorer than those already subjected to peer-
503 review process. This is not the case for the unpublished data included in our meta-analysis. We

504 can ascertain that the experimental procedure used in all the unpublished studies are of high-
505 quality standard. Majority of this studies are bound by confidentiality agreement with the
506 financing companies, therefore, they remained unpublished.

507 To understand the possible effect of literature source (published or unpublished) on the
508 studies included in the meta-analysis, we conducted a univariate meta-regression using
509 literature source (published *vs*. unpublished) as the co-variate. Based on MFH and SNV, the
510 results showed that literature source did not associate with the severity of enteritis (Table S8).
511 Conversely, based on LP and SC, the source of literature affected the severity of enteritis (Table
512 S8). However, the R^2 revealed that the literature source contributes between 9-10% of the
513 variation observed with these variables. Additionally, the Q and I^2 statistics showed other
514 factors rather than literature source are responsible for this variation. Based on this meta-
515 regression analysis, we recommend that the results on LP and SC in the current paper should
516 be interpreted with caution.

517 Certain decisions could influence the outcome of a meta-analysis. While some of these
518 decisions are clearly objective, some are very contentious and arbitrary (Higgins, et al., 2019).
519 In the current meta-analysis, certain decisions were made during the data standardization,
520 which may or may not influence the observed results. For example, data standardization in this
521 meta-analysis assumed that each score categories are similar between the studies. The
522 standardization was done to avoid bias of choosing inappropriate cut-off points between the
523 score categories. It was the reason for grouping the data into three, rather than two, which can
524 be directly meta-analyzed. However, it is uncertain whether all the cut-off points were captured
525 or whether there are some slightly missing cut-offs that were not addressed in this study.
526 Sensitivity analysis could be used to determine if the findings of a meta-analysis are not
527 dependent on such decision. Sensitivity analysis was not conducted in this meta-analysis
528 because there was no baseline study with established cut-off points to be used in the analysis.

529 The conversion of a continuous variable to nominal/categorical variable is always
530 questionable because it is assumed that in the absence of defensible cut-off point, such
531 conversion may lead to omitting meaningful information that can result in over-estimation or
532 under-estimation of intervention effect. However, continuous variable can be converted into
533 categorical variable for sub-group analysis when it is conducted as a complementary analysis
534 to a meta-regression (Higgins, et al., 2019), like we did in this meta-analysis. However, such
535 approach also has its limitation. Sub-group analyses are considered to be exploratory and their
536 findings may need to be tested in original studies (Kelley and Kelley, 2019). Additionally, the
537 conversion of continuous variable into categorical variable for sub-group analysis is prone to
538 false-positive results, which may led to misleading conclusion (Higgins, et al., 2019).
539 Therefore, the results of the sub-group analysis conducted with continuous variables (SBM
540 inclusion, water temperature and year of study) in the current study should be considered
541 exploratory and the findings need to be further substantiated in original studies. In addition, the
542 justification for partitioning the variables into different categories was based on the scatter plots
543 obtained after the meta-regression analysis (Fig. S1). Partitioning the studies based on these
544 graphs has its limitation, but that was the best possible approach we could think of when
545 conducting the meta-analysis. For instance, our idea of stratifying the study based on year of
546 study was to understand if the increase tolerance of fish is related to genetic strain of Atlantic
547 salmon developed over the years. Information on strain of fish used are generally lacking in
548 literature, and as such the best we could do was to stratify the studies based on year and develop
549 speculation based on the results. This additional limitation strengthens why the results of the
550 sub-group analysis with continuous variables should be considered exploratory. In addition, it
551 should be noted that unlike the meta-regression analysis, the linear and quadratic analyses
552 between fish production parameters and the LOR assumed equal variance across studies and

553 did not correct for heteroscedasticity, as such, the obtained coefficients with the meta-
554 regression may not be comparable with the regression analysis performed in Excel.

555

556

557 **Credit authorship contribution statement**

558 **Jeleel Opeyemi Agboola**: Conceptualization, Methodology, Formal analysis, Investigation,
559 Data curation, Writing – Original draft, Writing – Review & Editing, Visualization. **Elvis M.**
560 **Chikwati:** Methodology, Investigation, Resources, Writing – Review & Editing. **Jon Øvrum**
561 **Hansen:** Methodology, Resources, Data curation, Writing – Review & Editing, Supervision.
562 **Trond M. Kortner:** Methodology, Investigation, Resources, Writing – Review & Editing.
563 **Åshild Krogdahl:** Resources, Writing – Review & Editing. **Liv Torunn Mydland:**
564 Methodology, Resources, Data curation, Writing – Review & Editing, Supervision. **Brankica**
565 **Djordjevic:** Methodology, Resources, Writing – Review & Editing, Supervision. **Johan W.**
566 **Schrama:** Methodology, Resources, Writing – Review & Editing, Supervision. **Margareth**
567 **Øverland:** Methodology, Resources, Data curation, Writing – Review & Editing, Supervision,
568 Project administration, Funding acquisition.

569

570 **Declaration of Competing interest**

571 The authors declared no competing interest.

572

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792 **FIGURE CAPTION**
793 Fig. 1. A flow diagram showing the search strategy and literature selection for the meta-analysis.
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827 **TABLES**
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831 **Table 1**
832 Univariate meta-regression to determine possible study factors associated with the severity of enteritis in fish fed soybean meal
833 (SBM)-based diets. Log odds ratio is the dependent variable (effect size) of the equation generated with this meta-regression.

Study factors	Intercept	Coefficients	95% CI		R^2	Number of studies	Q	I^2 (%)	*P*-value
			Lower	Upper					
Reduction in mucosal fold height									
Production phase: seawater	1.99	1.52	0.80	2.24	0.25	92	238.6	61.9	< 0.0001
Feed type: with SCI[1]	3.34	-0.94	-1.69	-0.19	0.09	92	238.6	61.9	0.014
SBM Inclusion level (%)	4.42	-0.06	-0.10	-0.02	0.22	92	238.6	61.9	0.0013
Year of study	250.8	-0.12	-0.19	-0.06	0.22	92	238.6	61.9	0.0001
Temperature (ºC)	6.71	-0.33	-0.46	-0.20	0.42	82	201.5	59.8	< 0.0001
Exposure time (day)	3.19	-0.01	-0.02	0.01	0.00	87	225.2	61.8	0.305
Loss of supranuclear vacuolization									
Production phase: seawater	2.90	1.05	0.13	1.96	0.11	94	343.7	72.9	0.025
Feed type: with SCI	3.95	-0.94	-1.87	-0.02	0.06	94	343.7	72.9	0.044
SBM Inclusion level (%)	5.16	-0.07	-0.12	-0.02	0.15	94	343.7	72.9	0.007
Year of study	103.8	-0.05	-0.13	0.03	0.03	94	343.7	72.9	0.214
Temperature (ºC)	6.68	-0.28	-0.45	-0.11	0.19	84	289.0	71.3	0.001
Exposure time (day)	3.66	-0.005	-0.02	0.01	0.0	89	311.06	71.7	0.531
Infiltration of lamina propria									
Production phase: seawater	1.44	2.16	1.46	2.86	0.43	85	235.2	64.3	<0.0001
Feed type: with SCI	3.10	-0.67	-1.48	0.14	0.02	85	235.2	64.3	0.104
SBM Inclusion level (%)	4.14	-0.06	-0.10	-0.01	0.08	85	235.2	64.3	0.010
Year of study	365.87	-0.18	-0.24	-0.13	0.46	85	235.2	64.3	<0.0001
Temperature (ºC)	7.47	-0.40	-0.53	-0.28	0.50	77	199.5	61.9	<0.0001
Exposure time (day)	3.23	-0.01	-0.02	0.004	0.01	82	226.3	64.2	0.188
Submucosal cellularity									
Production phase: seawater	1.97	1.78	0.96	2.60	0.20	85	226.9	63.0	<0.0001
Feed type: with SCI	3.59	-1.15	-1.99	-0.32	0.11	85	226.9	63.0	0.007
SBM Inclusion level (%)	4.65	-0.07	-0.12	-0.02	0.09	85	226.9	63.0	0.007
Year of study	344.78	-0.17	-0.25	-0.11	0.34	85	226.9	63.0	<0.0001
Temperature (ºC)	7.49	-0.39	-0.54	-0.24	0.36	77	202.3	62.4	<0.0001
Exposure time (day)	3.49	-0.01	-0.02	0.004	0.01	82	226.3	64.2	0.170

834 [1]SCI – single cell ingredient.
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843 **Table 2**
844 The effect of production phase on the severity of enteritis in Atlantic salmon fed soybean meal (SBM)-based diets

Group/ Sub-Group[1]	Number of comparisons	Effect Size Estimates			Heterogeneity tests		
		Odds ratio[2]	95% CI	*P*-value[3]	Q	*P*-value[4]	I^2 (%)
Reduction in mucosal fold height							
Seawater	56	33.4[a]	21.02 – 52.9	***	110.2	***	50.1
Freshwater	36	7.4[b]	4.2 – 12.9	***	89.7	***	61.0
Loss of supranuclear vacuolization							
Seawater	56	50.0[a]	30.8 – 81.3	***	123.6	***	55.5
Freshwater	38	18.7[b]	8.1 – 43.1	***	189.3	***	80.5
Infiltration of lamina propria							
Seawater	51	39.8[a]	24.0 – 65.9	***	117.6	***	57.5
Freshwater	34	4.3[b]	2.8 – 6.7	***	49.9	*	33.8
Submucosal cellularity							
Seawater	52	43.7[a]	24.7 – 77.5	***	134.4	***	62.1
Freshwater	33	7.2[b]	4.2 – 12.4	***	61.0	***	47.6

845 [1]Subgroups analysis with production phase (seawater *vs.* freshwater).
846 [2]Odds ratio within the same subgroup, but with different superscript ([a, b]) are significantly different ($P < 0.05$).
847 [3]P-values of analysis comparing the odds ratio of the SBM treatments with the neutral reference group (Odds ratio = 1).
848 Asterisks denote level of significance (NS: not significant, *p < 0.05, **p < 0.01, ***p < 0.001).
849 [4]P-values of chi-square (Q) statistic used to detect heterogeneity across the studies. Significance level of the heterogeneity was
850 denoted with asterisks (NS: not significant, *p < 0.05, **p < 0.01, ***p < 0.001).
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866 **Table 3**

867 The effect of year of study on the severity of enteritis in Atlantic salmon fed soybean meal (SBM)-based diets in seawater

Group/	Number of	Effect Size Estimates			Heterogeneity tests		
Sub-Group[1]	comparisons	Odds ratio[2]	95% CI	*P*-value[3]	Q	*P*-value[4]	I^2 (%)
Reduction in mucosal fold height							
Before 2014	34	54.3[a]	28.6 – 103.0	***	67.9	***	51.5
After 2014	22	17.7[b]	9.7 – 32.3.	***	33.5	*	37.2
Loss of supranuclear vacuolization							
Before 2014	34	54.1	29.9 – 97.7	***	62.9	***	47.5
After 2014	22	43.7	18.9 – 101.1	***	60.5	***	65.3
Infiltration of lamina propria							
Before 2014	34	82.4[a]	48.3 – 140.8	***	51.0	*	35.3
After 2014	17	10.2[b]	5.2 – 19.9	***	28.2	*	43.2
Submucosal cellularity							
Before 2014	34	99.3[a]	51.1 – 193.0	***	68.9	***	52.1
After 2014	18	9.9[b]	5.0 – 19.6	***	27.0	NS	37.1

868 [1]Subgroups analysis with year of study (before or after 2014).
869 [2]Odds ratio within the same subgroup, but with different superscript ([a, b]) are significantly different ($P < 0.05$).
870 [3]P-values of analysis comparing the odds ratio of the SBM treatments with the neutral reference group (Odds ratio = 1).
871 Asterisks denote level of significance (NS: not significant, *p < 0.05, **p < 0.01, ***p < 0.001).
872 [4]P-values of chi-square (Q) statistic used to detect heterogeneity across the studies. Significance level of the heterogeneity was
873 denoted with asterisks (NS: not significant, *p < 0.05, **p < 0.01, ***p < 0.001).
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Table 4
The effect of water temperature on the severity of enteritis in Atlantic salmon fed soybean meal (SBM)-based diets in seawater

Group/ Sub-Group[1]	Number of comparisons	Effect Size Estimates			Heterogeneity tests		
		Odds ratio[2]	95% CI	*P*-value[3]	Q	*P*-value[4]	I² (%)
Reduction in mucosal fold height							
< 10 ºC	25	45.4[a]	25.4 – 81.2	***	32.9	NS	27.1
> 10 ºC	26	19.2[b]	9.8 – 37.6	***	52.8	***	52.7
Loss of supranuclear vacuolization							
< 10 ºC	25	54.8[a]	29.9 – 100.4	***	35.5	NS	32.4
> 10 ºC	26	33.0[b]	15.2 – 71.4	***	71.7	***	65.2
Infiltration of lamina propria							
< 10 ºC	25	70.4[a]	37.2 – 133.2	***	40.5	*	40.7
> 10 ºC	21	14.0[b]	7.3 – 27.0	***	38.4	**	48.0
Submucosal cellularity							
< 10 ºC	25	69.0[a]	37.2 – 127.8	***	34.0	NS	29.4
> 10 ºC	22	17.3[b]	7.0 – 42.4	***	62.7	***	66.5

907 [1]Subgroups analysis with water temperature (less than or greater than 10 ºC).
908 [2]Odds ratio within the same subgroup, but with different superscript ([a, b]) are significantly different (P < 0.05).
909 [3]P-values of analysis comparing the odds ratio of the SBM treatments with the neutral reference group (Odds ratio = 1).
910 Asterisks denote level of significance (NS: not significant, *p < 0.05, **p < 0.01, ***p < 0.001).
911 [4]P-values of chi-square (Q) statistic used to detect heterogeneity across the studies. Significance level of the heterogeneity was
912 denoted with asterisks (NS: not significant, *p < 0.05, **p < 0.01, ***p < 0.001).
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946 **Table 5**
947 Relationships (linear and quadratic) between the severity of enteritis and specific growth rate of fish fed SBM-
948 based diets[a]

Dataset	Regression type	Number of datapoints	Equation	P-value [b]	R^2 (adjusted)
Reduction in mucosal fold height					
Full	Linear	78	$Y = -0.062X + 1.9$	*	0.04
	Quadratic		$Y = -0.013X^2 - 0.184X + 2.5$	***	0.17
Freshwater	Linear	35	$Y = 0.026X + 2.9$	NS	-0.01
	Quadratic		$Y = -0.015X^2 - 0.161X + 3.4$	*	0.19
Seawater	Linear	43	$Y = -0.009X + 1.0$	NS	-0.02
	Quadratic		$Y = 0.036X^2 - 0.262X + 1.3$	***	0.25
Loss of supranuclear vacuolization					
Full	Linear	78	$Y = -0.119X + 2.2$	***	0.14
	Quadratic		$Y = -0.011X^2 - 0.177X + 2.6$	***	0.26
Freshwater	Linear	35	$Y = -0.04X + 2.9$	NS	0.01
	Quadratic		$Y = -0.015X^2 - 0.161X + 3.5$	***	0.40
Seawater	Linear	43	$Y = -0.045X + 1.1$	*	0.07
	Quadratic		$Y = 0.024X^2 - 0.240X + 1.4$	***	0.21
Infiltration of lamina propria					
Full	Linear	73	$Y = -0.089X + 2.1$	**	0.08
	Quadratic		$Y = 0.0006X^2 - 0.088X + 2.1$	*	0.07
Freshwater	Linear	35	$Y = -0.038X + 2.9$	NS	0.01
	Quadratic		$Y = -0.002X^2 - 0.041X + 3.0$	NS	0.001
Seawater	Linear	38	$Y = 0.072X + 0.6$	***	0.32
	Quadratic		$Y = 0.005X^2 + 0.37X + 0.6$	***	0.31
Submucosal cellularity					
Full	Linear	78	$Y = -0.085X + 1.9$	***	0.18
	Quadratic		$Y = -0.003X^2 - 0.115X + 2.0$	***	0.18
Freshwater	Linear	35	$Y = -0.072X + 2.8$	**	0.21
	Quadratic		$Y = -0.006X^2 - 0.173X + 3.1$	***	0.22
Seawater	Linear	43	$Y = -0.013X + 1.0$	NS	0.02
	Quadratic		$Y = 0.001X^2 - 0.006X + 0.9$	NS	0.004

949 [a] Y = specific growth rate and X = log-odds ratio.
950 [b] Asterisks denote level of significance (NS: not significant, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).
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1191 records identified from database:
478 ORIA
546 Scopus
167 Web of Science

5 additional records identified through other source: Google scholar

356 records after duplicates removal

356 records screened for titles and abstracts

310 records excluded with reasons:
- No histology scores reported
-Only morphometric scores such as length of mucosal folds reported
- Other plant protein ingredients used.
- Not Atlantic salmon
- Review paper
- Soybean meal subfractions such as saponins used instead of soybean meal

46 full-articles assessed for eligibility

31 full articles excluded with reasons:
- No dietary inclusion of soybean meal
- No neutral-reference diet used
- Only qualitative image of histology presented
- Semi-quantitative histological scores were presented in graphs
-Semi-quantitative histological scores were presented as means
- Unavailability of the raw-data file/actual number of fish in each histological ranking

16 articles + 10 unpublished studies included in the quantitative studies
(meta-analysis)
(n = 26)

Supplementary File

A meta-analysis to determine factors associated with the severity of enteritis in Atlantic salmon (*Salmo salar*) fed soybean meal-based diets

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Table S1
List of references excluded from the meta-analysis with reasons

Authors	Journals	Reasons for exclusion
Aslaksen, et al. (2007)	Aquaculture	Mean values of semi-quantitative data were presented
Baeverfjord and Krogdahl (1996)	Journal of Fish Diseases	Only histology (qualitative) images were presented, no semi-quantitative scoring; morphometry; mucosal fold height
Bakke-McKellep, et al. (2000)	Journal of Fish Diseases	Only histology (qualitative) images were presented, no semi-quantitative scoring
Bakke-McKellep, et al. (2006)	Journal of Fish Biology	Excluded due to cross-over experimental design
Bakke-McKellep, et al. (2007a)	Journal of Fish Diseases	Only histology (qualitative) images were presented, no semi-quantitative scoring
Bakke-McKellep, et al. (2007b)	British Journal of Nutrition	Mean values of semi-quantitative data were presented
Booman, et al. (2018)	Aquaculture	Mean/median values of semi-quantitative data were presented
Chikwati, et al. (2012)	British Journal of Nutrition	No fishmeal reference diet
Chikwati, et al. (2013a)	Cell and Tissue Research	Only histology (qualitative) images were presented, no semi-quantitative scoring
Chikwati, et al. (2013b)	Aquaculture	Only histology (qualitative) images were presented, no semi-quantitative scoring
Gu, et al. (2013)	British Journal of Nutrition	Only histology (qualitative) images were presented, no semi-quantitative scoring
Gu, et al. (2014)	PLoS One	Only histology (qualitative) images were presented, no semi-quantitative scoring
Gu, et al. (2015)	Aquaculture Nutrition	Mean values of semi-quantitative data were presented; soya saponins
Hu, et al. (2016)	PLoS One	Graphical presentation of the histological scores
Kiron, et al. (2020)	Frontiers in Immunology	Only histology (qualitative) images were presented, no semi-quantitative scoring
Knudsen, et al. (2007)	Journal of Agricultural and Food Chemistry	Mean/median values of semi-quantitative data were presented; soya saponins
Knudsen, et al. (2008)	British Journal of Nutrition	Mean values of semi-quantitative data were presented; soya saponins
Kortner, et al. (2011)	Aquaculture	Only histology (qualitative) images were presented, no semi-quantitative scoring
Kortner, et al. (2016)	BMC Veterinary Research	Graphical presentation of the histological scores
Krogdahl, et al. (2015)	Journal of Agricultural and Food Chemistry	Saponin, mean/values of semi-quantitative data were presented
Lilleeng, et al. (2007)	Comparative Biochemistry and Physiology	Only histology (qualitative) images were presented, no semi-quantitative scoring
Lilleeng, et al. (2009)	Fish and Shellfish Immunology	Only histology (qualitative) images were presented, no semi-quantitative scoring

Navarrete, et al. (2013)	Aquaculture Nutrition	Mean/median values of semi-quantitative data were presented
Nordrum, et al. (2000)	Comparative Biochemistry and Physiology	Only histology (qualitative) images were presented, no semi-quantitative scoring
Refstie, et al. (2000)	Aquaculture	Mean/median values of semi-quantitative data were presented
Refstie, et al. (2010)	Aquaculture	Mean/median values of semi-quantitative data were presented
Romarheim, et al. (2013)	British Journal of Nutrition	Mean/median values of semi-quantitative data were presented
Sahlmann, et al. (2013)	Fish and Shellfish Immunology	Only histology (qualitative) images were presented, no semi-quantitative scoring
Sahlmann, et al. (2015)	PLoS One	Only histology (qualitative) images were presented, no semi-quantitative scoring
Uran, et al. (2008)	Journal of Fish Biology	Mean/median values of semi-quantitative data were presented
Venold, et al. (2013)	Comparative Biochemistry and Physiology	Only histology (qualitative) images were presented, no semi-quantitative scoring

References excluded from the meta-analysis with reasons.

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Table S2

The description of studies included in the meta-analysis investigating the effect of soybean meal (SBM) on intestinal enteritis of Atlantic salmon

Reference	Year of study	SBM inclusion (%)	SBM types	Number of SBM treatment without and with single cell ingredients				Number of fish used for histological assessment	PP[1]	IFW[2] (g)	FFW[3] (g)	Temperature (º C)	Salinity (‰)	Exposure time (d)	Scoring system
				Total	without	with	Types[4]								
Krogdahl, et al. (2003)	2001	7.6, 11.7, 15.3, 19.4, 27	Solvent-extracted toasted SBM	5	5	0	No	6 fish each for 4 treatments, 10 for 1 treatment	SW	280	510	8.4	26	60	0 to 4
Refstie, et al. (2005)	2003	17.5	Solvent-extracted toasted SBM & Biotechnological processed SBM	3	3	0	No	15 fish per treatment	SW	187	453	NA	NA	68	0 to 4
Sanden, et al. (2005)	2002	12.5	non-GM & GM full fat SBM	2	2	0	No	9 fish per treatment	FW	0.21	108	12	2.1	224	0 to 4
Refstie, et al. (2006)	2003	25	Solvent extracted SBM	1	1	0	No	12 fish per treatment	SW	172	232	10	NA	21	0 to 4
Bakke-McKellep, et al. (2007)	2003	17.2	non-GM & GM full fat SBM	2	2	0	No	10 fish per treatment	SW	104	NA	11	33	84	0 to 4
(Uran, et al., 2008)	2005	20	Solvent-extracted HiPro	1	1	0	No	16 fish per treatment	SW	300	NA	8	34	20	1 to 5
Uran, et al. (2009)	2005	10, 20	Solvent-extracted HiPro	2	2	0	No	35 fish per treatment	SW	300	NA	12	34	56	1 to 5

Uran, et al. (2009a)	2005	20	Solvent extracted SBM	7	7	0	No	9 fish per treatment	SW	396	NA	12	34	28	1 to 5
Marjara, et al. (2012)	2007	20	Solvent extracted SBM	1	1	0	No	12 fish per treatment	SW	500	NA	10	NA	21	0 to 4
Grammes, et al. (2013)	2011	20	Solvent extracted SBM	5	1	4	SCI	15 fish per treatment	SW	107	142	10.3	32.5	28	0 to 2
Romarheim, et al. (2013)	2011	20	Solvent extracted SBM	7	1	6	SCI	15 fish per treatment	SW	107	140	7.5	34	28	0 to 2
Unpublished 4	2014	30	HiPro SBM	5	5	0	No	12 fish per treatment	SW	314	651	15	NA	48	0 to 10
Unpublished 5	2014	46	Solvent extracted SBM & high and low special soy	3	3	0	No	27 fish per treatment	FW	NA	NA	NA	NA	NA	0 to 10
Unpublished 6	2014	5.8	Solvent extracted SBM & HiPro SBM	2	2	0	No	47 and 45 fish in each treatment	FW	NA	NA	NA	NA	NA	1 to 10
Unpublished 10	2017	25	Full fat SBM & Triple null SBM	2	2	0	No	18 and 17 fish in each treatment	FW	40	115	14	NA	56	1 to 10
Unpublished 1	2017	20	Solvent extracted SBM	3	1	2	A	15 fish per treatment	FW	53	140	13.7	NA	56	0 to 4
Unpublished 2	2017	20	Solvent extracted SBM	2	1	1	A	15 and 14 fish in each treatment	SW	140	160	NA	35	47	0 to 4
Unpublished 3	2018	20	Solvent extracted SBM	5	1	4	A	15 fish per treatment	SW	107	145	11.5	35	49	0 to 4
Hansen, et al. (2019)	2017	40	Solvent extracted SBM	4	1	3	SCI	13 fish in 2 treatments; 14 in 1 treatment and 15 in 1 treatment	FW	4.4	14.8	15.2	NA	28	0 to 2

Reveco-Urzua, et al. (2019)	2017	20	Solvent extracted SBM	5	1	4	SCI	23 fish in 1 treatment and 8 fish in 4 treatments	SW	526	667	8	34.5	30	0 to 2
Unpublished 7	2018	25	Solvent extracted SBM	5	1	4	A	16 fish in 4 treatments and 28 fish in 1 treatment	FW	30	90	15	NA	52	0 to 4
Unpublished 8	2019	15, 25	Solvent extracted SBM	6	2	4	A	12 fish per treatment	FW	28	90	15	NA	50	0 to 4
Unpublished 9	2020	25	Solvent extracted SBM	1	1	0	No	23 fish per treatment	FW	38	85	15	NA	34	0 to 4
Krogdahl, et al. (2020)	2017	27	Full fat SBM & Triple null SBM	4	4	0	No	18 fish per treatment	FW	41	116	14	NA	56	1 to 10
Agboola, et al. (2021)	2019	40	Solvent extracted SBM	8	1	7	SCI	18 fish in 7 treatments and 17 fish in 1 treatment	FW	5	25	15	NA	37	1 to 5
Agboola, et al. (2022)	2020	30	Solvent extracted SBM	5	1	4	SCI	18 fish per treatment	SW	136	180	12	34	42	0 to 4

[1] PP – production phase (seawater (SW) and freshwater (FW).
[2] IFW – average initial fish weight.
[3] FFW – average final fish weight.
[4] SCI – single cell ingredients; A – other additives aside from SCI.
NA – not available.

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Unpublished studies 1, 2 and 3 – Professor Margareth Øverland's research group.
Unpublished studies 4, 5, 6, 7, 8, 9 and 10 – Professor Åshild Krogdahl's research group.

Table S3

Multivariate meta-regression to determine possible study factors associated with the severity of enteritis in fish fed SBM-based diets. Log odds ratio is the dependent variable (effect size) of the equation generated with this meta-regression

Study factors	Number of studies	Coefficients	95% CI Lower	95% CI Upper	P-values[1]	Overall Statistics R^2	Overall Statistics P-values[2]
Reduction in mucosal fold height							
Intercept	82	24.4	-161.7	210.5	0.797	0.44	< 0.001
Production phase: seawater	82	0.01	-1.2	1.2	0.989		
Feed type: with SCI[3]	82	-0.88	-1.8	0.002	0.050		
SBM Inclusion level (%)	82	0.006	-0.1	0.07	0.866		
Year of study	82	-0.008	-0.1	0.08	0.859		
Temperature (º C)	82	-0.32	-0.6	-0.08	0.009		
Loss of supranuclear vacuolization							
Intercept	84	-288.3	-527.3	-49.2	0.018	0.24	< 0.001
Production phase: seawater	84	0.1	-1.4	1.6	0.876		
Feed type: with SCI	84	-1.4	-2.6	-0.3	0.012		
SBM Inclusion level (%)	84	-0.03	-0.1	0.1	0.474		
Year of study	84	0.2	0.03	0.3	0.015		
Temperature (º C)	84	-0.4	-0.7	-0.1	0.008		
Infiltration of lamina propria							
Intercept	77	301.2	139.7	462.7	0.001	0.70	< 0.001
Production phase: seawater	77	-0.5	-1.8	0.8	0.454		
Feed type: with SCI	77	-0.004	-0.8	0.8	0.992		
SBM Inclusion level (%)	77	-0.01	-0.1	0.04	0.684		
Year of study	77	-0.2	-0.2	-0.1	0.001		
Temperature (º C)	77	-0.3	-0.5	-0.03	0.024		
Submucosal cellularity							
Intercept	77	216.6	18.5	414.8	0.032	0.54	< 0.001
Production phase: seawater	77	-1.0	-2.3	0.3	0.141		
Feed type: with SCI	77	-0.8	-1.7	0.2	0.107		
SBM Inclusion level (%)	77	-0.01	-0.1	0.1	0.698		
Year of study	77	-0.1	-0.2	-0.004	0.041		
Temperature (º C)	77	-0.4	-0.6	-0.1	0.008		

[1]P-values when individual variable are included in the model.
[2]Overall P-values of the meta-regression analysis.
[3]SCI – single cell ingredient.

Table S4a

The effect of feed type on the severity of enteritis in Atlantic salmon fed soybean meal (SBM)-based diets in seawater

Group/ Sub-Group[1]	Number of comparisons	Effect Size Estimates			Heterogeneity tests		
		Odds ratio[2]	95% CI	*P*-value[3]	Q	*P*-value[4]	I^2 (%)
Reduction in mucosal fold height							
SBM without SCI[5]	33	43.0	21.3 – 87.0	***	77.7	**	58.8
SBM with SCI	23	24.8	14.2 – 43.3	***	31.1	NS	29.3
Loss of supranuclear vacuolization							
SBM without SCI	33	46.2	23.3 – 91.7	***	78.7	***	59.3
SBM with SCI	23	55.6	28.0 – 110.2	***	44.5	***	50.6
Infiltration of lamina propria							
SBM without SCI	28	59.5	30.9 – 114.6	***	52.6	*	48.7
SBM with SCI	23	25.0	11.8 – 53.1	***	58.4	***	62.3
Submucosal cellularity							
SBM without SCI	29	78.8[a]	35.4 – 175.5	***	67.7	***	58.7
SBM with SCI	23	22.4[b]	10.6 – 47.5	***	53.6	***	59.0

[1]Subgroups analysis with feed type (SBM without SCI *vs* SBM with SCI).
[2]Odds ratio within the same subgroup, but with different superscript ([a, b]) are significantly different ($P < 0.05$).
[3]P-values of analysis comparing the odds ratio of the SBM treatments with the neutral reference group (Odds ratio = 1). Asterisks denote level of significance (NS: not significant, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).
[4]P-values of chi-square (Q) statistic used to detect heterogeneity across the studies. Significance level of the heterogeneity was denoted with asterisks (NS: not significant, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).
[5]SCI – single cell ingredients.

Table S4b
The effect of feed type on the severity of enteritis in Atlantic salmon fed soybean meal (SBM)-based diets in freshwater

Group/ Sub-Group[1]	Number of comparisons	Effect Size Estimates			Heterogeneity tests		
		Odds ratio[2]	95% CI	*P*-value[3]	Q	*P*-value[4]	I^2 (%)
Reduction in mucosal fold height							
SBM without SCI[5]	18	14.6[a]	5.6 – 38.1	***	54.9	***	69.0
SBM with SCI	18	3.8[b]	2.3 – 6.3	***	20.4	NS	16.7
Loss of supranuclear vacuolization							
SBM without SCI	19	66.3[a]	16.4 – 267.6	***	116.4	***	84.5
SBM with SCI	19	5.1[b]	2.5 – 10.4	***	36.1	***	50.1
Infiltration of lamina propria							
SBM without SCI	17	4.8	2.8 – 8.3	***	17.8	NS	10.0
SBM with SCI	17	4.0	2.0 – 8.0	***	31.6	**	49.3
Submucosal cellularity							
SBM without SCI	16	11.8	6.1 – 22.8	***	19.8	NS	24.3
SBM with SCI	17	4.7	2.1 – 10.5	***	35.9	**	55.5

[1]Subgroups analysis with feed type (SBM without SCI *vs* SBM with SCI).
[2]Odds ratio within the same subgroup, but with different superscript ([a, b]) are significantly different ($P < 0.05$).
[3]P-values of analysis comparing the odds ratio of the SBM treatments with the neutral reference group (Odds ratio = 1). Asterisks denote level of significance (NS: not significant, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).
[4]P-values of chi-square (Q) statistic used to detect heterogeneity across the studies. Significance level of the heterogeneity was denoted with asterisks (NS: not significant, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).
[5]SCI – single cell ingredients.

Table S5
The effect of soybean meal (SBM) inclusion level on the severity of enteritis in Atlantic salmon fed SBM-based diets in seawater

Group/ Sub-Group[1]	Number of comparisons	Effect Size Estimates			Heterogeneity tests		
		Odds ratio[2]	95% CI	*P*-value[3]	Q	*P*-value[4]	I² (%)
Reduction in mucosal fold height							
< 20% inclusion level	9	54.6	15.9 – 187.2	***	14.3	NS	44.0
20% inclusion level	34	26.6	17.4 – 50.5	***	59.8	**	44.9
> 20% inclusion level	13	32.7	9.6 – 112.7	***	34.7	**	65.5
Loss of supranuclear vacuolization							
< 20% inclusion level	9	52.9	14.4 – 195.0	***	16.03	*	50.1
20% inclusion level	34	40.0	25.2 – 62.7	***	46.0	NS	28.2
> 20% inclusion level	13	86.9	16.3 – 461.7	***	58.6	***	79.5
Infiltration of lamina propria							
< 20% inclusion level	9	59.9	21.0 – 170.7	***	10.4	NS	23.0
20% inclusion level	34	34.8	18.4 – 66.0	***	91.9	***	64.1
> 20% inclusion level	8	47.0	14.1 – 156.5	***	13.1	NS	46.5
Submucosal cellularity							
< 20% inclusion level	9	59.0	20.1 – 173.1	***	10.9	NS	26.9
20% inclusion level	33	47.9	22.5 – 102.1	***	97.8	***	67.3
> 20% inclusion level	10	24.7	6.7 – 91.9	***	23.6	**	61.8

[1]Subgroups analysis with SBM inclusion level (<20%, 20% or >20% inclusion level).
[2]Odds ratio within the same subgroup, but with different superscript ([a, b]) are significantly different (P < 0.05).
[3]P-values of analysis comparing the odds ratio of the SBM treatments with the neutral reference group (Odds ratio = 1). Asterisks denote level of significance (NS: not significant, *p < 0.05, **p < 0.01, ***p < 0.001).
[4]P-values of chi-square (Q) statistic used to detect heterogeneity across the studies. Significance level of the heterogeneity was denoted with asterisks (NS: not significant, *p < 0.05, **p < 0.01, ***p < 0.001).

Table S6
Relationships (linear and quadratic) between the severity of enteritis and final weight of fish fed SBM-based diets[a]

Dataset	Regression type	Number of datapoints	Equation	P-value [b]	R^2 (adjusted)
Reduction in mucosal fold height					
Full	Linear	76	$Y = 10.739X + 186.0$	*	0.05
	Quadratic		$Y = 1.454X^2 + 25.096X + 119.6$	***	0.10
Freshwater	Linear	35	$Y = -0.847X + 76.0$	NS	-0.01
	Quadratic		$Y = 0.499X^2 + 5.435X + 58.3$	*	0.13
Seawater	Linear	41	$Y = -3.505X + 346.6$	NS	-0.02
	Quadratic		$Y = 3.679X^2 – 13.525X + 306.3$	NS	-0.03
Loss of supranuclear vacuolization					
Full	Linear	78	$Y = 7.714X + 195.5$	NS	0.01
	Quadratic		$Y = 0.004X^2 + 7.735X + 195.2$	NS	-0.01
Freshwater	Linear	35	$Y = 1.90X + 73.3$	NS	0.02
	Quadratic		$Y = 0.478X^2 + 5.752X + 54.3$	***	0.28
Seawater	Linear	43	$Y = -31.14X + 464.9$	NS	0.06
	Quadratic		$Y = 7.77X^2 – 93.993X + 558.3$	NS	0.08
Infiltration of lamina propria					
Full	Linear	73	$Y = 8.831X + 165.5$	NS	0.03
	Quadratic		$Y = -0.088X^2 + 8.713X + 168.2$	NS	0.02
Freshwater	Linear	35	$Y = 0.543X + 75.4$	NS	-0.02
	Quadratic		$Y = 0.049X^2 + 0.644X + 73.6$	NS	-0.04
Seawater	Linear	38	$Y = 12.915X + 242.5$	NS	-0.01
	Quadratic		$Y = -5.879X^2 + 59.350X + 174.4$	NS	-0.02
Submucosal cellularity					
Full	Linear	78	$Y = 3.52X + 215.7$	NS	-0.00
	Quadratic		$Y = 0.012X^2 + 3.66X + 215.1$	NS	-0.01
Freshwater	Linear	35	$Y = 2.608X + 78.8$	**	0.18
	Quadratic		$Y = 0.191X^2 + 5.599X + 71.7$	*	0.17
Seawater	Linear	43	$Y = -8.803X + 353.6$	NS	0.02
	Quadratic		$Y = -0.356X^2 – 11.212X + 369.9$	NS	-0.00

[a] Y = fish final weight and X = log-odds ratio.
[b] Asterisks denote level of significance (NS: not significant, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Table S7

Relationships (linear and quadratic) between the severity of enteritis and thermal growth coefficients of fish fed SBM-based diets[a]

Dataset	Regression type	Number of datapoints	Equation	P-value [b]	R^2 (adjusted)
Reduction in mucosal fold height					
Full	Linear	73	$Y = 0.007X + 2.0$	NS	-0.01
	Quadratic		$Y = -0.0001X^2 + 0.005X + 2.0$	NS	-0.03
Freshwater	Linear	35	$Y = 0.002X + 1.9$	NS	-0.02
	Quadratic		$Y = -0.002X^2 – 0.024X + 2.0$	*	0.13
Seawater	Linear	38	$Y = 0.022X + 1.9$	NS	-0.02
	Quadratic		$Y = 0.043X^2 – 0.236X + 2.2$	NS	-0.01
Loss of supranuclear vacuolization					
Full	Linear	73	$Y = -0.013X + 2.0$	NS	-0.01
	Quadratic		$Y = -0.004X^2 – 0.033X + 2.2$	NS	0.05
Freshwater	Linear	35	$Y = -0.001X + 1.9$	NS	-0.03
	Quadratic		$Y = -0.002X^2 – 0.016X + 2.0$	**	0.21
Seawater	Linear	38	$Y = -0.118X + 2.5$	*	0.08
	Quadratic		$Y = -0.017X^2 + 0.016X + 2.3$	NS	0.07
Infiltration of lamina propria					
Full	Linear	68	$Y = 0.007X + 1.9$	NS	-0.01
	Quadratic		$Y = 0.0004X^2 + 0.008X + 1.9$	NS	-0.02
Freshwater	Linear	35	$Y = -0.011X + 1.9$	*	0.10
	Quadratic		$Y = -0.0002X^2 – 0.011X + 1.9$	NS	0.09
Seawater	Linear	33	$Y = 0.191X + 1.2$	***	0.20
	Quadratic		$Y = -0.059X^2 + 0.622X + 0.7$	**	0.28
Submucosal cellularity					
Full	Linear	73	$Y = -0.001X + 2.0$	NS	-0.01
	Quadratic		$Y = 0.02X^2 + 0.026X + 1.9$	NS	-0.003
Freshwater	Linear	35	$Y = -0.013X + 1.9$	***	0.28
	Quadratic		$Y = -0.001X^2 – 0.026X + 1.9$	***	0.28
Seawater	Linear	38	$Y = 0.014X + 2.0$	NS	-0.02
	Quadratic		$Y = 0.003X^2 + 0.038X + 1.9$	NS	-0.03

[a] Y = thermal growth coefficients and X = log-odds ratio.

[b] Asterisks denote level of significance (NS: not significant, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Table S8

Univariate meta-regression to determine the effect of literture source (published and unpublished) on studies included in the meta-analysis. Log odds ratio is the dependent variable (effect size) of the equation generated with this meta-regression.

Study factors	Intercept	Coefficients	95% CI		R^2	Number of studies	Q	I^2 (%)	*P*-value
			Lower	Upper					
Reduction in mucosal fold height									
Source: unpublished	3.2	-0.9	-1.65	-0.06	0.00	92	238.6	61.86	NS
Loss of supranuclear vacuolization									
Source: unpublished	3.4	0.4	-0.55	1.34	0.02	94	343.7	72.9	NS
Infiltration of lamina propria									
Source: unpublished	3.2	-1.3	-2.10	-0.45	0.09	85	235.2	64.3	**
Submucosal cellularity									
Source: unpublished	3.6	-1.3	-2.20	-0.49	0.10	85	226.9	63.0	**

Asterisks denote level of significance (NS: not significant, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Fig. S1
Scatter plots from the meta-regression used to convert continuous variables used for the sub-group analysis into categorical variables.

Soy-bean meal inclusion level was categorized into < 20%, 20% and > 20%; water temperature was partitioned into < 10 ºC and > 10 ºC; while year of study was partitioned into before 2014 and after 2014.

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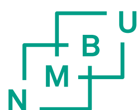

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