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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 re-suspended 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).

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

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 ($^{a\text{-}e}$) 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 ($^{a\text{-}d}$) 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 ($^{a\text{-}d}$) 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

.
[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 ($^{a\text{-}e}$) 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).

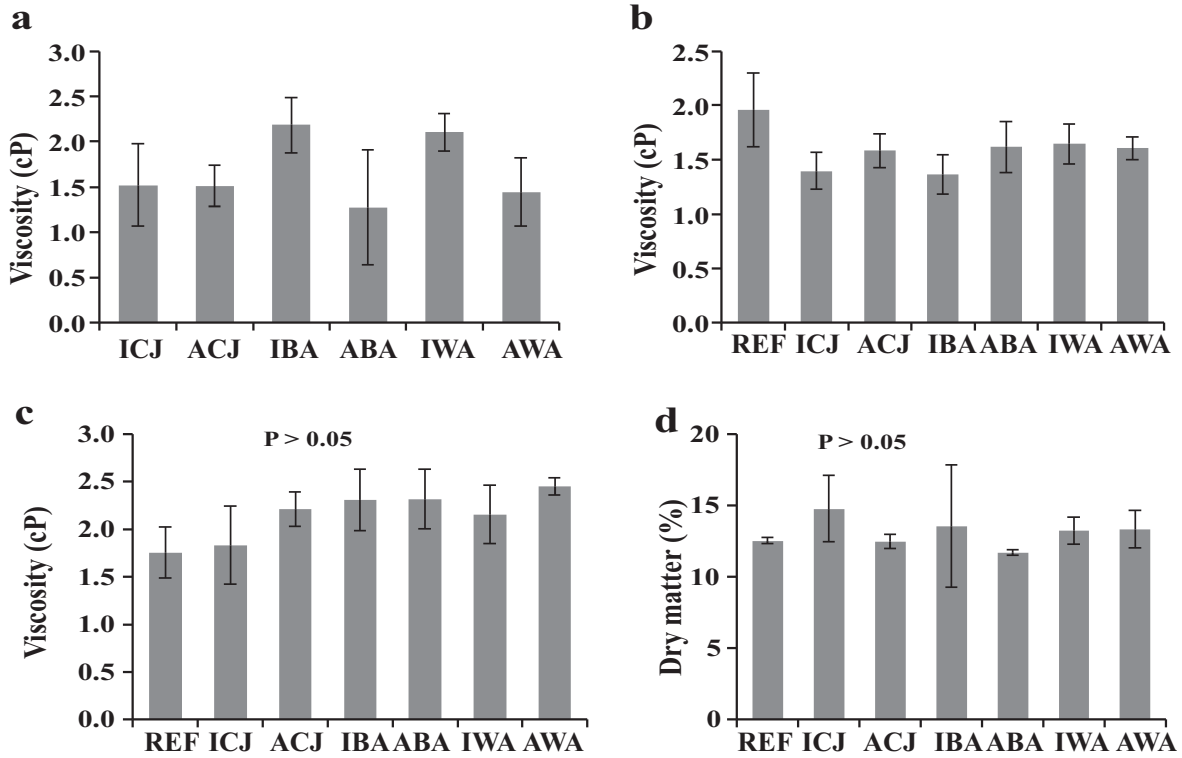

Fig. 1. Viscosity (centipoise; cP) of yeasts (a), diets (b) and digesta (c). REF is reference diet, Inactivated (ICJ) *vs* Autolyzed *Cyberlindnera jadinii* (ACJ); Inactivated (IBA) *vs* Autolyzed *Blastobotrys adeninivorans*; and Inactivated (IWA) *vs* Autolyzed *Wickerhamomyces anomalus* (AWA). The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

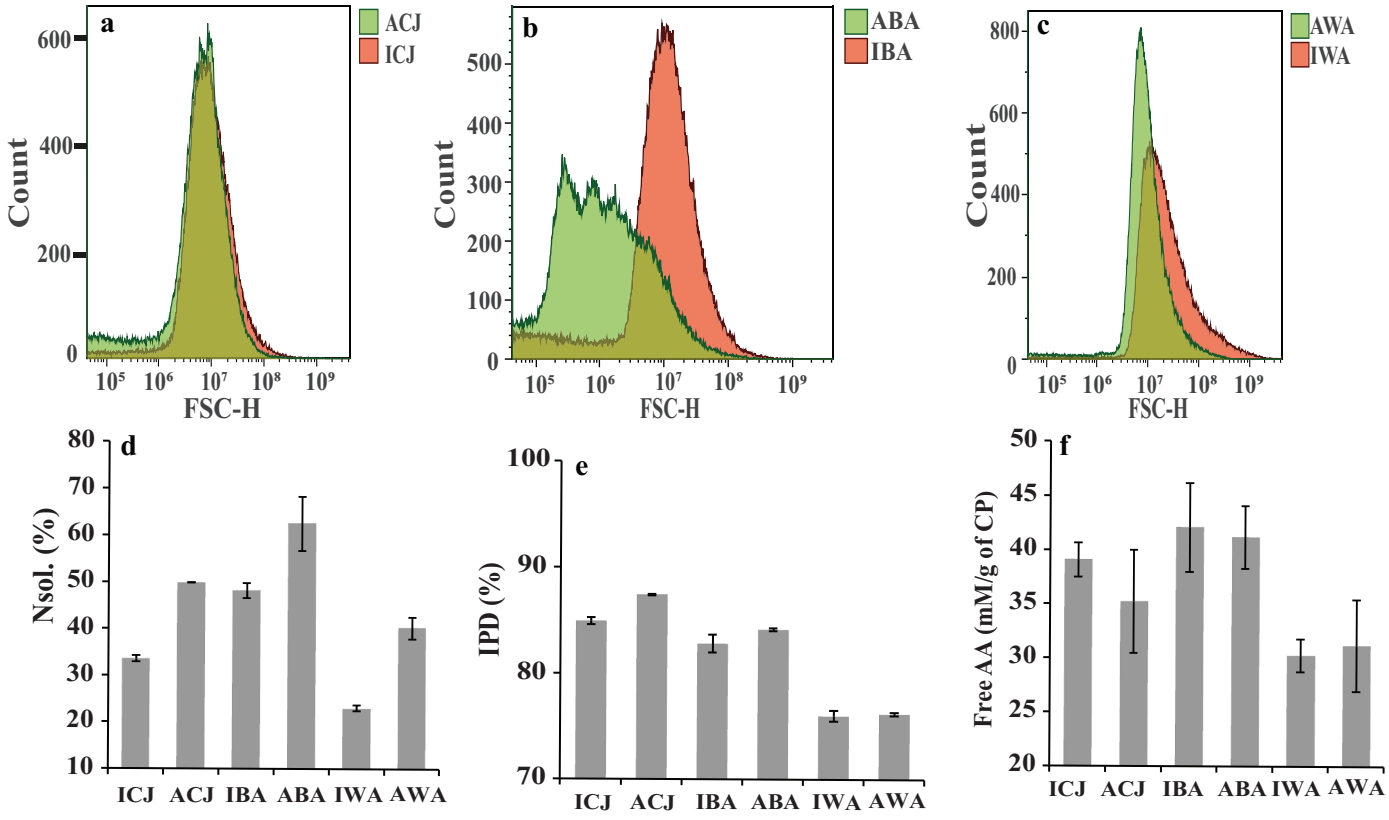

Fig. 2. Effects of processing on cell wall integrity of yeasts. Impact of processing on size distribution (a-c), nitrogen solubility (d) *in vitro* protein digestibility and release of free AA (e-f) of yeasts. Inactivated (ICJ) *vs* Autolyzed *Cyberlindnera jadinii* (ACJ); Inactivated (IBA) *vs* Autolyzed *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

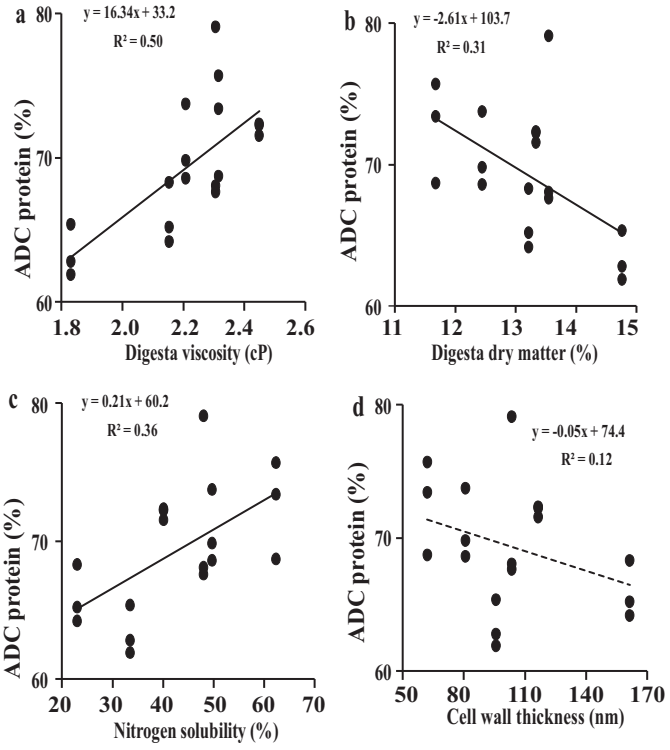

Fig. 3. Linear relationships between the apparent digestibility coefficients (% ADC) of protein from yeasts in juvenile Atlantic salmon and (a) digesta viscosity; (b) digesta dry matter (DM); (c) nitrogen solubility of yeasts; and (d) cell wall thickness of yeasts. Solid and dotted lines denote significant ($P < 0.05$) 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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