Down-stream processing of baker's yeast (*Saccharomyces cerevisiae*) – Effect on nutrient digestibility and immune response in Atlantic salmon (*Salmo salar*)

Jon Øvrum Hansen[a],[*], Leidy Lagos[a], Peng Lei[a], Felipe Eduardo Reveco-Urzua[a],[1], Byron Morales-Lange[c], Line Degn Hansen[b], Marion Schiavone[d], Liv Torunn Mydland[a], Magnus Øverlie Arntzen[b], Luis Mercado[c], Ricardo Tavares Benicio[a], Margareth Øverland[a]

[a] *Department of Animal and Aquacultural Sciences, Norwegian University of Life Sciences, P.O. Box 5003, NO-1432 Ås, Norway*
[b] *Faculty of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, P.O. Box 5003, NO-1432 Ås, Norway*
[c] *Instituto de Biología, Pontificia Universidad Católica de Valparaíso, 2373223 Valparaíso, Chile*
[d] *Lallemand SAS, 19 rue des Briquetoers, BP59, 31702 Blagnac, France*

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ABSTRACT

The increased demand for high-quality feed ingredients for the aquaculture industry has led to an increased focus on microbial ingredients as nutrient sources. Limited information exists, however, on the effect of down-stream processing of yeast on nutrient digestibility and health in Atlantic salmon (*Salmo salar*). In the present study, three laboratory-scale down-stream processing methods were used: direct inactivation by spray-drying, autolysis, or cell crushing. All yeast was inactivated with sray-drying post treatment. The yeast products were evaluated for their nutritional value and health effects in diets for Atlantic salmon. The experimental diets consisted of a high-quality fishmeal-based reference diet (FM) and six experimental diets were 300 g kg^{-1} of each of the processed yeast candidates were added to the FM control mixture. Fish were fed the diets for 21 days, and at the end of the experiment, feces were collected, and spleen, head kidney, distal intestine (DI) and plasma were sampled for assessment of immune response.

Processing of yeast resulted in increased level of soluble protein in the yeast cream, with the highest level for yeast disrupted by cell crushing (20 K PSI). This was confirmed with scanning electron microscopy, where disrupted and cracked cells could be observed. Significant lower protein digestibility of the directly inactivated yeast coincided with the increased detection of whole yeast cells by flow cytometry in the DI content of fish fed the directly inactivated yeast. Autolysis for 16 h resulted in the highest protein digestibility, which was similar to the FM control.

Interestingly, 16 h autolyzed yeast induced the secretion of IL-8, while cell crushed yeast induced the secretion of TNFα in the DI as analyzed by ELISA. To conclude, different down-stream processing of *S. cerevisiae* led to increased protein and β-glucan solubility that further increased protein digestibility in Atlantic salmon. Furthermore, different processing of the yeast triggered different immune stimulatory effects in Atlantic salmon.

1. Introduction

The global decline of captured marine fisheries for fishmeal and -oil production, along with an increased demand for aquaculture feeds, have led to increased use of non-marine ingredients in salmon feed (Ytrestøyl et al., 2015). The latter has led to an increased demand for high-quality novel feed ingredients, which further has led to increased interest in microbial ingredients as sources of protein and lipids. The production of ingredients such as single cell protein grown on waste water from starch production (ICell Sustainable Nutrition Co., Ttd) and microalgae with high concentrations of omega-3 long-chain fatty acids (AlgaePrime™, San Francisco, USA) have scaled up to industrial level,
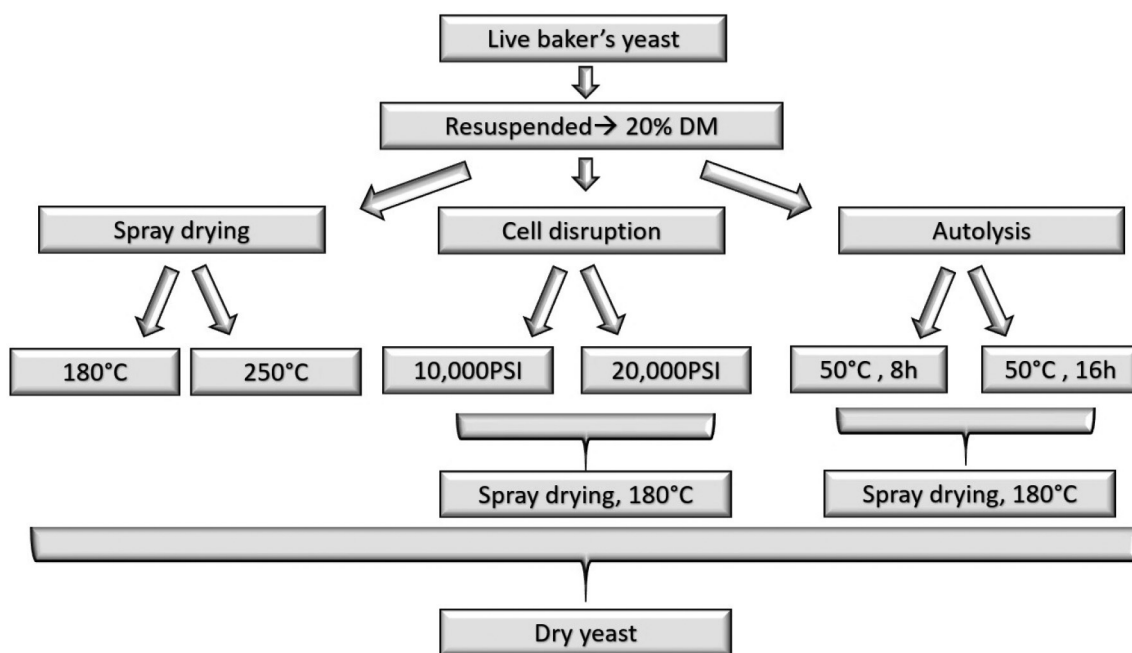

Fig. 1. Flow-sheet of down-stream processing of *Saccharomyces cerevisiae*.

providing products for commercial fish feed producers.

The scientific evaluation of yeast as a food and feed ingredient has lasted for more than 100 years (Osborne and Mendel, 1919). The recent focus regarding yeast as a feed additive is mainly related to health beneficial effects as an immune modulator and its positive effect on mucosal surfaces in fish and farmed animals (Hoseinifar et al., 2015; Vohra et al., 2016; Shurson, 2018). The interest in yeast has also increased due to the ability of using non-food lignocellulosic biomass as a sugar stream (Øverland and Skrede, 2017) for yeast production. Alternative nitrogen sources such as animal hydrolysates and seaweed have also been tested as fermentation medium (Sharma et al., 2018; Lapeña et al., 2019).

Different types of intact yeasts have different digestibility in fish. Inclusion of brewer's yeast (*Saccharomyces cerevisiae*) gave lower protein digestibility than fishmeal in diets for sea bass (*Dicentrarchus labrax*) (Oliva-Teles and Gonçalves, 2001) while similar protein digestibility was observed when fishmeal was substituted with 38% brewer's yeast in diets for Pacu (*Piaractus mesopotamicus*) (Ozório et al., 2010). Furthermore, low protein digestibility of the same type of yeast was reported for rainbow trout (*Oncorhynchus mykiss*) (Rumsey et al., 1991a). Nonetheless, limited information exists on the effect of down-stream processing of yeast on nutrient digestibility and immune response in fish. Rumsey et al. (1991b) demonstrated higher protein and energy digestibility in rainbow trout fed mechanically disrupted *S. cerevisiae* compared to intact yeast. This experiment was, however, conducted with a small number of fish that were force-fed and reared in individual metabolic tanks. Increased digestibility of protein and amino acids were also presented by Langeland et al. (2016) when comparing a commercial autolyzed yeast extract from *S. cerevisiae* with intact yeast cells fed to Arctic char (*Salvelinus alpinus*). However, in the latter experiment the different yeast products tested were obtained from different suppliers, hindering robust conclusions.

Aquaculture feeds are formulated with various types of ingredients which support animal's basic nutritional demands for maintaining its normal biological functions, including a robust immune system, growth, and reproduction. On the other hand, functional ingredients are usually associated with non-nutritive aspects, mainly immunological properties. These properties are linked to the non-digestible components usually present in the yeast cell walls which contribute to about 10–30% of the yeast cell biomass. For instance, *S. cerevisiae's* cell wall typically contains 5% protein and 95% polysaccharides, where 35–40% is mannoprotein, 5–10% is β-1,6-glucans, 50–55% is β-1,3-glucans and 2–5% is chitin (Klis et al., 2002; Schiavone et al., 2014). Several of these components are considered to have functional properties, and the relationship between β-glucans from *S. cerevisiae* have particularly been associated with increased immune resistance in Atlantic salmon (*Salmo salar*) (Robertsen et al., 1990; Jin et al., 2018). Together with β-glucans, mannan oligosaccharides have also shown positive effects on immunity, gut homeostasis and metabolic functions (Song et al., 2014; Torrecillas et al., 2014; Hoseinifar et al., 2015) as well as direct modulatory effects on the gut microbiota (Ringø et al., 2012). It has also been shown that fermentation conditions and down-stream processing methods of yeast can affect the chemical composition, biophysical properties and solubility of different cell wall components (Middelberg, 1995; Giovani et al., 2010; Schiavone et al., 2015; Dimopoulos et al., 2020), presumably exerting impact on its immune properties. Therefore, the aim of the study was to investigate the effect of three different down-stream processing methods on baker's yeast and their nutritional and immunological value in Atlantic salmon. Protein digestibility and immune responses were evaluated in this research, including the level of cytokines and B-cells, in distal intestine (DI), spleen and head kidney.

2. Materials and methods

2.1. Processing of yeast and protein solubility

Fresh baker's yeast (*S. cerevisiae*) (Idun industry AS, Norway) was suspended in 7 °C deionized water in a 30 l bioreactor system EINAR 30 l (Belach Bioteknik, Sweden), equipped with a helical impeller to obtain a slurry with 20% (*w/v*) dry matter (DM) content. Six kg of yeast (30% DM) was used for each of the following treatments (Fig. 1).

First, two batches were directly inactivated by drying in a SPX 150 MS (SPX Flow Technology, Denmark A/S) spray-dryer using an inlet and outlet temperature of 180 and 80 °C or 250 and 90 °C, respectively. Pump speed was set to auto and stabilized at around 35% and the spray-drier was fitted with a co-current nozzle.

Another two batches of yeast were treated with a high shear

processor LM20 Microfluidizer (Microfluidics corp. Boston, US) at a force of 10.000 PSI or 20.000 PSI to obtain cell disruption. Crushed ice was placed in a cooling bath covering the cooling coil (product outlet) to cool down the treated yeast to approximately 4 °C. Finally, autolysis was carried out with the last two yeast batches in the same 30 l bioreactor by incubating yeast at 50 °C for 8 or 16 h with constant stirring at 50 rpm using helical impellers. pH was not adjusted during autolysis. Both yeast preparations were spray-dried using an inlet and outlet temperature of 180 and 80 °C.

After spray-drying, 2 g of yeast was diluted with 10.5 g of deionized water followed by incubation in a water bath (20 °C) for 30 min. The supernatant was recovered after centrifugation at 6000 rpm (Mega Star 1.6R, VWR, Radnor, US) for 10 min, and the amount of soluble protein was determined by the Kjeldahl method.

2.2. Scanning electron microscope (SEM)

After autolysis and cell disruption, three yeast samples per treatment were fixed by adding 450 μL of yeast culture to 50 μL of formaldehyde (37%) in an Eppendorf tube that was incubated at room temperature for 15 min. Thereafter spun down for 5 min at 8000 rpm and re-suspended in a 0.1 M potassium phosphate and stored at 4 °C until imaging with an EVO 50 EP (Carl Zeiss AG, Oberkochen, Germany) scanning electron microscope.

2.3. Formulation and production of experimental diets

A fishmeal-based reference diet (Table 1) including yttrium oxide as a digestible marker was mixed in a ratio of 70:30 with the dry test yeast. Dry ingredients for the control diet were mixed in a 60 l twin shafted experimental mixer. The diets were produced using gelatin as the main binder. Gelatin was mixed in cold water and heated up to 60 °C in a microwave oven before mixing with dry ingredients and fish oil. The mash was cooled down to room temperature before pelleting (P35A, Carasco, Italy). The pellet was dried in small experimental dryers at approximately 60 °C drying temperature.

Table 1
Diet formulation, g kg^{-1}.

Reference diet	g kg^{-1}
Fishmeal[a]	481.35
Wheat gluten[b]	130
Potato starch[c]	120
Gelatin[d]	110
Fish oil[e]	150
Choline chloride[f]	2
Mineral and vitamin premix[g]	6.5
Yttrium oxide[h]	0.15
Analyzed composition, g kg^{-1}	
Crude protein	544
Ash	73
Dry matter	881

[a] LT fishmeal, Norsildmel, Egersund, Norway.
[b] Wheat gluten, Amilina AB, Panevezys, Lithuania.
[c] Lygel F 60, Lyckeby Culinar, Fjälkinge, Sweden.
[d] Rousselot® 250 PS, Rousselot SAS, Courbevoie, France.
[e] NorSalmOil, Norsildmel, Egersund, Norway.
[f] Choline chloride, 70% Vegetable, Indukern s.a., Spain.
[g] 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$_2$O 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.
[h] Y$_2$O$_3$. Metal Rare Earth Limited, Shenzhen, China.

2.4. Biological experiment and sampling procedure

The fish experiment was performed at the Norwegian University of Life Sciences. The experimental procedures were performed in accordance with the institutional and national guidelines for the care and use of animals (the Norwegian Animal Welfare Act and the Norwegian Regulation and Animal Experimentation).

A total of 840 fish with an average weight of 114 g were randomly divided into 21 fiberglass tanks (300 l) equipped with automatic feeders. The uneaten feed was sieved from the outlet water of each tank according to Shomorin et al. (2019), and feed intake was calculated according to Helland et al. (1996). Fish were fed 1.5% of body weight (about 110% of measured feed intake) twice a day for 21 days. The water flow was standardized to about 11 l min^{-1}, and the oxygen content of the outlet water was kept within 8.3–9.5 mg l^{-1}. Fish were exposed to a constant light regime and recirculated freshwater with an average temperature of 14.6 °C. All fish were anesthetized with metacaine (MS-222™; 50 mg l^{-1} water) and stripped for feces at day 14 and 21 during the experimental period. Feces from both days were pooled by tank and kept frozen at −20 °C prior to freeze-drying. On day 21, all fish were weighed, and tissue samples were dissected out for histology, intestinal content for flow cytometry and spleen, DI and head kidney for analyses of immune responses.

2.5. Chemical analysis

Freeze-dried feces and diets were ground with a mortar and pestle prior analysis. Diets and feces were analyzed for DM by drying to constant weight at 104 °C, and ash by combustion at 550 °C (Commission dir. 71/393/EEC), carbon, nitrogen, and sulfur by using an element analyzer (Vario El Cube elemental analyzer system GmbH, Hanau, Germany). Yttrium was determined in feed and feces by microwave digestion (Milestone UltraClave III; Milestone, Sorisole, Italy) using HNO$_3$ at 250 °C and analyzed using mass spectroscopy system (Agilent 8800 Triple Quadrupole; Agilent Technologies, Santa Clara, CA, United States).

2.6. Indirect ELISA

Samples of head kidney and DI from four 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). Then, the homogenate was centrifuged at 12000 *x g* for 25 min at 4 °C. The supernatant containing soluble proteins was stored at −20 °C until use. All protein samples were quantified by BCA protein assay kit (Thermo) following the manufacturer's instructions. Following the protocol from Morales-Lange et al. (2018), 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) at 45 ng μL^{-1} (100 μL) for overnight incubation at 4 °C. After blocking with 5% Blotting-Grade Block (Biorad) diluted in PBS for 2 h at 37 °C, the plates were incubated for 90 min at 37 °C with the first antibody anti-TNFα, IL-1β or IL-8 (1:200). Then, the second antibody-HRP (Thermo) was incubated for 1 h at 37 °C at 1:7000 dilution. Finally, chromagen substrate 3,3′,5,5′-tetramethylbenzidine single solution (Invitrogen) was added (100 μL), and incubated for 30 min at room temperature. The reaction was stopped with 50 μL of 1 N sulfuric acid and read at 450 nm on a Spectramax microplate reader (Molecular Devices).

2.7. Flow cytometry

From each fish, the head kidney was removed under aseptic conditions and passed through 100-μm pore size cell strainers (Falcon) in L-15 medium containing penicillin (10 U ml^{-1}), streptomycin (10 μg ml^{-1}), 2% fetal bovine serum (FBS), and heparin (20 U ml^{-1}). The resulting suspension was placed on a 25/54% discontinuous Percoll

gradient and centrifuged at 400g for 40 min at 4 °C. The cells at the interface were collected, washed twice in L-15 medium, and cell count and viability tests were performed by the Trypan blue method. Finally, the cells were resuspended in complete L15 (10% FBS and 1% penicillin-streptomycin) to a concentration of 2×10^6 cells ml^{-1}. For the labeling, the cells were washed with ice-cold PBS and incubated with anti-IgM monoclonal antibody (200-fold dilution) for 1 h in PBS, 5% FBS on ice. The secondary Alexa488 rabbit anti-mouse antibody was diluted to 1 μg ml^{-1} in PBS, 5% FBS and the cells were incubated for 30 min on ice before washing with PBS. The flow cytometry was performed using a Gallios Flow Cytometer (Beckman Coulter), and data were analyzed using Kaluza software v.2.1 (Beckman Coulter). Hoechst stain was combined with propidium iodide stain for the separation of dead and live cells.

The microbial flow cytometry was performed following the protocol described by Lambrecht et al. (2018). Briefly, fecal content was mashed and vortexed in 1 ml of PBS per 200 mg feces. Large debris was removed by centrifugation at 400 g for 5 min. Supernatants containing fecal bacteria and undigested whole yeast cells were fixed in 2% formaldehyde in PBS and incubated for 30 min at RT. Then, samples were centrifuged and resuspended in 70% ethanol and stored at −20 °C to further stain and analysis. For the staining, samples were washed with PBS, diluted to $OD_{600} = 0.035$ with PBS, stained with DAPI nuclear stain (Molecular Probes) and BacLight Red Bacterial Stain (Thermo Fisher Scientific) following the manufacture instructions. After washing twice with PBS, stained bacteria and yeast were analyzed on Gallios Flow Cytometer (Beckman Coulter) and Kaluza software v.2.1 (Beckman Coulter).

2.8. Histology

Formalin-fixed tissues were embedded in paraffin with an orientation to provide longitudinal sections. Sections (2 μm) were mounted on glass slides (Menzel Gläser, Thermo Scientific, Braunschweig, Germany) and stained with hematoxylin and eosin (HE). Digital images were captured with a Zeiss AxioCam ERc5s camera connected to a light microscope (Zeiss Axio Lab.A1, Carl Zeiss, Germany. Evaluation of the DI, 12 fish per treatment, was performed according to changes being associated with the development of SBMIE as previously described for Atlantic salmon (Baeverfjord and Krogdahl, 1996).

2.9. In vitro pre-digestion of yeast

The *in vitro* digestion model was used to mimic the *in vivo* gastro-intestinal digestion. The model included two steps: 1) Gastric hydrolysis and 2) Intestinal hydrolysis. The procedure used was as follow: 2 g of down-stream processed yeast was added into 50 ml tubes together with 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) and 0.4 ml Chloramphenicol-solution (0.1% w/v, Sigma-Aldrich) and incubated in a shaking water bath at 37 °C for 24 h. Then, 675 μl of NaOH 1 M was added to inactivate pepsin activity, and pH was adjusted to 7.8 by adding approximately 30 ml of 10 mM Phosphate buffer (PBS, pH 7.8, Sigma-Aldrich). The tubes were incubated in a shaking water bath at 37 °C for 1 h. Further, added 0.6 ml of the intestinal enzyme cocktail (Trypsin 2100 U ml^{-1}, Chymotrypsin 100 U ml^{-1} and Elastase 0.2 U ml^{-1} in 10 mM Phosphate buffer, pH 7.8, Sigma-Aldrich), incubated in the shaking water bath at 37 °C for another 18 h. Finally, total digesta were centrifuged at 10,000g for 5 min at room temperature to separate the pellet and supernatant fractions for sugar composition analysis.

2.10. Composition analysis of yeast cell wall

Composition analysis was carried out to evaluate the effects of down-stream processing on the yeast cell wall composition. The DM concentration (mg mL^{-1}) was estimated for both pellet and supernatant

fractions after *in vitro* digestion, using gravimetry after overnight incubation at 100 °C. For the pellets, roughly 80 mg slurry was weighed and diluted with H_2O to 500 μL for compositional analysis, while for the supernatants, 500 μL was used directly. 96% H_2SO_4 was added to a final concentration of 4% and the samples were autoclaved at 121 °C for 1 h. Precipitate was removed by centrifugation at 16,000g for 5 min and supernatants were subjected to sugar analysis using high-performance anion exchange chromatography coupled to pulsed amperometry detection (HPAEC-PAD). For this, an ICS3000 system (Dionex, Thermo Scientific, San Jose CA, USA) was used equipped with a CarboPAC PA1 column (2.5 × 250 mm) with guard (2.5 × 50 mm) and coupled to an electrochemical detector operated with a disposable gold electrode utilizing carbohydrate-specific waveforms. The mobile phase was 1 mM KOH and generated by an inline eluent generator (EG Reagent-Free IC module with Continuously Regenerating Trap Column-technology) and monosaccharides (Glc, Man) were eluted isocratically over 35 min. Quantification was done using standards purchased from Sigma-Aldrich and further converted to polymeric concentrations (β-glucan and mannan) using the following formula: $C = (C_{HPAEC} \times D \times V_{sample} \times P) (R \times DM_{load})^{-1}$, where C is the polymeric concentration, C_{HPAEC} is the measured concentration of the monomer, D is the dilution factor, V_{sample} is the sample volume, P is the polymeric conversion factor (0.9 for Glc/Man), R is the recovery as estimated using sugar recovery standards and DM_{load} is the amount of DM weighed in. β-1,3-glucans were determined by an enzymatic method, as described in Schiavone et al. (2014).

2.11. Calculations and statistical analysis

Feed utilization was evaluated by feed conversion ratio (FCR); feed consumed x weight gain^{-1}. Specific growth rate (SGR) was calculated as: SGR = 100 × (ln(end wt) − ln(start wt)/Δt), where end wt = end weight of fish, start wt = start weight of fish, Δt = number of experimental days. The nutrient apparent digestibility coefficients (ADCs) were calculated by using Eq. (1) (Cho and Slinger, 1979).

$$ADC \ (\%) = 100 * \frac{(a - b)}{a} \tag{1}$$

where a represents (nutrient in feed/ yttrium in feed) and b represents (nutrient in feces/yttrium in feces). The apparent digestibility coefficients of test ingredients was calculated using Eq. (2) (Bureau and Hua, 2006).

$$ADC_{ingredients} (\%)$$
$$= ADC_{testfeed} + (ADC_{testfeed} - ADC_{control\ feed}) \times \frac{0.7 \times Nutrient_{ref}}{0.3 \times Nutrient_{ingredient}}$$

All parameters were analyzed using a one-way ANOVA followed by Tukey HSD as a *post hoc* test conducted with the General Linear Models procedure in SAS software package (SAS/STAT Version 9.4. SAS Institute, Cary, NC, USA). Differences were considered significant when $p < 0.05$. Growth and digestibility parameters were based on tank ($n = 3$) as statistical unit. Numbers of bacteria and yeast in the DI were calculated using three fish per tank (9 fish per treatment), whereas ELISA were measured using two fish per tank (6 fish per treatment). The effect of the tank within diet was investigated but was not significant for either of the parameters tested.

3. Results

3.1. Processing of yeast and diets

The yeast was processed as depicted in Fig. 1 by spray-drying, autolysis or cell crushing without any unexpected problems. It is worth to mention that the consistency of the re-suspended yeast after processing differed: autolysis treatments yielded a foamy surface and more
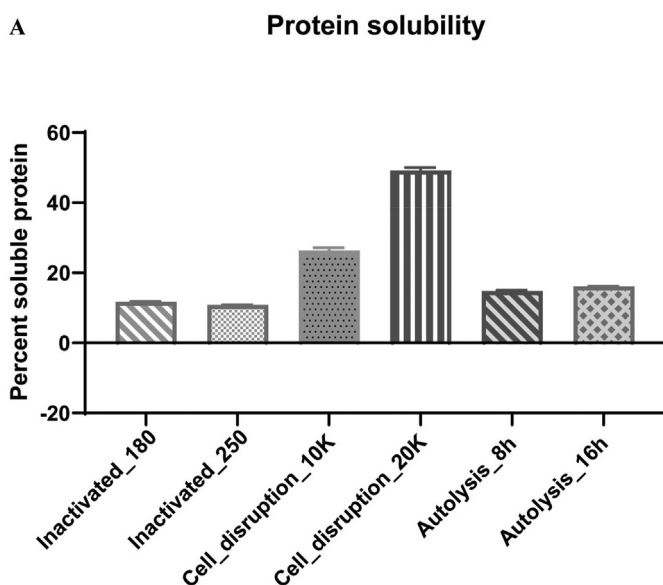
A

Protein solubility

Fig. 2. Percent of soluble protein (% of total protein) in *Saccharomyces cerevisiae* after down-stream processing. The data are presented as means ± SD.

characteristic yeast odor compared to the untreated and cell disrupted yeast. These differences in consistency and odor, however, did not influence the spray-drying process in terms of clogging or visual stickiness of the yeast powder post-drying. When the dry yeast was mixed with water and other ingredients during feed processing, the differently treated yeasts affected the color and consistency of the feed mash. The treated yeast, especially the crushed cells, gave the mash visually higher plasticity and firmness prior to pelleting compared to the untreated yeast that was inactivated. The final pellet from the crushed yeast diets also had a more shiny and compact shape than other pelleted yeast based products. The level of yttrium in the control diet was 0.11 ± 0.005 g kg^{-1} ($n = 3$), wereas the yttrium level in the test diets ranged from 0.0787 to 0.0797 g kg^{-1}, indicating an even distribution of marker in all experimental diets.

3.2. Protein solubility and scanning electron microscopy

The levels of soluble protein and free amino acids were measured (Kjeldahl method) post-treatment as shown in Fig. 2. In the directly inactivated yeasts, 10.8 and 11.8% of the total protein was soluble, whereas 26.3 and 49.2% of the protein were soluble in the 10,000 and 20,000 PSI cell disruption treatment, indicating that cell crushing increased the release of internal protein. Yeast autolyzed for 8 and 16 h had 14.8 and 16.1% soluble protein, respectively. The yeast processing was not performed with replication, thus, no statistics was performed regarding the protein solubility. Nevertheless, a numerically increase in protein solubility was seen for the autolyzed cells compared to the inactivated yeast. This result may indicate a moderate level of autolysis under the present laboratory conditions.

The SEM images show that the non-treated yeast cells, before spray drying, had circular shapes and a smooth surface (Fig. 3A). The shape of the autolyzed yeast appeared to be more uneven with some shrunk and punctured cells (Fig. 3B-C), yet the cell membrane integrity seems intact; this is also further strengthened by the low amount of soluble proteins measured in autolyzed yeast biomass compared to crushed cells. Yeast treated with cell disruption using different units of pressure, gave visually a higher degree of crushed cells with intracellular content leaking out, compared to the other treatments (Fig. 3D-E).

3.3. Yeast cell wall sugar composition

Accordingly, in this study, the cell wall of *S. cerevisiae* was primarily composed of β-glucans and mannans, representing up to 30% of the yeast dry weight (Table 2). The analyses of both total β-glucan, β-1,3-glucan and mannans of the differently down-stream processed yeasts show similar composition of the given carbohydrates (Table 2). The β-glucan content ranged from 221 to 242 g kg^{-1}, and the β-1,3-glucan accounted for approximately 50% of the total β-glucan content. The level of mannan was between 124 and 131 g kg^{-1} for the different down-stream processed yeast. The relative amount of β-glucan and mannan present in the soluble and insoluble fractions after *in vitro* digestion did not fully add up to the expected values presented for the undigested yeast and had some variation for the different processed yeast (Table 2). Nevertheless, there was a visually trend for increased level of β-glucan found in the soluble fraction for the autolyzed yeast post *in vitro* digestion. Enhanced β-glucan solubility was coupled with the deformation degree of processed yeast, where the autolyzed yeasts with the highest destruction degree of single-cells had the highest amount of soluble β-glucan. The other major components of the yeast cell wall, mannan, showed comparable total yields from all six processed yeast, where solubility of mannan was increased in physically disrupted yeast.

3.4. Fish growth and digestibility

There was no mortality during the experimental period and no fish showed signs of abnormal behavior. No abnormal changes were observed in the DI of fish fed the differently treated yeast based on the histology examination (data not shown). Fish grew from an average initial weight of 114 g to between 141 and 148 g during the experimental period (Table 3). Fish fed the reference diet showed significantly higher weight and increased SGR compared to fish fed the two directly inactivated yeasts, whereas the fish fed the inactivated yeast at 250 °C showed worst FCR compared to all dietary treatments.

Digestibility of organic matter, protein, and carbon were significantly affected by dietary treatments (Table 4). Organic matter digestibility on diet level ranged from 89.7 to 74.8%, with lowest levels in fish fed the directly inactivated yeasts. Digestibility of carbon followed the same trends as for the organic matter. Crude protein digestibility in fish fed the reference diet was 92.7%, which was significantly higher than the other treatments, except for in the fish fed the 16 h autolyzed yeast. Digestibility on ingredient level showed the same trends for organic matter, and carbon with lower digestibility in fish fed the inactivated yeasts and highest for the 16 h autolyzed yeast. Crude protein digestibility on ingredient level increased from 51.0 to 89.2% for the inactivated yeast (250 °C) and the 16 h autolyzed yeast, respectively.

3.5. Quantification of yeast by flow cytometry

The number of whole yeast cells detected in the content of DI was significantly higher in fish fed diets containing inactivated yeast, followed by the diets containing mildly processed yeast (cell disruption 10 K and autolysis 8 h) (Fig. 4). According to the present gatting strategy, no yeast was detected in the DI content of fish fed the control diet. Interestingly, the content of fish fed cell disrupted (20 K) and autolyzed (16 h) yeast presented a similar pattern than fish fed the control diet, where whole yeast cells were poorly detected. These results correlated with the results of digestibility, where the highest digestibility was detected for the fish fed autolyzed 16 h yeast. The number of yeast cells was determined by cell size and amount of DNA, as those parameters are higher in yeast compared to bacteria. The gating strategy is described in detail in Supplementary Fig. 1.
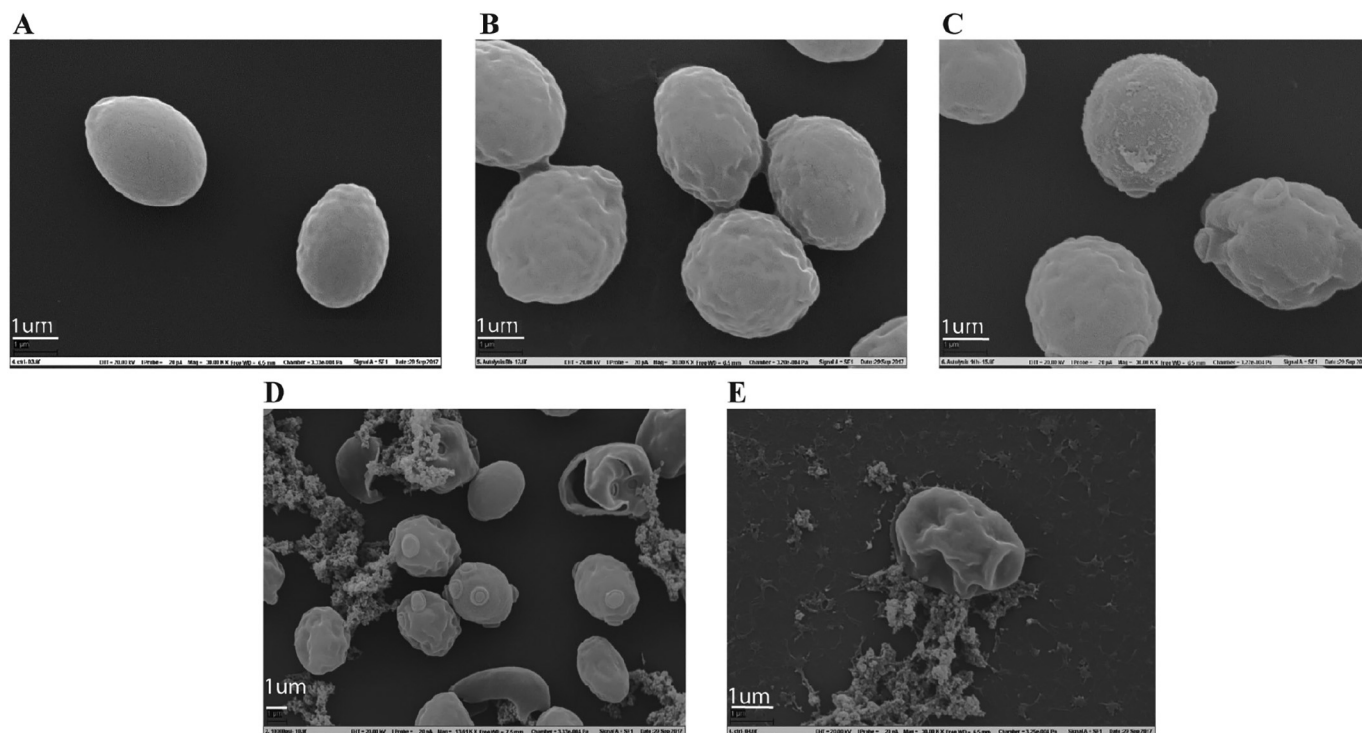
Fig. 3. Scanning electron microscopy (SEM) of down-stream processed *Saccharomyces cerevisiae*: **A**) non-treated yeast, **B**) autolyzed 8 h, **C**) autolyzed 16 h, **D**) cell disruption 10.000 PSI, **E**) cell disruption 20.000PSI.

Table 2
Total β-glucan, β-1,3-glucans and mannans in differently down-stream treated *Saccharomyces cerevisiae*, and the soluble and insoluble *in vitro* digested fractions of these yeasts.

	Total β-glucans	β-1,3-glucans	Mannans
Dry yeast, mg g^{-1}			
Inactivated 180	242	121	130
Inactivated 250	222	107	125
Cell crushing 10 K	228	127	124
Cell crushing 20 K	221	119	127
Autolysis 8 h	222	107	128
Autolysis 16 h	236	90	131
In vitro digested			
Unsoluble, mg g^{-1}			
Inactivated 180	191 ± 21		110 ± 8
Inactivated 250	191 ± 17		94 ± 1
Cell crushing 10 K	181 ± 19		95 ± 8
Cell crushing 20 K	126 ± 19		77 ± 8
Autolysis 8 h	234 ± 16		115 ± 6
Autolysis 16 h	158 ± 19		62 ± 8
Soluble, mg g^{-1}			
Inactivated 180	2 ± 0.4		1 ± 0.1
Inactivated 250	2 ± 0.5		2 ± 0.1
Cell crushing 10 K	9 ± 2		3 ± 0.1
Cell crushing 20 K	14 ± 2		5 ± 0.1
Autolysis 8 h	34 ± 2		3 ± 0.1
Autolysis 16 h	54 ± 2		3 ± 0.1

3.6. Immune responses

After 21 days of feeding, indirect ELISA analyses showed that the presence of cytokines in the head kidney of salmon was not affected (Fig. 5A) by the dietary treatment. The level of TNFα in the DI of fish fed disrupted yeast was significantly higher than in fish fed the control diet. In contrast, autolyzed yeast resulted in the lowest level of TNFα in DI (Fig. 5 B). No difference was observed in the level of IL1β, while an increased level of IL-8 was observed in the distal intestine of fish fed autolyzed yeast compared to the disrupted yeast. There were no

statistical differences in the number of B cells observed in the head kidney and spleen of fish fed the differently treated yeast (Fig. 5C-D).

4. Discussion

A major part of the research concerning technical and nutritional properties of yeast has mainly been linked to the food segment (Gierhart and Potter, 1978; Cooney et al., 1980; Lasztity, 2017). The feasibility of using yeast as a protein source in the aquafeed industry strongly depends on the cost and the scale of production, processing conditions, and the resulting nutritional value (Nasseri et al., 2011). High pressure homogenization and autolysis are the most commonly employed methods for large scale breakage of microorganisms and high recovery of by-products. However, mechanical methods lead to a final product with cell debris which complicates the following down-stream processes such as drying and filtration. Furthermore, the cost of down-stream processing of yeast could vary from 10 to as high as 70% of the total production cost (Straathof, 2011). It is also expected that the final processed yeast presents nutrient digestibility coefficients similar to those of high-quality protein feed ingredients. Therefore, the chosen down-stream method of yeast plays a key role in the nutritional value of the yeast for the food and feed industry.

In the present study, a higher release of soluble proteins from crushed yeasts cytoplasm compared with directly inactivated and autolyzed yeast was observed, which corresponds to the morphological changes, *i.e.* cell rupture and shrunken porous cells for cell crushing and autolysis, respectively. This finding is in line with Hedenskog and Mogren (1973), who found that protein solubility in processed baker's and brewer's yeast increased with cell crushing. High pressure homogenization and autolysation have also shown to increase protein solubility in processed baker's yeast (Dimopoulos et al., 2020; Takalloo et al., 2020).

Increased protein solubility with increased processing was also observed in microalgae (Agboola et al., 2019; Teuling et al., 2019). Further, autolysis is a process where the intracellular enzymes of yeast break down internal components in the cell, resulting in reduced chain

Table 3

Specific growth rate (SGR) and feed conversion rate (FCR) of Atlantic salmon (*Salmo salar*) fed reference diet and test diets with different down-stream processed *Saccharomyces cerevisiae*[1].

	Ref diet	Inactivated 180	Inactivated 250	Cell disruption 10 K PSI	Cell disruption 20 K PSI	Autolysis 8 h	Autolysis 16 h	s.e.m.[2]	*p*-Value
Start weight, g	116	115	116	114	116	113	113	3.5	0.81
End weight, g	148	141	141	143	144	142	141	4.4	0.47
Weight increase, g	31.9 [A]	25.4 [B]	24.8 [B]	29.0 [AB]	28.3 [AB]	29.5 [AB]	28.3 [AB]	2.0	0.01
FCR	0.65[C]	0.78 [AB]	0.82[A]	0.69[BC]	0.73 [BC]	0.73[BC]	0.74[ABC]	0.03	0.004
SGR	1.16 [A]	0.95 [B]	0.93 [B]	1.08 [AB]	1.04 [AB]	1.11 [AB]	1.07 [AB]	0.07	0.010

[1] Test diets were mixed in a ratio of 70:30 (reference diet:down-stream processed dry yeast).
[2] Pooled standard error of the mean. Different letters denote significant ($P < 0.05$) difference among diets. n = 3 replicates per treatment.

length of protein and peptides, and reduced content of nucleotides and free amino acids (Babayan and Bezrukov, 1985; Hernawan and Fleet, 1995; Babayan and Latov, 2003). Moreover, based on the present results, yeast after *in vitro* digestion showed higher solubility of β-glucan in the autolyzed yeast compared with the directly inactivated yeast. Similarly, Janusz et al. (1986) demonstrated higher β-glucan solubility in *S. cerevisiae* post autolysation. A reason for this could be that intracellular β-1,3-glucanases and proteases have degraded the inner layer of the cell wall during the autolysis and increased the solubility of β-glucans. In addition, higher disruption pressure and longer time of autolysis were associated with higher yields of both soluble β-glucan and mannan, which is in line with the release of glycosylated proteins found on wine yeasts after thermic and high-pressure treatments (Comuzzo et al., 2015). The autolysis conditions used in the present study may, however, not reflect industrial autolysis/hydrolysis where exogenous enzymes are frequently used and the duration of the treatment often extends above 16 h (Lallemand, personal communication). Thus, by adding exogenous enzymes or increasing the incubation time, a higher soluble fraction can be expected.

The fish experiment was designed as a digestibility experiment, however, data for growth and feed intake are presented as a quality control of the experiment. The overall specific growth rate was lower than given in standarised growth tables for Atlantic salmon (Melberg and Davidrajuh, 2009) and is reflected by the short feeding period and the handling of fish during stripping. Both fish growth and FCR were, however, highly supported by the digestibility values for each treatment. Fish fed the fishmeal control obtained similar growth and FCR as fish fed the crushed and autolyzed yeast. This is in line with the higher digestibility for both organic matter, protein and carbon for fish fed the processed cells compared to the directly inactivated cells. Low digestibility of intact *S. cerevisiae* as seen in the present experiment is in line with results presented for sea bass (Oliva-Teles and Gonçalves, 2001), rainbow trout (Cheng et al., 2004; Hauptman et al., 2014), and Atlantic salmon (Øverland et al., 2013). Regarding the digestibility effect of cell crushing, Rumsey et al. (1990, 1991b) showed increased protein and energy digestibility in lake trout (*Salvelinus namaycush*) and rainbow trout fed brewer's yeast cells treated with a cell homogenizer and

further increased digestibility by extracting the proteins, which is in line the increased protein digestibility for the crushed yeast. This increased protein digestibility is also in line with increased protein solubility of the crushed cells. The highest protein digestibility was seen in fish fed yeast autolyzed for 16 h, even though the protein solubility was numerically lower than for the crushed yeast. Further, the SEM pictures confirm that the autolyzed yeast has kept their round shape and visually appear intact. A reason for this high digestibility could be, as mention previously, that intracellular β-1,3-glucanases and proteases have degraded the inner layer of the cell wall during the autolysis, which made the cell more porous as described by Middelberg (1995). This porosity may lead to increased accessibility of the digestive enzymes in the fish and, thus, to increased protein digestibility. The increased degradation of the cell wall is also supported by the increased solubility of β-glucans after the present *in vitro* digestion, which also may lead to better digestibility of the protein that is integrated into the cell wall.

The present results could reveal that inactivation of the yeast with spray-drying had a severe impact on nutrient availability, however, the processed yeast was also spray-dried, which indicates a low effect of the drying itself. Hence, the lower digestibility of the inactivated yeast seems to be the lack of processing rather than the spray-drying itself. There was, nevertheless, a significant reduction in protein digestibility by increasing the drying temperature from 180/80 °C to 250/90 °C. Spray-drying is widely used as a drying method for different food and feed ingredients (Patel et al., 2014). It is a gentle drying method where liquid material can be dried in a normal atmosphere at fairly low temperatures. Spray-dried blood cells and plasma for instance, have often a high digestibility in fish (Bureau et al., 1999). Several studies have focused on the viability of different strains of *Saccharomyces* yeasts after different spray-drying conditions and carrier materials (Luna-Solano et al., 2005; Aponte et al., 2016). There is, though, to the author's knowledge, no literature covering the effect of spray-drying temperature on nutrient availability from yeast.

The present digestibility results are supported by the detection of whole yeast cells by flow cytometry, where a low level of whole cells was detected in the content of DI of fish fed yeast disrupted 20 K or

Table 4

Apparent digestibility (%) of organic matter, crude protein and carbon on both diet- and ingredient level in Atlantic salmon (*Salmo salar*) fed diets with differently down-stream processed *Saccharomyces cerevisiae*[1].

Apparent digestibility, %	Ref diet	Inactivated 180	Inactivated 250	Cell disruption 10 K PSI	Cell disruption 20 K PSI	Autolysis 8 h	Autolysis 16 h	s.e.m.[1]	*p*-Value
Diet level									
Organic matter	89.7 [A]	75.3 [D]	74.8 [D]	78.6[C]	79.0[C]	78.7[C]	81.7 [B]	0.73	< .0001
Crude protein	92.7 [A]	83.9 [C]	82.4 [D]	89.4 [B]	89.8 [B]	89.4 [B]	91.8 [A]	0.15	< .0001
Carbon	91.3 [A]	78.6 [D]	77.8 [D]	81.3[C]	81.7[C]	81.2[C]	83.9 [B]	0.58	< .0001
Ingredient level									
Organic matter		45.0[C]	43.2[C]	55.1 [B]	57.1 [B]	55.3 [B]	63.6 [A]	2.41	< .0001
Crude protein		56.2[C]	51.0 [D]	79.1 [B]	81.0 [B]	79.4 [B]	89.2 [A]	1.25	< .0001
Carbon		37.6 [DE]	35.8 [E]	40.9 [BC]	43.2 [A]	39.0 [CD]	41.5 [AB]	0.78	< .0001

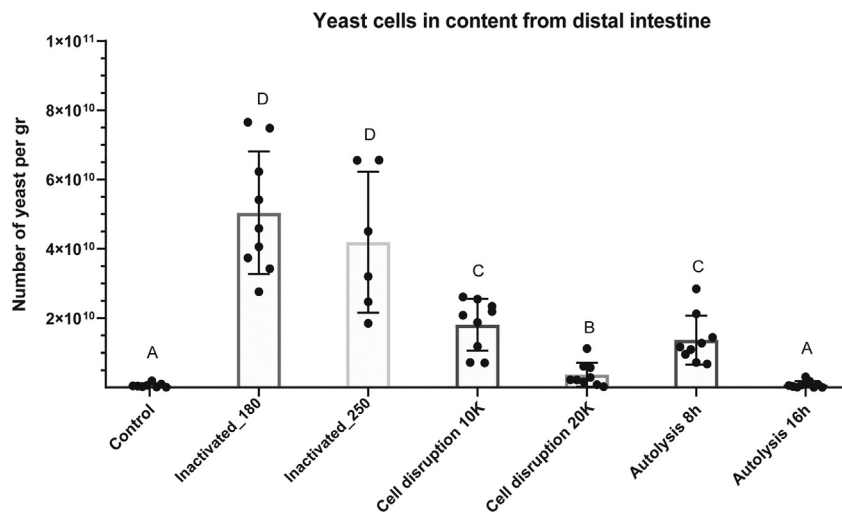[1] Test diets were mixed in a ratio of 70:30 (reference diet:down-stream processed dry yeast).

Fig. 4. The number of the whole *Saccharomyces cerevisiae* cells detected per g of DI content using flow cytometry. Values are presented as the mean ± SD of nine fish per diet; letters denote a significant difference between treatments.
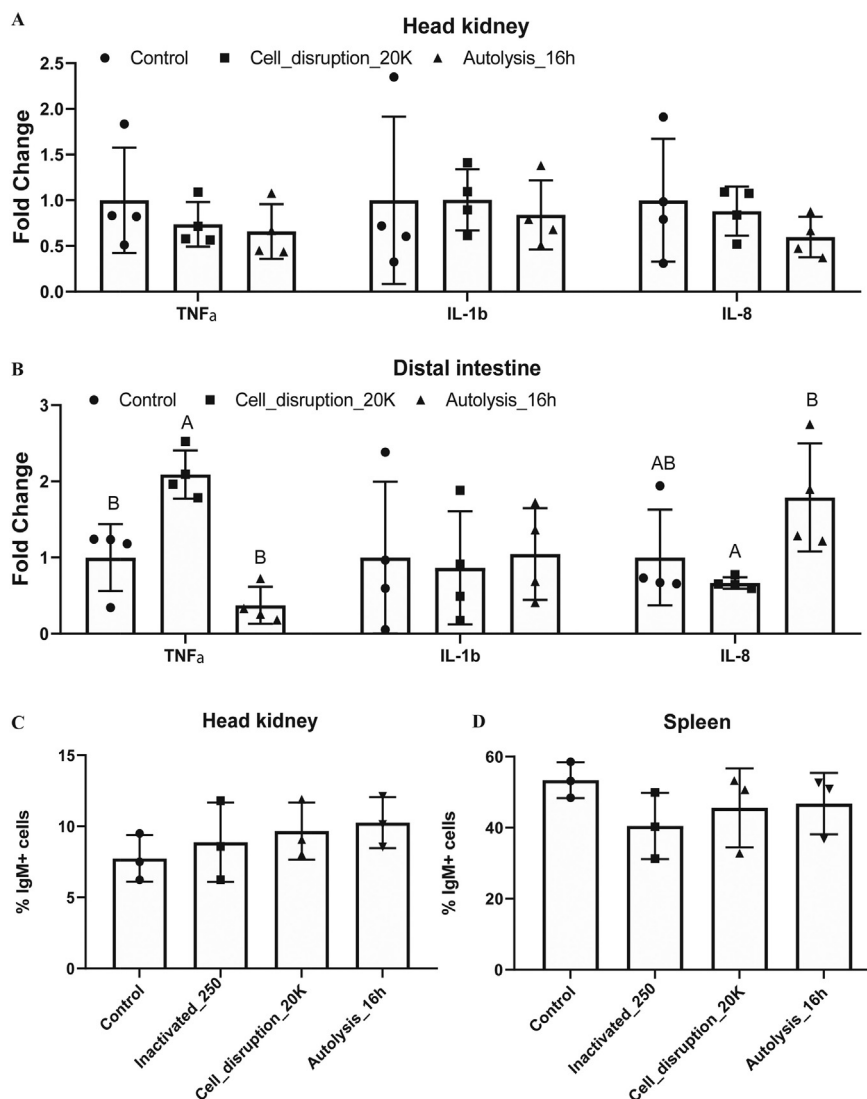

Fig. 5. Effect of processed *Saccharomyces cerevisiae* cells on the immune response of Atlantic salmon (*Salmo salar*). **A.** Head kidney and **B** DI protein expression of TNFα, IL-1β and IL-8, as measured by indirect ELISA. Values are presented as the mean fold change in relation to control ± SD of four fish per diet; letters denote a significant difference between dietary treatment. Percentage of B cells in head kidney **C** and spleen **D**, as measured by flow cytometry. Values are presented as the mean ± SD of three fish per diet.

autolyzed 16 h. Whereas a higher level of intact yeast was found in fish fed the directly inactivated yeast. To the authors knowledge, this is the first-time that flow cytometry has been used to count and detect yeast cells in intestinal content of fish. Further work is needed to be able to quantify and separate different strains of bacteria or yeast.

In order to study the immune response induced by the differently treated yeasts, the concentration of TNFα, IL-1β, and IL-8 were evaluated by ELISA, and quantification of IgM+ cells was done by flow cytometry. The present fish experiment was designed as a digestibility trial with 30% yeast inclusion, and thus, with high dietary inclusion levels of β-glucans and mannans. β-glucans derived from yeast are the most tested immunostimulant in fish with documented increased resistance for different pathogens (Ringø et al., 2012). A reason for this could be that β-glucan binding proteins (β-GBP) are one of the most important recognition receptors in fish, which facilitate recognition of β-glucan in the host and, thus, stimulate the innate immune system (Magnadóttir, 2006; Petit and Wiegertjes, 2016; Zhang et al., 2019). Production of cytokines, as a part of the innate immune system, did not increase in the head kidney of fish fed the different treated yeasts, as compared to fish fed the control diet. This is contrary to what was previously reported in fish fed yeast β-glucans (Robertsen et al., 1994; Meena et al., 2012). The reason for this discrepancy might be due to differences in down-stream processing of the yeast used in this study compared to industrially produced cell wall products. Nevertheless, an elevated level of TNFα in the DI of fish fed the disrupted yeast at 20 K and increased level of IL-8 in DI of the fish fed the autolyzed yeast was observed, suggesting a higher stimulation of the local immune system in the DI compared to more systemic response such as in the head kidney.

The increased solubility of the β-glucans post *in vitro* digestion from the crushed and autolyzed yeast could be expected to have increased immune-stimulatory effect. This is in line with Lee et al. (2001) who showed that mice fed soluble β-glucans from *S. cerevisiae* had increased macrophage activity and TNF-α secretion. The latter is also in line with Schiavone et al. (2019) who found increased detection of β-1,3-glucans on the surface of protease-treated yeast cells using atomic force microscope equipped with functionalized dendritips. Nevertheless, there was no clear systematic increased immune response with increased β-glucan solubility/degree of processing in the present study. Although no statistical differences in the number of IgM positive cells in the head kidney were observed, there was a numerically increased IgM with increased level of processing. Additionally, a study by Schiavone et al. (2015) showed that even though no alteration of the chemical composition in the yeast cell wall was detected after autolysis, the nanomechanical properties changed with a 2–4 increase in cell surface roughness for the yeast treated with autolysis. In agreement with this, several other studies have shown that nanomechanical properties of the yeast cell surface are more dependent on the molecule architecture of the cell wall and not its chemical components (Canetta et al., 2006; Pillet et al., 2014). This increased roughness or surface of the cell could have led to increased branching and available reactive molecules on the autolyzed yeast cell wall, therefore, leading to increased immune stimulation.

In conclusion, different down-stream processing methods of the yeast *S. cerevisiae* led to increased protein and β-glucan solubility that further increased protein digestibility in Atlantic salmon. Yeast that was autolyzed for 16 h obtained the highest protein digestibility that was supported by the poor detection of intact yeast cells in the distal intestine content. Finally, the different processing of the yeast triggered different immune stimulatory effects in Atlantic salmon.

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CRediT authorship contribution statement

Jon Øvrum Hansen: Conceptualization, Methodology, Formal analysis, Investigation, Resources, Data curation, Writing - original draft, Visualization, Project administration. **Leidy Lagos:** Methodology, Formal analysis, Investigation, Data curation, Visualization, Writing - original draft, Writing - review & editing. **Peng Lei:** Methodology, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing. **Felipe Eduardo Reveco-Urzua:** Conceptualization, Methodology, Project administration, Validation, Writing - review & editing. **Byron Morales-Lange:** Methodology, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing. **Line Degn Hansen:** Methodology, Project administration, Validation, Writing - review & editing. **Marion Schiavone:** Methodology, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing. **Liv Torunn Mydland:** Conceptualization, Methodology, Writing - review & editing, Supervision, Funding acquisition. **Magnus Øverlie Arntzen:** Methodology, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing. **Luis Mercado:** Methodology, Writing - review & editing, Funding acquisition. **Ricardo Tavares Benicio:** Formal analysis, Project administration, Validation, Writing - review & editing. **Margareth Øverland:** Conceptualization, Methodology, Writing - review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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