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Yeast as a protein source during smoltification of Atlantic salmon (*Salmo salar* L.), enhances performance and modulates health

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ABSTRACT

Yeast produced from lignocellulosic biomass has the potential to serve as a high-quality protein source with health benefits, especially during critical stages of the Atlantic salmon life cycle, such as during seawater transfer (SWT). In this study, we evaluated the effect of adding 25% *Candida utilis* yeast to salmon feed on growth performance and overall health by using morphometry, immunohistochemistry, cytokine enzyme-linked immunosorbent assays (ELISA) and gene expression analysis during and after SWT. There were four dietary treatments: 1) control diet in freshwater (FW) and seawater (SW) (Control); 2) control diet in FW and a yeast-based diet in SW (Control/Yeast); 3) yeast-based diet in FW and SW (Yeast); 4) yeast-based diet in FW and a control diet in SW (Yeast/Control). Our results showed that fish fed the yeast diet throughout the FW and SW period achieved higher feed intake and higher growth rate than fish fed the control diet. Morphometric and immunochemical analyses of the distal intestine (DI) revealed decreased length and number of CD3 labeled cells in the simple folds of fish fed control diet, while no changes were observed in fish fed the yeast diet. Furthermore, yeast significantly decreased the secretion of IFNγ, TNFα, IL-1β, IL-8, and modulated the gene expression of aquaporin 8 (*aqp8ab*), superoxide dismutase (*sod1*) and major histocompatibility complex 1 (*mhc1*) in DI, suggesting reduced inflammatory processes in yeast fed fish. These findings indicate that *Candida utilis* yeast is a promising alternative protein source with functional properties in diets for smolting Atlantic salmon before and after SWT.

1. Introduction

Atlantic salmon (*Salmo salar* L.) is the main species in Norwegian aquaculture, and as an anadromous fish undergoes a series of physiological, structural and functional changes in the transition from freshwater (FW) to seawater (SW). During this period, fish are more susceptible to stress, physical damage and infectious diseases (Stefansson et al., 2008). High mortality during seawater transfer (SWT) and the first months at sea represents a significant economic loss and a major challenge for the aquaculture industry (Hjeltnes et al., 2018). Current disease control strategies in aquaculture include the use of chemotherapeutics, vaccination and selective breeding programs. These measures may decrease smoltification stress, but additional action is required to increase salmon smolt robustness during SWT. The use of functional ingredients in fish feeds and adapted feeding protocols are a possible solution to these challenges.

The rapid growth in the aquaculture industry, but stable production of the main protein source, fishmeal (FM), has led to the increasing use of alternatives, resulting in significant changes in the composition of aquafeeds. Microbial ingredients such as bacterial meal (Aas et al., 2006; Romarheim et al., 2013; Romarheim et al., 2010; Vasanth et al., 2015) and yeast (Couture et al., 2019; Grammes et al., 2013; Meena et al., 2013; Micallef et al., 2017; Øverland et al., 2013), represent potential sustainable alternative protein sources in aquafeeds due to their high nutritional value, in addition to their content of a wide range of bioactive components with potential as functional ingredients. Feed ingredients are considered functional when they have health beneficial properties beyond their nutritional value (Montalban-Arques et al.,

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2015). The use of functional feeds to modulate the immune system has received increasing attention in fish farming (Herman and Schmidt, 2016; Kiron, 2012). The positive effects of yeast or its components have been documented in several studies for salmonids (Øverland and Skrede, 2017). However, yeast strain, processing technology, and inclusion level can result in varying effects on the immune system (Hauptman et al., 2014; Øverland and Skrede, 2017). Yeast produced from lignocellulosic biomass is a high-quality protein source with beneficial effects on fish health (Grammes et al., 2013). Yeast contains a wide range of components such as α-glucan, β-glucan, α-mannan, nucleic acids (known as microbe-associated molecular patterns; MAMPs) and antioxidants (damage-associated molecular patterns; DAMPs) (Navarrete and Tovar-Ramírez, 2014). The immune modulation functions of glucans have been demonstrated at transcriptional and protein level in salmonids, mainly the expression of pro-inflammatory cytokines such as TNFα, IL-8 or IL-1β (Morales-Lange et al., 2015; Salah et al., 2017). The latter compounds can strengthen the gut barrier of fish, improve immune status and increase resistance to infectious pathogens (Meena et al., 2013). When fish is fed a potential challenging plant-based diets combined with exposure to other stressors such as sub-optimal environmental condition (Mosberian-Tanha et al., 2018) or SWT, this could lead to adverse effects on intestinal function and an intensified immune response.

In the present study, we evaluated immunomodulatory effect of yeast under two challenging conditions (SWT and plant-based diet). We propose that the inclusion of *Candida utilis* yeast in fish diets has the potential to enhance immunity and alleviate stress related to smoltification and SWT. The objective of the present study was to investigate potential functional feed effects of moderate dietary levels of *Candida utilis* on Atlantic salmon growth rate, intestinal morphology, cytokine secretion, and transcriptional levels of genes involved in intestinal homeostasis and immune responses during the stressful SWT period.

2. Materials and methods

2.1. Experimental design, diets and feeding

Atlantic salmon (*Salmo salar* L.) pre-smolts were fed a commercial salmon feed (Skretting, Stavanger, Norway) prior to the start of the experiment, at the fish facility at the Norwegian University of Life Sciences, Aas, Norway. There were two diets, a plant-based control diet and an experimental diet with 25% *Candida utilis* (Table 1). The diets were produced by cold pelleting with a pasta machine (P35A Carasco, Genova, Italy) at the Norwegian University of Life Sciences, Aas, Norway. All dry ingredients (except gelatine), were mixed by a Morette Foreni kneading machine (Spiry 25, Mondofolo, Italy). The gelatine was dissolved in cold water and subsequently heated to 60 °C in a microwave oven and thereafter mixed with fish oil. Liquid and dry ingredients were mixed into a dough, which was processed in the pasta machine to form 3 mm pellets. These pellets were dried to a moisture content of 7–9%.

The experiment lasted for 56 days and consisted of two periods: FW (0–28 days) and SW (28–56 days). During the FW period, fish (n = 600) were placed in two 2800 l tanks with 300 fish per tank. One tank received the control diet and the other, the yeast-based diet. Continuous 24 h light was provided during FW and SW periods. Average water temperature during the FW period was 12 °C, water flow approximately 40 l min^{-1} and the oxygen saturation between 85 and 90%. Automatic belt feeders distributed feed continuously with a feeding level of 2% of the body weight plus 20% in excess per day. During SWT on day 28, 480 fish were transferred to the SW facility at the Norwegian Institute for Water Research (NIVA). Fish were distributed into 16 tanks of 300 l water capacity with 30 fish per tank. Fish fed the control diet during the FW period were randomly assigned to one of eight tanks. Four tanks were continued on the control diet, and the other four were switched to the yeast-based diet. Accordingly, fish fed the yeast-based diet during FW were either continued on the yeast-based diet or were switched to the control diet. This resulted in four dietary treatments, where fish were either fed: 1) control diet in FW and SW (Control), 2) control diet in FW and yeast-based diet in SW (Control/Yeast), 3) yeast-based diet in FW and SW (Yeast) and 4) yeast-based diet in FW and control diet in SW (Yeast/Control) (Fig. 1). Seawater was supplied directly from a depth of 60 m (salinity: 35 ppt; average temperature 7.7 °C). Intake water was passed through an UV filter and a drum filter to remove larger particles. Average water flow in each tank was adjusted to 7 l min^{-1} and oxygen saturation between 85 and 90%. In the SW period, fish were fed with electric belt feeders, once per day for 120 min in the morning. The amount of feed was based on 0.5–1% of the body weight, adjusted by the average feed consumption in each tank over the last seven days with 10% excess. Uneaten feed was collected for two weeks during the SW period. The recovery values for each feed (pellets) were measured per tank to ensure correct calculations of uneaten/eaten feed (dry matter (DM), g) according to Helland et al. (1996). The experiment was

Table 1
Diet composition.

Ingredient	Control	Yeast
Formulation %		
Fish meal[a]	15	10.65
Wheat gluten[b]	20	14.24
Soy protein concentrate[c]	20	14.24
Pre-gel potato starch[d]	8.9	5.3
Candida utilis[e]	0	25
Field beans[f]	3	2.14
Gelatin[g]	8	8
Cellulose[h]	4	0
Corn gluten[i]	3	2.13
Rapeseed oil[j]	8	8.25
Fish oil[k]	8	8.25
Threonine[l]	0.2	0
Lysine[m]	0.2	0
Rhodimet[n]	0.13	0.2
MCP[o]	0.8	0.8
Choline chloride[p]	0.15	0.15
Yttrium oxide[q]	0.01	0.01
Vitamins/mineral premix[r]	0.6	0.65
Chemical composition g kg $^{-1}$		
Crude protein	491	495
Crude lipids	161	176
Starch	80	62
Ash	47	55
Gross energy, MJ	22	22

[a] LT fishmeal, Norsildmel, Egersund, Norway.
[b] Wheat gluten, Amilina AB, Panevezys, Lithuania.
[c] SPC, Lyckeby Culinar, Fjälkinge, Sweden.
[d] Lygel F 60, Lyckeby Culinar, Fjälkinge, Sweden.
[e] *Candida utilis*, LYCC 7549, Lallemand Yeast Culture Collection.
[f] Dehulled field bean, Copenhagen Merchants, Denmark.
[g] Rousselot® 250 PS, Rousselot SAS, Courbevoie, France.
[h] Alpha-Cel™ C100, International Fibre Europe NV, Belgium.
[i] Heinz & Co. AG, Zurich, Switzerland.
[j] AAK, Karlshamn, Sweeden.
[k] NorSalmOil, Norsildmel, Egersund, Norway.
[l] L-Threonine, CJ Biotech CO., Shenyang, China.
[m] L-Lysine CJ Biotech CO., Shenyang, China.
[n] Rhodimet NP99, Adisseo ASA, Antony, France.
[o] Monocalsium phosphate, Bolifor® MCP-F, Oslo, Norway.
[p] Choline chloride, 70% Vegetable, Indukern s.a., Spain.
[q] Yttrium, Metal Rare Earth Limited, Shenzhen, China.
[r] 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.

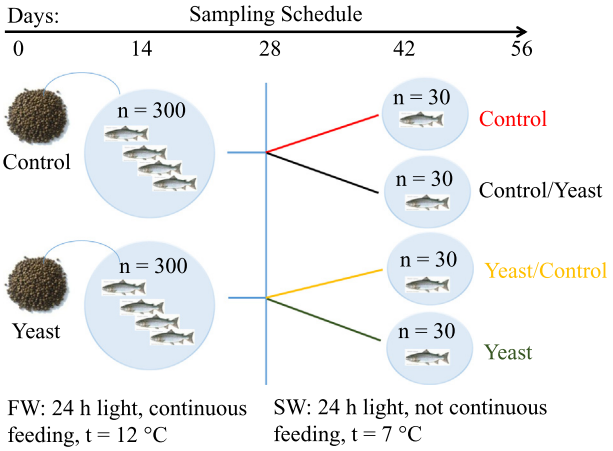

Fig. 1. Experimental design. The experiment consisted of two periods: the experimental freshwater period (FW) lasted for 28 days and included two diets: plant-based control diet (Control) and a plant-based diet with 25% yeast (*Candida utilis)* inclusion (Yeast). At seawater transfer (SWT) on day 28 (end of FW period), the Control group was divided into two groups, each in four tanks, receiving either control diet (Control) or yeast diet (Control/Yeast). Similarly, the Yeast group was divided into two groups, receiving either control diet (Yeast/Control) or yeast diet (Yeast). The SW period lasted for 28 days.

performed according to the guidelines established by the Norwegian Animal Research Authority. All animals were cared for, according to laws and regulations for experiments on live animals in EU (Directive 2010/637EU) and Norway (FOR-2015-06-18-761).

2.2. Yeast production

Carbohydrate substrate, containing monosaccharides (C5 and C6 sugars), was produced at Borregaard pilot plant, Sarpsborg, Norway), using wood chips from Norwegian spruce trees in a biorefinery process (BALI process; Sjöde et al., 2011; Sjöde et al., 2013; Sjöde et al., 2015). The BALI-sugars were mixed with beet molasses sugars in the ratio 1:1 in a 42,000-l fermenter (Lallemand plant, Salutaguse, Estonia), and were used as the main carbon source to grow yeast (*Candida utilis;* LYCC 7549; Lallemand Yeast Culture Collection). After fermentation, the yeast cells were washed, centrifuged and heat-inactivated before drum drying.

2.3. Sampling

Fish were sampled in FW on day 0, 14 and 28 (d0, d14 and d28) and in SW on day 42 and 56 (d42 and d56). Total weight of fish biomass was recorded per tank on d0, d28 and d56. During the FW period, 12 fish per tank were randomly sampled, anesthetized (80 mg l^{-1} MS222) and weighed individually. Then fish were dissected for tissue samples. For histology and immunohistochemistry, samples of distal intestine (DI) were placed in 10% neutral buffered formalin for 48 h at room temperature and further processed according to routine histological procedures. For gene and protein expression DI, spleen and head kidney of approximately 5x5x5 mm size were placed in cryotubes containing 1.5 ml RNAlater, placed at 4 °C for 24 h and stored at −80 °C until further analysis. During the SW period, three fish per tank (12 fish per treatment) were sampled using the procedure described above.

2.4. Histology and Morphometry

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). For morphometric measurements, 12 fish per dietary treatment, sampled in FW on d0, d14 and d28, and in SW on d42 and d56 were analyzed. Digital images were captured with a Zeiss AxioCam ERc5s camera connected to a light microscope (Zeiss Axio Lab.A1, Carl Zeiss, Germany). For the measurement of simple fold length, only fish in the Control or the Yeast diet treatment were examined. Morphometric measurements of the length of simple folds in the DI were performed by one individual (CP) on digital images of longitudinally orientated HE sections at 2.5× magnification using ImageJ software, version v1.51r (Schneider et al., 2012). The fold height was measured from stratum compactum to the tip of the epithelium lining the fold. Three simple folds were measured from each fish using the segmented line tool. A selected fold was the first full-length simple fold with an intact epithelium adjacent to a complex fold. Only one simple fold was selected for measurement between a pair of complex folds. Means were calculated based on three measurements.

2.5. Immunohistochemistry (IHC)

CD3 T lymphocytes were identified in DI tissue sections by IHC using a monoclonal anti-CD3ε antibody (dilution 1:600) (Boardman et al., 2012). Twelve fish from all dietary treatment collected at FW (d28) and SW (d42) were subjected to IHC analysis. Briefly, formalin-fixed, paraffin-embedded sections (4 μm) were mounted on poly-L-lysine-coated glass slides (Superfrost Plus, Thermo Scientific, Braunschweig, Germany) and left to dry at 37 °C. The slides were incubated at 58 °C for 30 min, deparaffinized in xylene, and rehydrated in graded alcohols to distilled water. Antigen retrieval was performed by using hydrated autoclaving at 121 °C for 15 min in 0.01 M citrate buffer, pH 6. Unless otherwise stated, sections were washed between steps in phosphate-buffered saline (PBS). The sections were incubated in phenyl hydrazine at 37 °C for 40 min to quench endogenous peroxidase activity. An automated immunostainer (Lab Vision™ Autostainer 360) was used for the IHC procedure. Non-specific antibody binding was blocked by incubating in normal horse serum. The sections were incubated at room temperature with the primary antibody for one hour. The tissue sections were incubated with the biotinylated secondary antibody and then with ABC/PO reagent (ABC Vectastain Elite kit). Immunolabelling was revealed using the 3-amino-9-ethylcarbazole solutions (ImmPACT® AEC, Vector Laboratories). Sections were counterstained in Alcian blue for 1 min and coverslips were mounted using a mounting medium (Aquatex). For negative control, the primary antibody was replaced with an irrelevant antibody.

For the calculation of the percentage of simple fold area occupied by CD3 T cells and goblet cells, images of immunohistochemically labeled sections at 10× magnification were analyzed by one individual (CP) using ImageJ. Three simple folds were selected as described for HE sections above. The freehand selection tool was used to trace the simple fold area from stratum compactum to the tip of the epithelium lining the fold, and a region of interest (ROI) defined. The fold area was measured from stratum compactum, including the middle of the fold base on each side, and the whole simple fold. The colour deconvolution plugin was used to extract the red (Colour 2) and blue (Colour 1) grey scale images. For the measurement of the area labeled for CD3, the red image was segmented, and threshold settings and particle size settings for detection of immunolabelling were then determined, and area measurements within the ROIs of each image were performed. The measurement of threshold particles was confined to the ROI (simple fold) and the percentage of area calculated. A mean for each individual was calculated based on the three measurements. The same procedure was performed on the blue image to determine the mean percentage of simple fold area occupied by Alcian blue stained goblet cells.

2.6. RNA extraction, cDNA synthesis, quantitative real time PCR

Total RNA was extracted using RNeasy® Plus Universal protocol

Table 2
Primer used in qPCR analyses.

Gene name	Genes	Fwd/Rwd	Primer sequence	GenBank accession.no	Ref.
Hypoxanthine phospho-ribosyltransferase 1	HPRT 1	Fwd	CCGCCTCAAGAGCTACTGTAAT	XM_014212855.1	(Kortner et al., 2013; Kortner et al., 2012)
		Rev	GTCTGGAACCTCAAACCCTATG		
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	Fwd	AAGTGAAGCAGGAGGGTGGAA	XM_014141819.1	(Kortner et al., 2013; Kortner et al., 2012)
		Rev	CAGCCTCACCCCATTTGATG		
Superoxide dismutase	SOD1	Fwd	CCACGTCCATGCCTTTGG	NM_001123587.1	This study
		Rev	TCAGCTGCTGCAGTCACGTT		
Aquaporin-8ab	AQP8ab	Fwd	GTTGGCATAGTTCTCCTTTGATG	XM_014179685.1	(Kortner et al., 2013; Kortner et al., 2012)
		Rev	TTTCAACCCTCCCTTCACC		
Mucin 2	MUC2	Fwd	TCTGTCCTGATGGGATGAAAC	XM_014183074.1	(Sahlmann et al., 2013)
		Rev	GGACTCCAAACAAACGCAAT		
Interleukin 1 beta	IL1b	Fwd	TCAGGGTCTGGATCTGGAGG	XM_014170479.1	This study
		Rev	CACAGCACTCTCCAGCAAGA		
Heat shock protein 70	HSP70	Fwd	CCCCTGTCCCTGGGTATTG	XM_014137172.1	This study
		Rev	CACCAGGCTGGTTGTCTGAGT		
Glutathione S-transferase 3	GSTA3	Fwd	AACGCCCAGAAATAGCCTCT	NM_001140755.1	This study
		Rev	GACACGATTCATCCTCAGCA		
Matrix metallopeptidase 13	MMP13	Fwd	CCTTCCAAGTCCGAGGCTTT	NM_001140524.1	This study
		Rev	GGCTCATGAGGGTCGATGTT		
Major histocompatibility complex 1	MHC1	Fwd	TGCACTCTGTTCAGCAAACC	XM_014177344.1	This study
		Rev	ATACGTCCAACCAGCCTCAC		
Interleukin 8	IL8	Fwd	GGAAAGCGGCTCTCTTCTCAT	NM_001140710.2	This study
		Rev	AGTCTGTTGTTATCTCGCTGGT		

(Qiagen). The concentration of total RNA was determined using a NanoDrop TM 8000 spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). The RNA quality was checked using an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). Only samples of high quality (RIN ≥ 7) were included in the analyses. Purified total RNA was stored at −80 °C until further analysis. All samples were normalized to the same concentration (364 ng μl^{-1} for DI and 396 ng μl^{-1} for spleen), prior to cDNA synthesis. The cDNA synthesis was performed using an AffinityScript QPCR cDNA Synthesis kit (Agilent Technologies). In a total volume of 20 µl, 6 µl of RNA was added together with 3 µl random primers, 1 µl AffinityScript RT / RNase Block Enzyme Mixture and 10 µl cDNA Synthesis Master Mix. The conditions for the cDNA synthesis were: 25 °C for 5 min, 42 °C for 45 min, 95 °C for 5 min, 4 °C. A set of genes involved in intestinal homeostasis and epithelial integrity, oxidative stress responses and immune responses were analyzed by quantitative real-time PCR (qPCR), using LightCycler® 480 System (Roche). Two reference genes (*hprti* and *gapdh*) met the qualifications of transcriptional stability in DI, while in spleen, only *hprti* met the qualifications of transcriptional stability. The sequences of all primers used in this study are provided in Table 2. The qPCR reactions were conducted in a total volume of 20 µl using 10 µl of LightCycler 480 SYBR Green I Master, 2 µl of gene specific primers, 3 µl of Milli-Q® water and 5 µl of cDNA previously diluted 1:10. The qPCR condition were: 95 °C for 5 min, 95 °C for 10 s, 60–64 °C for 10–15 s depending on the primers, 72 °C for 15 s, in total 40–45 cycles. At the end of the program, a melting curve analysis was performed to confirm the absence of primers dimers or unspecific products.

2.7. Cytokine assay by indirect ELISA

For the detection of IFNγ, TNFα, IL-1β and IL-8 samples of head kidney, spleen and DI were homogenized in an automatic homogenizer using metal beads and lysis buffer (Tris 20 mM, NaCl 100 mM, Triton X-100 0.05%, EDTA 5 mM, and protease inhibitor cocktail 1 ×). 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 a Pierce BCA Protein Assay Kit (ThermoFisher Scientific) following the manufacturer's instructions. 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 (Boltana et al., 2018). 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 anti-epitope primary antibodies (IFNγ, IL1β, TNFα and IL8) at 1:200 dilution. Then, the second antibody-HRP (ThermoFisher Scientific) was incubated for 1 h at 37 °C at 1:7000 dilution. Finally, chromagen substrate 3,3′,5,5′-tetramethylbenzidine single solution (Thermo Fischer Scientific, USA) was added (100 µl) and incubated for 30 min in the dark, at room temperature. The reaction was stopped with 50 µl of 1 N sulphuric acid and read at 450 nm on a Spectramax microplate reader (Spectra Max M2, Molecular Devices).

2.8. Statistical analysis

The effects of dietary treatment on weight gain, feed intake and relative weight gain in SW period were determined using one-way ANOVA. Tukey post hoc comparisons were used for pairwise comparisons for differences of means between groups (dietary treatments). The described analyses were performed using Minitab 18. For group analysis and graphical presentation of the results for qPCR, histology, morphometry, IHC and cytokine levels, the statistical software GraphPad Prism® 7.0[8] was used for calculation of statistical measures, including mean values, standard deviation, D'Agnostino Pearson omnibus normality test, *t*-tests and ANOVA. In addition, two-way ANOVA was run for interaction by two main factors, factor 1 (diet composition before SWT) and factor 2 (diet composition after SWT). Statistical significance was declared at P-value < .05.

3. Results

3.1. Growth rate and feed intake

During the eight weeks experimental period, no mortalities were observed, and fish appeared to be in good health in both FW and SW period. Growth rate and feed intake estimates during FW and SW period are presented in Tables 3 and 4, respectively. Generally, the feed intake after SWT was low for both dietary groups. One way ANOVA (Table 4) revealed that fish fed yeast-based diet during the entire experiment had higher weight gain and feed intake (average eaten DM (g) per g fish) per treatment, for a two weeks period compared with fish fed the control diet during the same period. Two-way ANOVA revealed no interaction between diets in FW and SW on growth parameters; however, a significant interaction was found for feed intake. (Table 4).

Table 3
Mean initial and final fish weight for fish fed control or yeast-based diets during the FW phase. Calculated weight gain is presented as mean weight gain per fish and relative weight gain (RWG) is presented in percentage terms.

Freshwater	Control	Yeast
Initial fish weight (g)	80	85
Final fish weight (g)	105	115
Weight gain (g)	25	30
RWG %	31	35

3.2. Histology and Morphometry

DI of fish examined during the FW and SW period showed normal morphology, as described previously by others (Løkka et al., 2013). Simple folds were slender with a thin lamina propria. Intestinal epithelial cells were tall with the nucleus located basally, large vacuoles located apically and many intraepithelial lymphocytes. Goblet cells and rodlet cells were scattered among the epithelial cells. The lamina propria adjacent to the stratum compactum was thin and numerous eosinophilic granule cells were present (Fig. 2A). We did not observe differences between diets. The length of simple folds in the DI was measured in fish that received either the control diet (Control) or the yeast-based diet (Yeast) throughout the experiment, i.e., from day 0 to day 56 in both FW and SW periods. There was no difference in the length of simple folds between dietary treatments at the start of the experiment (Fig. 2B). Two weeks after transfer to SW (Fig. 2B; SW (d42)) there was a significant decrease in length of simple folds in the DI of fish on the control diet compared with the fish under the same diet before SWT (Fig. 2B; FW (d28)) ($P < .05$). There was no change in the length of the simple folds in fish fed the yeast-based diet in any of the sampling points (Fig. 2B).

3.2.1. Immunohistochemistry

The change in simple fold morphology that was detected with transfer to SW was investigated further by the morphometric examination of immunohistochemical sections labeled to identify CD3 T cells and counterstained with Alcian blue to identify goblet cells. At FW (d28), CD3 positive cells in Control and Yeast fed fish, showed an abundant presence at the base of the epithelium, and along the entire length of simple folds (Fig. 3A). Only a few CD3 labeled cells were observed in the lamina propria adjacent to the stratum compactum. CD3 labeled cells were rare in the lamina propria of the simple fold. Further some rodlet cells showed labeling for CD3. There was a diffuse labeling of smooth muscle, which was interpreted as background labeling. The percentage of simple fold area occupied by CD3 labeled cells and Alcian blue-stained cells were estimated for all dietary groups at FW (d28) and SW (d42) (Fig. 3B). There was no difference between the percentage of area occupied by CD3 labeled cells in the simple folds of fish fed the control and yeast diets in FW. Two weeks after transfer to SW (d42), there was a significant decrease in the presence of CD3 labeled cells in the simple folds of fish fed the control diet ($P < .05$) (Fig. 3B). However, there was no change in CD3 labeled cells in fish fed yeast diet. Two weeks after SWT coupled with a shift in diet, a difference ($P < .05$) in CD3 labeled cells between the fish that changed diet from control to yeast (Control/Yeast) and the fish that changed from yeast to control (Yeast/Control) was observed (Fig. 3B). Two-way ANOVA revealed an interaction between diets given in FW and SW on CD3 labeled cells. There were no differences detected between the fish groups in the presence of Alcian blue labeled goblet cells (Fig. 3C).

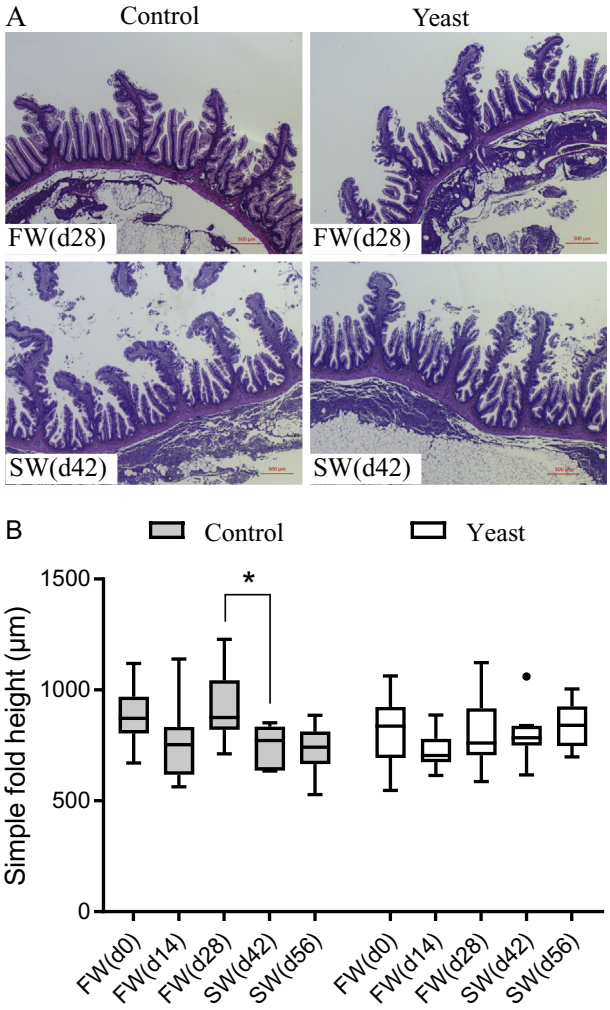

Fig. 2. Histological evaluation of distal intestine of Atlantic salmon. (A) Representative histological images of distal intestine from plant-based control diet (Control; images to the left) and plant-based diet with 25% yeast inclusion (Yeast; images to the right) at four weeks in freshwater FW (d28) and two weeks in seawater SW (d42). (B) Morphometric measurements of simple fold height of the distal intestine in Control and Yeast group at FW and SW. Data are expressed as mean and standard deviation, $n = 12$ for all groups. The black point outside the whisker range of SW (d42) is displayed as an outlier. Significant differences are denoted with * ($P < .05$).

Table 4
Fish weight at the start and the end of the SW period (g/fish), feed intake (g/fish/2 weeks period), and relative weight gain percentage (RWG %) for the four dietary treatments in the SW phase. Data are presented as means ± standard error per fish per dietary treatment (three fish per tank; four tanks per treatment). Means with different superscript letters in a row are significantly different ($P < .05$).

Seawater	Control	Control/yeast	Yeast/control	Yeast	*P*-value
Initial weight (g)	117 ± 0.91^{a}	115 ± 0.8^{a}	128 ± 0.41^{b}	129 ± 1.2^{b}	< 0.0001
Final weight (g)	120 ± 2.7^{a}	122 ± 3.5^{a}	140 ± 0.51^{b}	142 ± 1.5^{b}	< 0.0001
Weight gain (g)	3 ± 2.6^{a}	6 ± 3.8^{ab}	12 ± 0.42^{ab}	14 ± 1.2^{b}	0.017
Feed intake (DM, g)	1.54 ± 0.05^{a}	1.96 ± 0.06^{b}	1.85 ± 0.06^{b}	2.22 ± 0.03^{c}	< 0.0001
RWG %	3 ± 2.2^{a}	6 ± 2.7^{ab}	9 ± 0.34^{ab}	11 ± 0.95^{b}	0.041

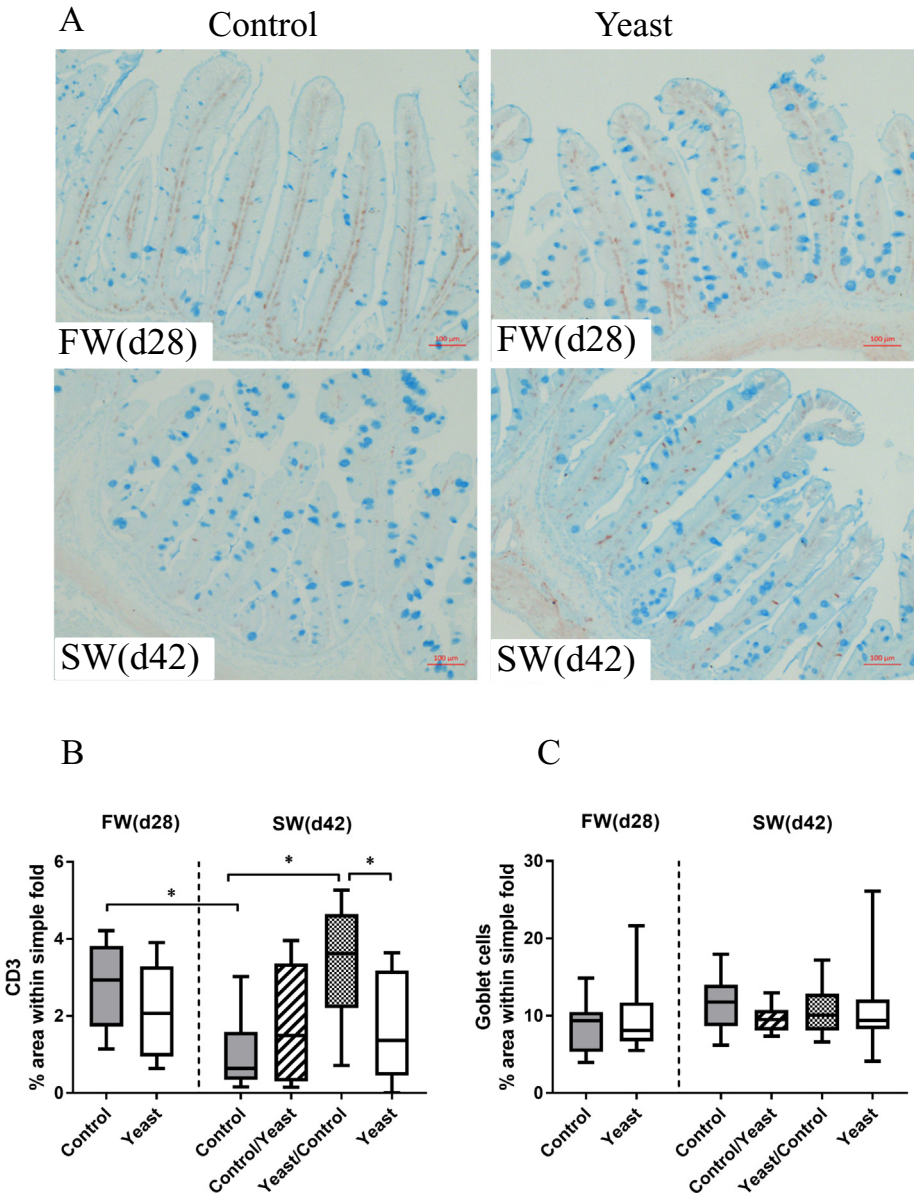

Fig. 3. Immunohistochemical evaluation of distal intestine of Atlantic salmon. (A) Representative immunochemistry images of DI tissue from plant-based control diet (Control; images to the left) and plant-based diet with 25% yeast inclusion (Yeast; images to the right) at four weeks in freshwater FW (d28) and two weeks in seawater SW (d42). Red colour indicates the area of simple folds stained for CD3-T cells and blue colour indicates the area of simple folds stained for Goblet cells. (B) CD3-T cell % area within the simple fold. (C) Goblet cell percentage area within the simple fold. Data are expressed as mean and standard deviation, $n = 12$ for all groups. Significant differences are denoted with * ($P < .05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. Gene expression in DI and spleen

The expression of several genes representing various regulatory and immune functions were analyzed. The results in DI revealed increases ($P < .05$) in the expression levels of aquaporin 8 (*aqp8ab*) in Yeast treatment compared with the Yeast/Control treatment at the final sampling (d56) (Fig. 4A). The comparison between FW and SW for Yeast and Control, showed increases in the expression of *aqp8ab* in SW (d56) compared to FW (d28) for both dietary treatments, with significant interaction between diets given before and after SWT. At the same time point (d56), expression of superoxide dismutase (*sod1*) in the DI was lower in the Yeast treatment compared with the Control/Yeast and Yeast/Control treatments, and a trend for decreased expression compared with Control with significant interaction between diets given before and after SWT (Fig. 4B). Expression levels of the pro-inflammatory marker interleukin 1 beta (*il1b*) showed a trend for decreased expression in Yeast and Control/Yeast compared with Control treatment in SW (d56) (Fig. 4C). No differences were observed in the expression levels of interleukin 8 (*il8*) at any time point or in any treatments for DI, although, there was a trend for decreased expression of *il8* in Yeast and Control/Yeast compared with Control in DI in SW (d56) (Fig. 4D). On the other hand, the results revealed a significant upregulation ($P < .05$) of major histocompatibility complex 1 (*mhc1*) gene in DI in fish fed the Yeast diet in FW (d28) compared to fish fed the Control (Fig. 4E), whereas the expression level of *mhc1* was lower in the SW period as compared to the FW period for both Control and Yeast. Mucin 2 (*muc2*) gene expression in DI did not reveal significant differences between the dietary treatments (Fig. 4F).

3.4. Protein levels of cytokines

Indirect ELISA was performed on DI, spleen and head kidney from fish in SW (d56). There was a significantly decreased protein level of IFNγ, TNFα, IL-1β, IL-8 and in DI of fish fed Yeast compared to Control (Fig. 5A). No significant changes were observed in the protein level of any of the cytokines evaluated in head kidney or spleen (Fig. 5B-C).

4. Discussion

In the present study, we investigated the potential of using 25% *Candida utilis* yeast as a functional feed ingredient to improve feed intake, growth rate and reduce stress during the critical period of SWT on

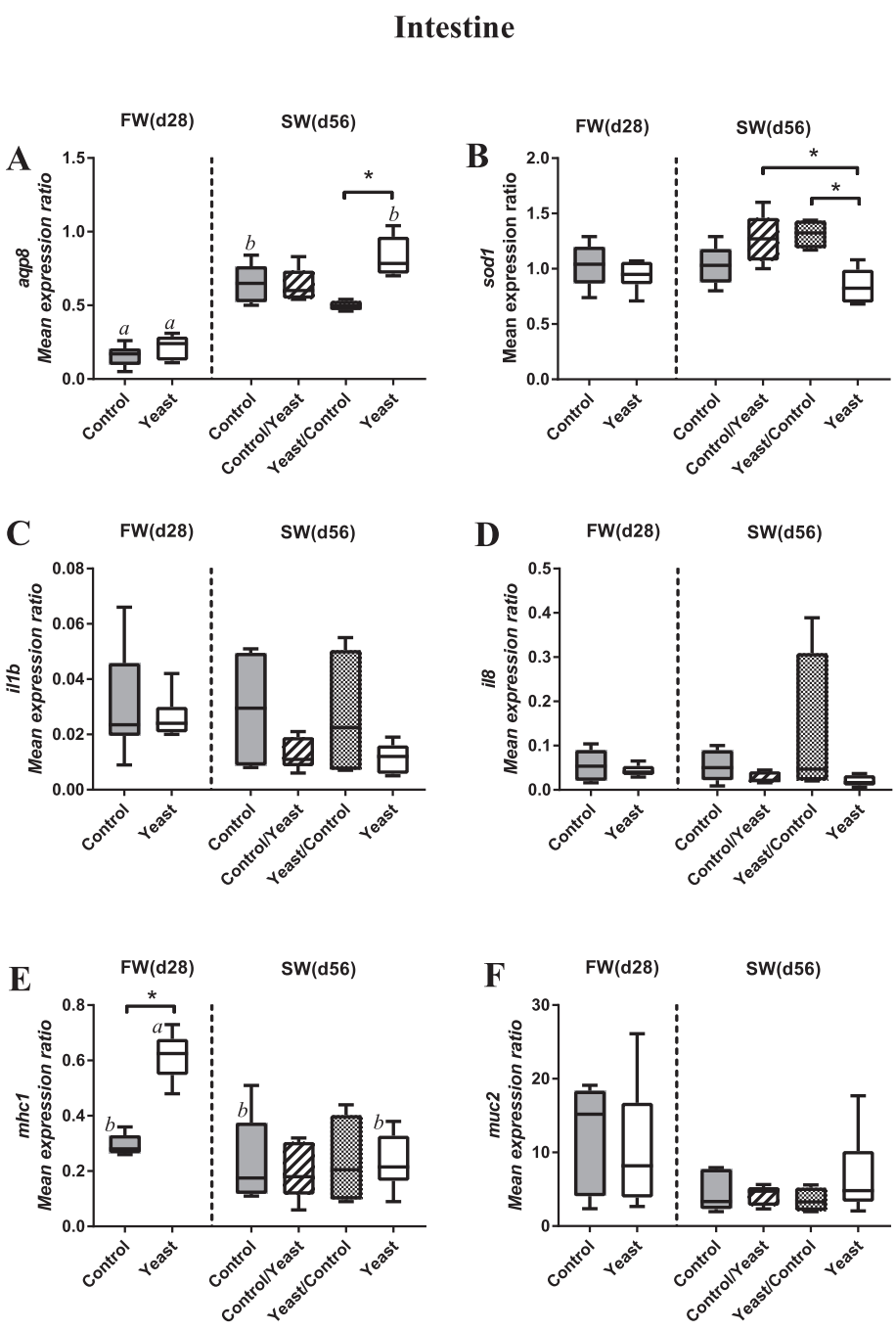

Fig. 4. Quantitative real-time PCR data of selected genes in distal intestine: (A) Aquaporin-8ab (*aqp8*) (B) Superoxide dismutase (*sod1*) (C) Interleukin 1 beta (*il1b*) (D) Interleukin 8 (*il18*) (E) Major histocompatibility complex 1 (*mhc1*) (F) Mucin 2 (*muc2*). Data are presented as mean expression ratio with box and whisker plots. Boxes mark the interquartile range, the line dividing the boxes denotes the median, and whiskers extend to 1.5 x interquartile range. A significant difference between dietary treatments during the whole experiment is denoted with * ($P < .05$). A significant difference between FW and SW period for Control and Yeast treatment are denoted with different letters ($P < .05$).

Atlantic salmon. The rationale of including 25% of yeast was to evaluate yeast as a protein source with functional properties and not only as an additive. Lower levels of yeast inclusion have also demonstrated beneficial health effects on Atlantic salmon (Hansen et al., 2019). Many yeast strains contain several bioactive components such as β-glucans, mannan, chitin and nucleotides, which have been shown to induce a general immune response, protect against bacterial infections, improve gastrointestinal barrier function and disease resistance (Mohan et al., 2018). The transition from FW to SW exposes salmon to large environmental changes that cause stress and can have detrimental effects on growth performance and survival. Some of the challenges of SWT includes osmotic changes and changes in naturally occurring bacteria in water. To the best of our knowledge, this is the first investigation of the effect of yeast inclusion during SWT and its effect on smolting Atlantic salmon.

In the present study, fish fed diets containing 25% yeast during the FW and SW period, grew faster and were more robust during the first weeks in SW. Moreover, fish fed yeast through the entire period also had a higher feed intake and the results demonstrated a significant interaction between feeding yeast in FW on the feed intake in SW. This result could be due to increased palatability of the diet containing yeast. Yeast possesses feed enhancing properties, including nucleotides and glutamate, which has proven to be taste enhancers in fish (Kasumyan and Døving, 2003). These properties could be an advantage when used in combination with plant-based ingredients which can lead to reduced feed intake due to their content of a wide range of antinutritional factors (Krogdahl et al., 2010). Immediately after SWT, fish usually do not exhibit typical feeding behavior and it can take several weeks until fish start eating normally. In our study, the fish presented this characteristic behavior of low feed intake after SWT. Fish fed yeast-based diet through the entire experimental period (Yeast) or yeast in the FW period and then the plant-based control diet in SW (Yeast/Control) had a higher

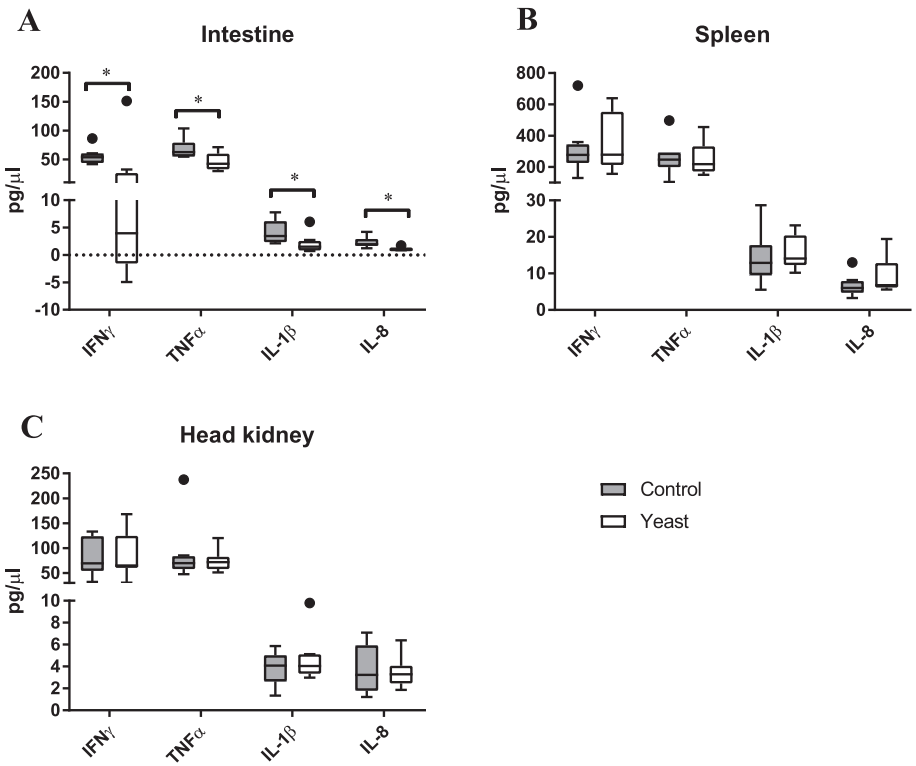

Fig. 5. The protein level of IFNγ, TNFα, IL-1β and IL-8 measured by indirect ELISA in (A) distal intestine (B) spleen and (C) head kidney. Data are presented in pg/µl, as mean and standard deviation. Black points outside whisker range are displayed as outliers. Significant differences between treatment are denoted with * ($P < .05$).

weight gain than fish in Control or the Control/Yeast treatment. No significant interaction between feeding yeast in FW and fish growth in SW was observed. Long term feeding trial might be an option to assess the effect of yeast on growth. Our histology results demonstrated significantly reduced length of simple folds after SWT in Control but not in Yeast treatment. This reduction has also been reported in fish fed diets with high levels of soybean meal (Baeverfjord and Krogdahl, 1996; Booman et al., 2018), pea protein concentrate (Kortner et al., 2012; Penn et al., 2011) and in fish fed diets where fish oil was substituted with plant oils (Moldal et al., 2014). In our study, we used a plant-based control diet, which could have induced mild inflammation when fish were transferred to SW. Starvation could also be a possible cause of reduced simple fold length in SW (d42), as reported in earlier studies (Baeverfjord and Krogdahl, 1996). The decreased levels of CD3 positive T cells at the same time point in DI of the Control treatment could indicate an immunosuppression after SWT as observed by Johansson et al. (2016). In contrast, fish in the Yeast group did not show a decrease in CD3 positive T cells. Furthermore, fish that were switched from yeast to control diet in SW presented higher levels of CD3 positive T cells in the DI epithelium (Yeast/Control), compared with the Control in SW, suggesting that yeast can reduce changes in immune cells induced by SWT or plant-based diets. Earlier studies showed that the DI of salmon reacts uniquely when challenged with a new diet. Soybean meal-induced enteritis is probably the best described condition for intestinal inflammation in salmon, causing inflammation only in the DI, while other intestinal regions and organs are unresponsive (Baeverfjord and Krogdahl, 1996; Chikwati et al., 2013). Our findings also confirmed this, having the effects of a change in the diet most prominently observed in the DI. The increased expression of aquaporin (*aqp8ab*) in the SW period compared to FW period in DI was in accordance with previously reported findings and as the expected response to increased salinity. Engelund et al. (2013) indicated the physiological role of *aqp8ab* in transcellular water uptake across intestinal enterocytes upon SWT. We observed the trend of higher *aqp8ab* expression in SW (d56) in Yeast treatment indicating beneficial yeast effect on the regulation of water and ion transport during smoltification. In some studies, the inclusion of new microbial ingredients into fish diets, greatly altered mucus production and epithelial morphology (Ouwehand et al., 2005; Torrecillas et al., 2014). Herein, however, we did not detect differences in mucin 2 (*muc2*) expression at the selected sampling points. This finding correlates with histological observations where no differences between dietary treatments regarding the abundance of goblet cells were seen. Several metabolic processes produce reactive oxygen species (ROS) that may cause oxidative stress when the balance between ROS and antioxidant is disturbed. Therefore, the expression of superoxide dismutase (*sod1*) plays a key role in controlling oxidative stress. In the present study, *sod1* was significantly lower in the Yeast treatment compared with the Control/Yeast and Yeast/Control in SW. As discussed earlier, immune-modulating abilities of feed are widely recognized and dietary changes can trigger an immune response that can be either beneficial or detrimental. Cytokines are secreted by activated immune-related cells and play pivotal roles as immune modulators. In our study, we show that the inclusion of yeast to diets triggered an intestinal immune modulation decreasing the secretion of IFNγ, TNF-α, IL-1β, and IL-8 locally in DI, but apparently without inducing a systemic response (i.e., no difference was observed in spleen or head kidney). Cytokine autocrine and paracrine modes of action have been previously documented in fish (Wang et al., 2011). Similarly, gene expression of the pro-inflammatory cytokines *Il1b* and *Il8* in DI were decreased in Yeast compared with the Control group in SW (d56). *Il1b* facilitates the organism to react quickly to infection by inducing the cascade of responses leading to inflammation (Zou and Secombes, 2016). Besides its role in inflammatory regulations, it has other diverse physiological functions, as affecting muscle metabolism and growth (Pooley et al., 2013; Valenzuela et al., 2017). Interestingly, a significant up-regulation of *mhc1* in the DI of yeast fed fish was observed right before SWT. In mammals, major histocompatibility complex class I (MHC-1) molecules are key players in discriminating self from non-self. If foreign elements,

such as viruses, are present in the cytoplasm, they are prone to degradation and presentation by MHC-1 molecules. It is assumed that presenting a larger repertoire of pathogen epitopes activates a wider range of T cell clones, a response needed for protection against pathogens (Tregaskes et al., 2016). Atlantic salmon possesses unique genome duplication, turning duplicated MHC-1 region into a remarkable target to study functional advantages of different immune activation in salmonids (Grimholt, 2016). The upregulation of *mhc1* induced by yeast in this study may be of advantage in boosting the immune system before SWT. However, the upregulation of *mhc1* was followed by a slight downregulation on d56 in SW in the Yeast group, suggesting an immune system depression post SWT that is consistent with the findings by Johansson et al. (2016) and Jarungsriapisit et al. (2018). Nevertheless, the immune suppression responses at the transcriptome level were not as pronounced as reported previously. It may be that the peak of immune suppression is occurring immediately after SWT and within the first week post-SWT and, therefore, the gene expression levels may already have normalized.

5. Concluding remarks

This study described the effects of 25% *Candida utilis* yeast inclusion in diets for Atlantic salmon pre and post SWT. *Candida utilis* inclusion improved weight gain and feed intake, triggered a lower secretion of cytokines in DI (IFNγ, TNF-α, IL-1β and IL-8), as well as decreased the expression of its genes. Yeast successfully modified immunosuppressive responses related to SW acclimation. In contrast to the control fish, the yeast fed fish did not get reduced length of simple folds or decreased levels of CD3 cells after SWT. The potential beneficial effects of yeast merit further investigation towards specific nutritional programming and lower inclusion levels of yeast to improve health and robustness during SWT. Overall, the study suggests that yeast is a high-quality protein source with beneficial health properties in diets for smoltifying Atlantic salmon.

Conflict of interest

The authors declare that they have no conflict of interest.

Submission declaration

All authors read and approved the final manuscript for submission. The content of the manuscript has not been published, or submitted for publication elsewhere.

Authors' contributions

CS, MØ, LTM and JØH designed the experiment. CS coordinated the execution of the experiment as well as samplings and coordinated qPCR and did the primer design and quality check. CS also performed data analysis and statistics of the qPCR. JH and CS designed feed and performed feed production. MB was helping during the practical part of the feeding experiment and laboratory analysis. LM designed antibodies and BML performed Elisa assay. RÅ performed the qPCR analysis. CMP performed histology and immunohistochemistry. BD was involved in manuscript writing and reviewing, data analysis, producing figures and tables for the manuscript as well as statistical analysis and quality checking. MØ was involved in writing and reviewing the manuscript. LL data analysis for ELISA, manuscript reviewing. LTM was involved in manuscript reviewing.

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