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Effect of *Candida utilis* on growth and intestinal health of Atlantic salmon (*Salmo salar*) parr

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ABSTRACT

In this study, *Candida utilis* was included as alternative protein source in diets for Atlantic salmon (*Salmo salar*) parr and the effects on growth performance and distal intestinal (DI) health were assessed. The potential of *C. utilis* to counteract possible adverse effects of a high soybean meal (SBM) diet was additionally assessed by using graded levels of *C. utilis* in combination with a 40% SBM diet. Six experimental diets were formulated: Fishmeal control (FM); FM with 20% *C. utilis* (FM20CU); SBM-based diet containing 40% SBM (SBM); three SBM-based test diets, where wheat gluten and starch were substituted with increasing levels of *C. utilis* of 5%, 10% and 20% (SBM5CU, SBM10CU and SMB20CU, respectively). A total of 2700 Atlantic salmon parr with an average weight of 4.4 g were distributed into 18 tanks and the diets were fed in triplicates. The experiment lasted 28 days and sampling for histology and gene expression analysis was done at the end of the experiment.

The fish grew to an average weight of 14.8 g in 28 days. Fish fed FM20CU obtained a significantly higher specific growth rate (SGR) of 4.59 than the other dietary groups. While fish fed FM and FM20CU displayed normal morphology of the DI, fish fed SBM-based diets showed mild histological changes in the DI that can be related to classical SBM induced enteritis (SBMIE). These changes included reduced height of simple folds and decreased presence of supranuclear vacuoles. The severity of these changes was not altered with increasing levels of *C. utilis* in the SBM diet. The expression of four of the five selected genes analyzed in the DI were altered by 40% dietary SBM inclusion. Aquaporin 8ab demonstrated the clearest changes among dietary groups, showing down-regulation independent of dietary inclusion of *C. utilis*. The strong down-regulation of aquaporin has been observed before and may be an indicator for intestinal barrier dysfunction. In conclusion, adding *C. utilis* to diets for Atlantic salmon parr gave high growth performance without any obvious adverse effects on intestinal health, but were unable to counteract the mild histology changes seen in DI of the SBM fed fish.

1. Introduction

Microbial ingredients (MI) produced from yeast or bacteria have shown to be a high-quality protein source for Atlantic salmon (*Salmo salar* L.) (Storebakken et al., 2004; Øverland et al., 2013) and rainbow trout (*Oncorhynchus mykiss* W.) (Martin et al., 1993). *Candida utilis*, commercially called Torula yeast, has been produced and evaluated in food and animal feeds for decades (Peterson et al., 1945). There has also been increased interest for using *C. utilis* in production of amino acids and enzymes (Liang et al., 2008; Ikushima et al., 2009).

While global finfish production has increased during the last decades, annual fishmeal (FM) production has remained steady. This imbalance has encouraged increased use of plant ingredients in finfish diets. Increased use of plant ingredients has reduced fish growth and welfare due to both unknown factors and a wide range of antinutritional factors (ANF) in plant ingredients (Krogdahl et al., 2010). The distal intestine (DI) is the target organ for changes following exposure to dietary anti-nutrients in Atlantic salmon (van den Ingh et al., 1991; Baeverfjord and Krogdahl, 1996). The use of high levels of extracted soybean meal (SBM) in diets for Atlantic salmon can induce inflammation in the DI (Van den Ingh et al., 1991), a condition referred to as soybean meal induced enteritis (SBMIE). Generally, SBMIE is thought

Abbreviations: Distal intestine, DI; Fishmeal, FM; Microbial ingredients, MI; Soybean meal, SBM; soybean meal induced enteritis, SBMIE; specific growth rate, SGR

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to be caused by the various antinutritional factors (ANFs) present in SBM. The effects of ANFs are often linked to sub-optimal digestibility of nutrients and a lack of proper barrier function in the gut that increases susceptibility to diseases (Becker et al., 2001; Knudsen et al., 2007; Knudsen et al., 2008; Krogdahl et al., 2010). Similar intestinal inflammatory processes have also been reported in salmon fed diets containing 40% pea protein concentrate (Penn et al., 2011) and for both salmon parr and post-smolt fed faba bean protein concentrate (De Santis et al., 2016; De Santis et al., 2015).

Previous work from the present group has shown that certain MI in various dietary inclusion levels have the ability to counteract SBMIE in Atlantic salmon. For example, bacterial meal produced from *Methylococcus capsulatus* grown on natural gas (Romarheim et al., 2011). Further investigations revealed that this ability seemed to be linked to the cell wall fraction of this bacterium (Romarheim et al., 2013). Various yeast strains have also shown similar effects on SBMIE in Atlantic salmon (Grammes et al., 2013). It is important to point out that SBMIE is not currently an issue in Norwegian salmon farming as SBM is not included in commercial compound feeds, in contrary to soy protein concentrate (Ytrestøyl et al., 2015). However, farmed salmon are under risk of developing SBMIE-like intestinal disorders due to the dietary inclusion of other plant-based ingredients containing saponins and other ANFs (Kortner et al., 2012). Thus, feeding MI represents a feeding strategy to improve the intestinal health and create a more robust fish in the presence of plant-based diets.

It has been suggested that the severity of SBMIE might be related to the life stage of salmon (Sahlmann et al., 2015). Further, limited information exists on using high dietary levels of SBM for salmon in the early life phases with respect to growth and health. De Santis et al. (2015) have shown that feeding a diet containing 36% SBM to salmon parr as a positive control increased the presence of goblet cells and decreased levels of supranuclear vacuoles in the DI compared with that seen in the healthy fish group fed a FM-based negative control diet. In addition, there is scarce documentation available on including *C. utilis* in diets for salmon (Grammes et al., 2013; Øverland et al., 2013), and to our knowledge, none concerning the early parr stage. To elucidate the relationship between nutrition and health in young salmon in freshwater, the aim of the present study was to evaluate: 1) effects of high dietary inclusion of *C. utilis* and SBM on growth and general intestinal health in Atlantic salmon parr; 2) the ability of the *C. utilis* strain to maintain the intestinal health of salmon parr fed diets containing high SBM inclusion.

2. Materials and methods

2.1. Diets

Six experimental diets were pelleted using gelatin as main binder (Tables 1 and 2). The diets were: a FM based diet as a negative control containing 50% FM; a FM-based test diet containing 20% *C. utilis* (FM20CU); a SBM based diet as a positive control containing 40% SBM (SBM); and three SBM-based test diets, where wheat gluten and starch were substituted with increasing levels of *C. utilis* of 5%, 10% and 20% (SBM5CU, SBM10CU and SMB20CU, respectively). The diets were formulated to have a similar ratio of digestible protein to digestible energy (Table 2). To adjust for the large span in protein and amino acid levels in FM, SBM and *C. utilis,* it was necessary to add the crystalline amino acids, threonine, lysine and methionine. All dry ingredients were mixed in a Morette Foreni machine (Spiry 25, Mondolfo, Italy). Gelatin was mixed in cold water and heated up to 60 °C in a microwave oven before mixing with dry ingredients and fish oil. The mash was cooled down to room temperature prior to pelleting (P35A, Carasco, Italy). The pellets were dried in small experimental dryers at approximately 60 °C drying temperature and stored at 5 °C prior to feeding.

Table 1
Chemical composition of fishmeal (FM), soybean meal (SBM), *Candida utilis* and wheat gluten used in the experimental diets.

	FM	SBM	*Candida utilis*	Wheat gluten
Composition, g/kg				
Dry matter	923	889	920	919
Crude protein	676	457	391	746
Crude lipid	91	12	21	6
Ash	146	60	67	9
Amino acids[a]				
Total amino acids[a], *g/16 g N*	74.4	82.4	60.0	82.6
Essential amino acids, *g/16 g N*				
Lysine	7.1	5.8	5.0	1.3
Threonine	3.8	3.6	3.8	2.1
Methionine	2.5	1.3	0.93	1.3
Valine	4.4	4.4	3.7	3.5
Isoleucine	3.7	4.2	3.1	3.2
Leucine	6.5	7.3	4.9	6.3
Phenylalanine	3.4	4.9	3.0	4.8
Histidine	1.9	2.6	1.4	2.0
Arginine	5.7	7.0	3.6	2.9
Non-essential amino acids, *g/16 g N*				
Cysteine	0.8	1.4	0.70	1.8
Aspargine	8.3	10.7	6.5	2.8
Serine	3.8	4.6	3.5	4.2
Glutamine	12.1	16.9	12.0	30.4
Proline	3.6	4.7	2.3	11.6
Glycine	5.2	3.5	2.7	2.7
Alanine	5.0	3.6	4.0	2.0
Tyrosine	2.3	2.9	2.5	2.6

[a] Total sum of amino acids without tryptophan.

2.2. Biological experiment and facilities

The fish trial was performed at the Norwegian University of Life Sciences (NMBU), Ås, Norway. The experimental procedures were performed in accordance to the institutional and national guidelines for the care and use of animals (the Norwegian Animal Welfare Act and the Norwegian Regulation on Animal Experimentation).

In total, 2700 Atlantic salmon with average weight of 4.4 g and age of 23 weeks post-hatch were distributed into 18 tanks. The 80-l tanks were receiving 4 l recirculated freshwater per min, and oxygen and water temperature were measured daily. The feeding experiment lasted for 28 days with a mid-weighing after day 16 and with an average water temperature of 15.2 °C throughout the experiment. Each diet was fed to triplicate tanks in excess of appetite, 120% of expected feed intake, to ensure maximum voluntary feed consumption.

2.3. Sampling

All fish were pooled and weighed at the start of the experiment and at day 16 and 28. At the end of the experiment, 10 fish per tank were randomly selected and anaesthetized with Tricaine mesylate (Finquel®, Scan Aqua, Årnes, Norway; 50 mg/l) and euthanized with a blow to the head. Body weight (BW) were recorded and included in total tank mean. Tissues were collected from five fish for histology. The abdominal cavity was opened and the DI was removed. The DI was defined as extending from where the diameter of the intestine increased and annular rings were visible to the rectum (Sanden et al., 2005). The DI was opened longitudinally and rinsed in PBS, and then fixed in 10% phosphate-buffered formalin for 24 h before storage in 70% ethanol until further processing (Løkka et al., 2013). The DI tissue from another 5 fish was sampled and snap frozen in liquid nitrogen and stored at −80 °C for gene expression analysis.

2.4. Chemical analyses

The ingredients and diets were analyzed for dry matter by drying to constant weight at 104 °C, crude protein using Kjeldahl nitrogen

Table 2
Diet formulation and chemical composition of the fishmeal control (FM), fishmeal control including 20% *Candida utilis* (FM20CU), soybean meal diet (SBM) and 3 diets with 40% SBM and increasing level of *C. utilis* (SBM5CU, SBM10CU, SBM20CU).

g/kg	FM	FM20CU	SBM	SBM5CU	SBM10CU	SBM20CU
Fishmeal[a]	502	502	200	200	200	200
Soybean meal[b]			400	400	400	400
Candida utilis[c]		200		50	100	200
Wheat gluten[d]	200	74	143	112	76	6
Potato starch[e]	130	47	64	40	30	
Gelatin[f]	60	60	60	60	60	60
Fish oil[g]	100	100	110	110	110	110
MCP[h]		7.6	8.7	14.3	11.3	14
L-Threonine[i]			2	1	1	
L-Lysine[j]			4.5	3.3	2.3	0.7
Methionine[k]			1.6	1.6	1.6	1.6
Choline[l]	1.5	1.5	1.5	1.5	1.5	1.5
Mineral and vitamin premix[m]	6.3	6.3	6.3	6.3	6.3	6.3
Composition. g/kg						
Dry matter	912	901	918	919	917	921
Crude protein	543	517	485	477	469	453
Crude lipid	136	141	114	127	126	122
Starch	127	63	67	55	48	29
Ash	79	94	65	71	71	78
Phosphorus	11.4	13.5	8.9	8.9	10.3	9.5
Calcium	18.4	18.8	11.2	12.8	12.4	13.9
Sodium	4.0	3.8	1.9	1.8	1.7	1.7
Magnesium	1.3	1.5	1.8	1.9	1.9	2.0
Potassium	4.0	6.6	7.1	7.8	8.7	10.4
Gross energy MJ/kg	21.1	20.4	20.8	20.6	20.6	20.6
DP: DE ratio[n]	24.1	24.2	23.6	23.8	23.7	23.8

[a] LT fishmeal, Norsildmel, Egersund, Norway.
[b] Non-dehulled hexane extracted soybean meal, Non-GMO, Denofa AS, Fredrikstad, Norway.
[c] Lake States® Torula, Lallemand, USA.
[d] Wheat gluten, Amilina AB, Panevezys, Lithuania.
[e] Lygel F 60, Lyckeby Culinar, Fjälkinge, Sweden.
[f] Rousselot® 250 PS, Rousselot SAS, Courbevoie, France.
[g] NorSalmOil, Norsildmel, Egersund, Norway.
[h] Monocalsium phosphate, Bolifor® MCP-F, Oslo, Norway Yara.
[i] L-Threonine, CJ Biotech CO., Shenyang, China.
[j] L-Lysine CJ Biotech CO., Shenyang, China.
[k] Rhodimet NP99, Adisseo ASA, Antony, France.
[l] Choline chloride, 70% Vegetable, Indukern s.a., Spain.
[m] 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.
[n] DP:DE = digestible protein: digestible energy ratio. Calculated based on internal values.

(Commission dir. 93/28/EEC) × 6.25, crude lipid by Accelerated Solvent Extractor (ASE200, Dionex, California, USA), ash by incineration at 550 °C (Commission dir. 71/250/EEC). Gross energy content was determined with an adiabatic bomb calorimeter (Parr 1281; Parr Instruments, Moline, IL, United States) according to ISO (1998). Amino acids were analyzed according to Commission dir. 152/2009/EC on a Biochrom 30 amino acid analyzer (Biochrom Ltd., Cambridge, UK).

2.5. Histology

Formalin-fixed tissue was dehydrated in ethanol, equilibrated in xylene and embedded in paraffin using standard histological techniques. Longitudinal sections of approximately 2 µm in thickness were prepared. The sections were stained with haematoxylin and eosin (HE), and blinded evaluation of the DI was performed according to changes being associated with development of SBMIE as previously described for Atlantic salmon (Baeverfjord and Krogdahl, 1996). Briefly, the evaluation was based on the width of the lamina propria and the length of simple and complex folds, infiltration of leucocytes in the lamina propria and the appearance of the intestinal epithelium including the height of the enterocytes, position of the nucleus, supranuclear vacuoles and intraepithelial lymphocytes. Each criteria was given a score ranging from 0 to 2 and half scores were included (i.e. 0, 0.5, 1, 1.5 and 2) as described in Grammes et al. (2013). Score 0 represented normal morphology and score 2 represented marked changes.

2.6. Morphometry

Images of HE stained intestinal tissue were captured using a Leica DMLS light microscope (Leica Microsystems, Wetzlar, Germany) equipped with a Leica E3 digital imaging camera and LAS EZ v4.9 software. Images were captured with a 10× objective magnification. In Image J (ImageJ software, version v1.51r) (Schneider et al., 2012), the scale was calibrated to give measurements in micrometers (2.19 pixels/µm) and the freehand selection tool was used to measure the length of the simple folds in the DI. The folds were measured from the stratum compactum to the tip of the fold. Only folds that appeared long, not bent and with epithelium attached to the basement membrane all the way to the tip of the fold were considered appropriate for measurement. From each individual, 3 measurements were performed. Individuals with < 3 simple folds suitable for measurement, either due to sectioning angle or detachment of the epithelium, were omitted from the morphometric measurements. At least 30 measurements were obtained from each dietary group (Løkka et al., 2013).

2.7. Quantitative real-time PCR (qPCR)

A set of genes involved in membrane transport (Aquaporin 8ab, *aqp8*), intestinal epithelial barrier protection (Mucin 2, *muc2*) and stress responses (Superoxide dismutase (*sod1*), Glutathione-s-transferase a3 (*gsta3*), heat shock protein 70 (*hsp70*)) were analyzed by quantitative real-time PCR. Total RNA from DI of 90 fish were extracted using the RNeasy® 96 QIAcube® HT kit, following the Pretreatment Protocol in the RNeasy® 96 QIAcube® HT Handbook (Qiagen, Venlo, Netherlands). On-column DNAse treatment was performed using PureLink ™ DNase kit (Invitrogen, Carlsbad, California). The total RNA concentration was measured using NanoDrop ™ 8000 spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). Samples were stored at −80 °C until further analysis.

Prior to cDNA synthesis, all samples were normalized to 500 ng/µl. The cDNA synthesis was performed using the AffinityScript QPCR cDNA Synthesis kit and protocol (Agilent Technologies, Santa Clara, USA). To give a total volume of 10 µl, 3 µL of RNA was added together with 5 µl of cDNA Synthesis Master Mix, 1.5 µl random primers, and 0.5 µl AffinityScript RT/ RNase Block Enzyme Mixture. The conditions for the cDNA synthesis were as followed: 25 °C for 5 min, 42 °C for 45 min, 95 °C for 5 min, 4 °C.

Each qPCR reaction was conducted in a total volume of 20 µl using 10 µl SYBR Green I Master (LightCycler® 480 SYBR Green I Master), 2 µl of gene specific primers, 3 µl of Milli-Q ® water and 5 µl of cDNA. The qPCR conditions were as followed: 95 °C for 5 min, 95 °C for 10 s, 60 °C for 10 s, 72 °C for 15 s. The last three steps were repeated 45 times. At the end of the program, melting curve analysis was carried out to confirm only one product. All samples were analyzed using LightCycler® 480 System (Roche Diagnostics, Mannheim, Germany). Sequences for all primers included in this study are provided in Table 3. HPRT1 and GAPDH were used as reference genes (Table 3).

Table 3
Primer sequences used for quantitative real-time PCR.

Genes	Fwd / Rev	Primer sequences	GenBank accession.no	Ref
HPRTI	Fwd	CCGCCTCAAGAGCTACTGTAAT	XM_014212855.1	(Kortner et al., 2012)
	Rev	GTCTGGAACCTCAAACCCTATG		
GAPDH	Fwd	AAGTGAAGCAGGAGGGTGGAA	XM_014141819.1	(Kortner et al., 2012)
	Rev	CAGCCTCACCCCATTTGATG		
SOD	Fwd	CCACGTCCATGCCTTTGG	NM_001123587.1	(Olsvik et al., 2005)
	Rev	TCAGCTGCTGCAGTCACGTT		
AQP8ab	Fwd	GTTGGCATAGTTCTCCTTTGATG	XM_014179685.1	(Kortner et al., 2012)
	Rev	TTTCAACCCTCCCTTCACC		
MUC2	Fwd	TCTGTCCTGATGGGATGAAAC	XM_014183074.1	(Sahlmann et al., 2013)
	Rev	GGACTCCAAACAAACGCAAT		
HSP70	Fwd	CCCCTGTCCCTGGGTATTG	XM_014137172.1	(Olsvik et al., 2007)
	Rev	CACCAGGCTGGTTGTCTGAGT		
GSTA3[a]	Fwd	AACGCCCAGAAATAGCCTCT	NM_001140755.1	(Sahlmann et al., 2013)
	Rev	GACACGATTCATCCTCAGCA		

[a] GSTA3 is referred to as GSTA4 in Sahlmann et al. (2013) however, after blasting against Atlantic salmon (taxid:8030) the resulting alignment were 100% with *Salmo salar* glutathione S-transferase alpha 3 (GSTA3) (NM_001140755.1).

2.8. Calculation and statistical analysis

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. Growth parameters were analyzed using a One-way ANOVA followed by Tukey HSD as a post hoc test. Growth parameters were based on tank (n = 3) as statistical unit and the fish performance analyses were 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 $p < .05$.

Gene expression analysis was performed using the ΔΔCt method (Pfaffl, 2001). Genes and histological evaluation were analyzed using a non-parametric Kruskal-Wallis test by ranks followed by Dunn's multiple comparisons test. Significance was set to $p < .05$. Data from morphometric measurements were tested for normality by D'Agostino-Pearson test and homogeneity of variance using a Brown-Forsythe's test, and further analyzed using a One-way ANOVA followed by Tukey HSD test. Morphometric analysis was performed at individual level using the mean of measurements of 3 simple folds per fish. Statistical analysis was performed using GraphPad Prism, version 7.0 (GraphPad software, San Diego, California, USA).

3. Results

The fish grew from approximately 4.4 g and were close to tripling their weight within the 28 days of feeding (Table 4). Fish fed the FM20CU diet obtained significantly higher final weight and SGR compared with those fish fed the FM control and the SBM based diets. Fish fed SBM-based diets with increasing levels of *C. utilis* showed a trend for a dose-dependent reduction in growth, and obtained similar SGR and final weight to fish fed the SBM-based positive control diet. There was no mortality observed during the experimental period.

3.1. Histology and morphometric measurements

Fish fed FM, either alone or in combination with 20% *C. utilis*, displayed normal morphology of the DI where the complex and simple folds were tall with a thin lamina propria (Fig. 1A and B, Fig. 2). The enterocytes were tall with nucleus located in a basal position with numerous supranuclear vacuoles. Intraepithelial lymphocytes were positioned between the enterocytes along the entire length of the folds (Fig. 1 G and H)

The morphology of the DI of salmon fed diets containing SBM displayed mild changes associated with SBMIE (Fig. 2) and the fish with the most severe changes are presented in Fig. 1 C, D, E and F. In general, the lengths of the simple and complex folds were reduced when compared with the FM control. The width of the lamina propria was slightly increased and there was a mild infiltration of leucocytes. The enterocytes appeared to have a more basophilic cytoplasm due to the decreased presence of supranuclear vacuoles (Fig. 1 I and J). There were no observable differences between SBM group and the groups receiving SBM diet with increasing levels of *C. utilis*.

Morphometric measurements of the simple folds showed that the mean lengths of simple folds of fish groups fed FM and FM20CU were significantly longer than the mean lengths of simple folds of fish fed SBM alone or combined with 10% *C. utilis* (Fig. 3). Length of simple folds in fish fed SBM diet with *C. utilis* inclusion was not significantly different from fish fed SBM alone.

3.2. qPCR

Aqp8 showed the clearest differences between diet groups. While there was no significant difference between the standard FM and the

Table 4
Body weights and specific growth rate (SGR) of Atlantic salmon fed a fishmeal control (FM), FM including 20% *C. utilis* (FM20CU), soybean meal diet (SBM) and three diets with 40% SBM and increasing level of *C. utilis* (SBM5CU, SBM10CU, SBM20CU).

	FM	FM20CU	SBM	SBM5CU	SBM10CU	SBM20CU	s.e.m.[a]	*p*-value
Start weight (g) 0d	4.37	4.39	4.42	4.40	4.40	4.42	0.035	0.99
Mid weight (g) 16d	8.90	9.32	9.00	9.13	8.89	8.85	0.14	0.346
Final weight (g) 28d	14.1 ^{B}	15.8^{A}	14.7 ^{AB}	15.1 ^{AB}	14.7 ^{AB}	14.5 ^{AB}	0.37	0.049
SGR 0–16 d	4.45 ^{BC}	4.71 ^{AB}	4.45 ^{BC}	4.56 ^{ABC}	4.40^{C}	4.34^{C}	0.009	0.0006
SGR 16–28 d [b]	3.83	4.38	4.08	4.17	4.21	4.08	0.013	
SGR 0–28 d	4.19 ^{D}	4.57 ^{A}	4.29 ^{CD}	4.39 ^{BC}	4.32 ^{BCD}	4.23 ^{D}	0.003	< 0.0001

a,bIndicate significant ($P \leq .05$) differences among diets within a row.
[a] Pooled standard error of mean. n = 3 replicate tanks per treatment.
[b] Statistics is omitted for SGR 16–28 d due to uneven start point in period 2.

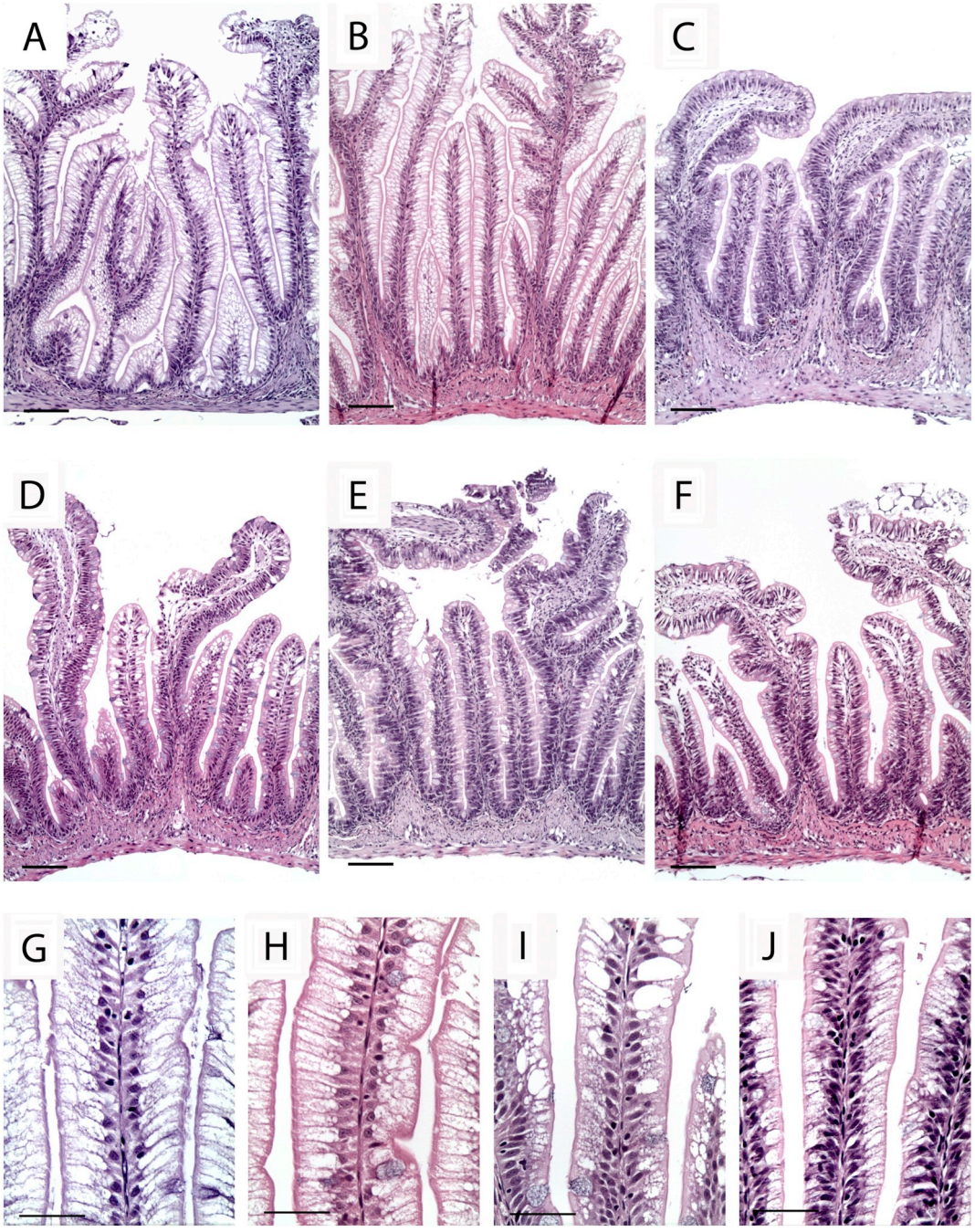

Fig. 1. The distal intestine (DI) of Atlantic salmon fed FM, either alone **(A)** or in combination with 20% *C. utilis* **(B)**, showed normal morphology. Salmon fed diets containing SBM had mild changes in the DI which are associated with SBMIE **(C:** SBM; **D:** SBM5CU; **E:** SBM10CU; **F:** SBM 20CU). Detail picture of simple folds of the distal intestine of Atlantic salmon. Fish fed (FM) **(G)** or FM combined with 20% *C. utilis* **(H)** had long enterocytes with basally located nuclei and abundant amount of supranuclear vacuoles. Atlantic salmon fed soybean meal (SBM) **(I)** or SBM combined with *C. utilis* (**J:** SBM5CU) have a reduced amount of supranuclear vacuoles giving the enterocytes a more basophilic appearance.
FM = fishmeal diet; CU = *C. utilis*; SBM = soybean meal diet; SBMIE = soybean meal-induced enteritis. Images A-F captured with 10 X magnification with scale bar 100 μm. Images G-J captured with 40 X magnification with scale bar 50 μm.

FM20CU groups, gene expression of *aqp8* significantly decreased in all diets containing SBM when compared with the FM diet group. In contrast, expression of a gene responsible for mucin secretion, *muc2*, did not differ between diet groups (Fig. 4). Three genes involved in stress response were tested (*sod1, gsta3* and *hsp70*). Sod1 expression was lower in fish fed SBM and SBM20CU diets compared to fish fed the FM diet (Fig. 4). *Gsta3* expression did not differ significantly between FM, FM20CU, SBM and SBM20CU but SBM and SBM20CU showed a pattern of decreased expression compared with FM (Fig. 4). Fish fed with SBM5CU and SBM10CU showed a significantly decreased expression of *gsta3* compared with the FM control. *Hsp70* expression did not differ significantly between FM and FM20CU but increased significantly in SBM compared with FM. While SBM with 5 and 10% *C. utilis* did not differ significantly compared with FM, it increased significantly in SBM20CU.

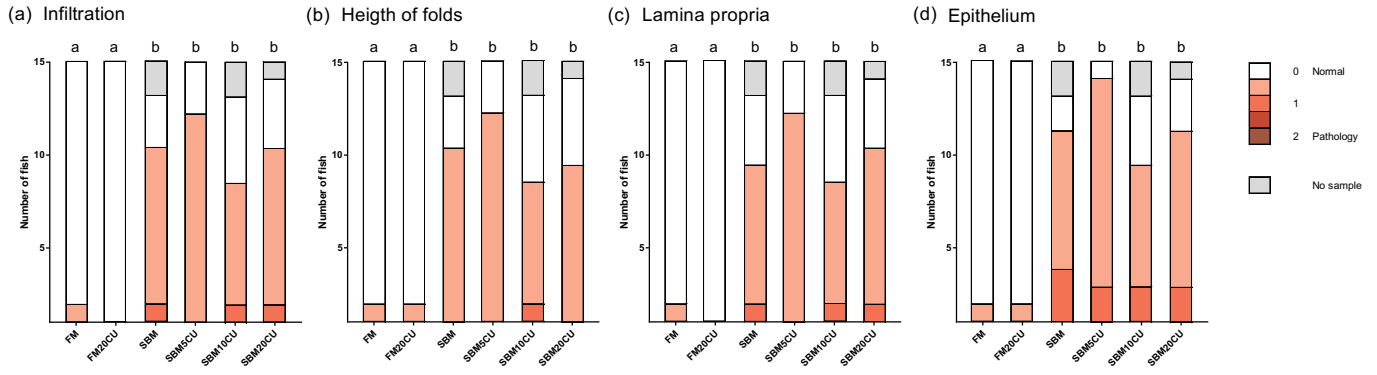

Fig. 2. Morphological evaluation of the distal intestine of Atlantic salmon fed a fishmeal (FM) diet, a FM diet combined with 20% *C. utilis* (FM20CU), a soybean meal (SBM) diet and three SBM diets with inclusion levels of 5%, 10% and 20% *C. utilis* (SBM5CU, SBM10CU, SBM20CU). The evaluation is based on (a) infiltration of leucocytes in the lamina propria, (b) reduction of the height of the intestinal folds, (c) increased width of the intestinal folds and (d) changes of the intestinal epithelium. Each parameter were given a grade from 0 to 2 including half grades (i.e. 0, 0.5, 1, 1.5, 2). Score 0 indicates normal morphology, and score 2 indicates pathological changes related to soybean meal induced enteritis. FM (n=15); FM20CU (n = 15), SBM (n = 13); SBM5CU (n = 15); SBM10CU (n = 13); SBM20CU (n = 14). Groups with different letters on the upper x-axis are significantly different (p < .05). FM = Fishmeal; SBM = Soybean meal; CU = *C. utilis*.

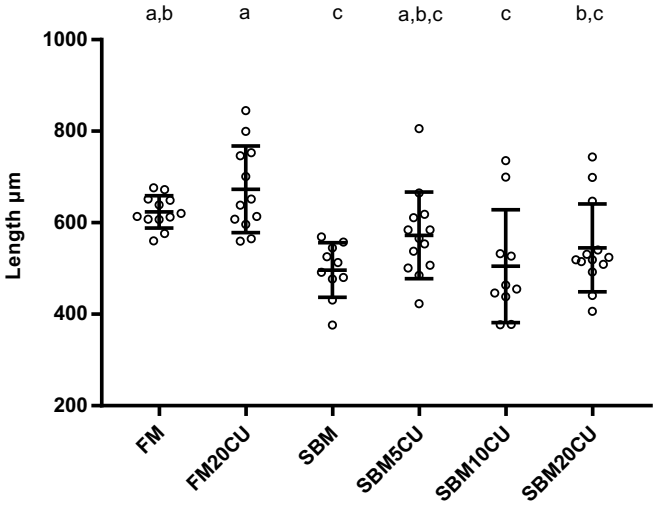

Fig. 3. Morphometric measurements of the simple folds (µm) in the distal intestine of Atlantic salmon expressed as mean and standard deviation (SD) for each individual: FM (n = 12); FM20CU (n = 12), SBM (n = 10); SBM5CU (n = 13); SBM10CU (n = 10); SBM20CU (n = 13). Groups with different letters on the upper x-axis are significantly different (p < .05). FM = Fishmeal; SBM = soybean meal; CU = *C. utilis*.

4. Discussion

The present study investigated growth and DI health in Atlantic salmon fed diets containing *C. utilis* in combination with FM or high levels of SMB. There are few studies that have used *C. utilis* in diets for Atlantic salmon (Øverland et al., 2013; Grammes et al., 2013), and to our knowledge none concerning the early parr stage. Fish fed FM20CU obtained significantly higher SGR compared with all other dietary treatments. In comparison, Øverland et al. (2013) described similar growth and feed utilization when Atlantic salmon (BW; 28–89 g) were fed 28.3% *C. utilis,* substituting 40% of the protein from FM compared with a FM control. A study with rainbow trout (*Oncorhyncus mykiss*) also showed similar growth when *C. utilis*, grown on peat and fish offal, replaced 35% of the FM protein (Martin et al., 1993). These latter experiments did not show any clear positive effect on growth when adding *C. utilis* to diets for salmonids, as shown in the present experiment. However, partially replacement of FM with *Saccharomyces cerevisiae* has increased growth of rainbow trout (Rumsey et al., 1991), sea bass (*Dicentrarchus labrax*) (Oliva-Teles and Gonçalves, 2001), and Pacu (*Piaractus mesopotamicus*) (Ozório et al., 2010). Yeast is considered to have high palatability for humans because it has the umami taste, which is often related to high glutamate content in combination with high levels of nucleic acids (Sarower et al., 2012). In accordance with this, glutamate and nucleic acids have also separately proven to be taste enhancers in fish feed (Kasumyan and Døving, 2003; Mamoto et al., 1993). Because yeast often contains high levels of both these components, this indicates that yeast can act as a taste enhancer to improve feed intake and growth in fish. Despite the lack of recorded feed intake in the present experiment, increased feed intake can be an explanation of the high SGR in fish fed FM20CU compared with the other experimental diets.

Due to challenges in replacing FM with high levels of both SBM and *C. utilis* the level of the level of pre-gelatinized potato starch and wheat gluten were varying among diets. This led to increased level of starch in the FM diet compared to the FM20CU diet and could be seen as a dietary difference. However, Hemre et al. (1995) showed no reduced growth performance in salmon fed up to 22% starch, and thus the increased starch level should not be a major factor explaining the increased growth in FM20CU compared to the FM diet.

There is limited information available on the effect of feeding high levels of SBM to Atlantic salmon parr, specifically the effects on growth and intestinal health. Here, salmon parr fed a diet with 40% solvent extracted SBM obtained similar high growth as the FM control. Generally, SBM has been reported to reduce growth and feed conversion rate in Atlantic salmon post-smolts (Olli et al., 1995; Refstie et al., 1998). Interestingly, De Santis et al. (2015) demonstrate similar or improved growth of the SBM (37%) fed fish compared with the growth of fish fed different mixtures of soy protein concentrate, faba bean protein concentrate and FM. Furthermore, in rainbow trout, Heikkinen et al. (2006) report similar growth of fish fed SBM (45%) when compared with FM control diet. Thus, there is evidence that SBM is an ingredient that gives acceptable growth in Atlantic salmon parr.

Histology and morphometric evaluation of intestine from salmon parr fed high levels of yeast has to the authors' knowledge not been published. Inclusion of high levels of *C. utilis* did not cause marked changes in the morphology of DI, which was found to be comparable to the morphology of the DI of parr fed only FM. This finding implies that *C. utilis* had no negative effect on the DI structure of parr when fed at high concentration in combination with FM.

SBM is known to induce SBMIE in the DI of seawater-adapted salmon (Baeverfjord and Krogdahl, 1996; Van den Ingh et al., 1991) where a T-cell-like response is central in SBM-induced intestinal changes (Bakke-McKellep et al., 2007). In contrast, the dietary exposure of SBM to salmon during early freshwater stage has not been

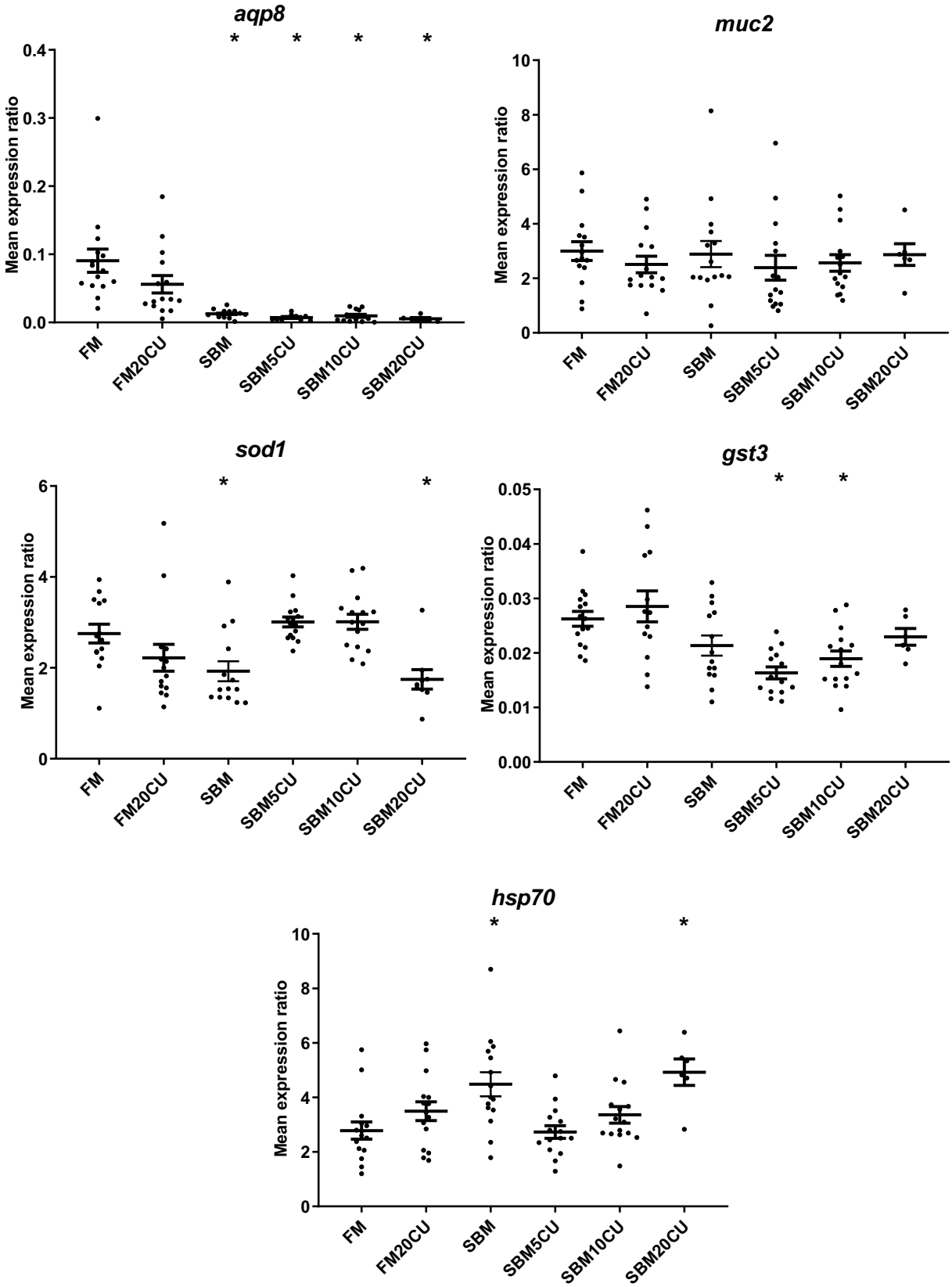

Fig. 4. Mean gene expression ratio of aquaporin 8 (*aqp8*), mucin 2 (*muc2*), superoxide dismutase 1 (*sod1*), glutathione-s-transferase (*gsta3*) and heat shock protein 70 (*hsp70*) in distal intestine of up to five Atlantic salmon per tank fed given diets; FM: Fishmeal control (n = 14); FM20CU: FM with 20% *C. utilis* (n = 14); SBM: soybean meal (n = 14); SBM5CU: SBM with5% *C. utilis* (n = 15); SBM10CU: SBM with 10% *C. utilis* (n = 15); SBM20CU: SBM with 20% *C. utilis* (*n* = 9). Asterisk denotes significantly different compared to control ($p < .05$).

demonstrated to elicit morphological changes of the same severity in the DI (DeSantis et al., 2015; Gu et al., 2015; Król et al., 2016; Sanden et al., 2005; Sahlmann et al., 2015). Histological findings of the current study are in line with previous studies performed in juveniles (DeSantis et al. 2015; Gu et al., 2015; Sanden et al., 2005; Sahlmann et al., 2015) as only mild changes were detected. However, morphometric measurements show that the lengths of simple folds were significantly shorter in SBM group compared with FM and FM20CU groups. Nevertheless, despite the reduction in the length of simple folds due to SBM, the weight gain of the fish was not significantly altered compared with fish fed the traditional FM diet. It would appear that parr possess sufficient biological plasticity to overcome the adverse effect on morphology caused by SBM and still maintain good growth. This difference between the seawater and freshwater stages suggests that there is a higher threshold level for SBM inclusion in the diets of parr compared with seawater-adapted salmon (Krogdahl et al., 2003). Additionally, due to the lack of inflammatory response seen in seawater-adapted salmon in exposure to SBM, it has been speculated that the immune system and intestinal function in juveniles are under-developed (Gu et al., 2015; Sahlmann et al., 2015). Interestingly, studies indicate that exposure to SBM early in life improves acceptance and utilization of the same diet at later life stages in rainbow trout (Geurden et al., 2013; Sealey et al., 2009) and Atlantic salmon (Clarkson et al., 2017).

It has previously been observed that a change in diet may affect intestinal homeostasis on a molecular level, even though an effect on morphology or a growth effect was not evident (Sahlmann et al., 2015). Thus, the addition of yeast alone or in combination with SBM was evaluated for underlying, molecular effects in DI tissue of salmon parr. The detailed characterization of dietary effects on the digestive and immunological system in freshwater salmon could reveal key aspects in finding the reason why freshwater salmon are more tolerant to plant proteins. As such, a detailed molecular study using RNA sequencing would be the next step to follow-up recent findings. Our gene expression analysis in this study was meant to get an indication of possible underlying effect in some genes related to intestinal function. Results from our study indicate that the expression of most of the investigated genes were affected by 40% SBM in the diet. *Aqp8* gave the largest response with a strong decrease in expression in all SBM diets. Aquaporin is a water channel with important osmoregulatory functions in the DI tract of salmonids (Tipsmark et al., 2010). Interestingly, this effect, which we observed in freshwater salmon parr, is in line with findings by Kortner et al. (2012), who observed decreased expression of *aqp8* in seawater-adapted salmon when exposed to soy saponins, a key ANF in SBM. A similar down-regulation of *aqp8* was seen in freshwater raised salmon parr fed 36% SBM (Król et al., 2016). Decreased *aqp8* expression could be a factor causing diarrhea, as potentially fewer water channels are present in the brush border, thus decreasing the amount of water that could be reabsorbed in the intestine. The gene expression of *muc2*, for example, was not affected by either *C. utilis* or SBM. Mucin 2, encoded by the gene *muc2,* is a glycoprotein and main component of the mucus secreted from goblet cells present in the intestinal epithelium. In previous studies using post-smolt salmon, however, muc2 was up-regulated in SBM fed fish (Sahlmann et al., 2013) but not in freshwater stage salmon (Sahlmann et al., 2015). The mucus layer of the intestinal epithelium plays an important role in intestinal barrier functions (Johansson and Hansson, 2016). Two genes related to oxidative stress were investigated. Both genes, *sod1* and *gsta3,* showed a trend to be down-regulated in SBM fed fish. These findings could indicate that the defense against oxidative stress in intestinal cells was affected by 40% SBM in the diet as has been observed previously with post-smolt salmon (Sahlmann et al., 2013). The significant up-regulation of *hsp70* in fish fed SBM observed in the present study could be an indication for a stress reaction of intestinal cells towards SBM, and *hsp70* may have a protective role during a stress related response. Heat shock proteins are acute phase chaperon proteins that under physiological circumstances interact with many signal transduction pathways controlling cell homeostasis, proliferation, differentiation and cell death (Mayer and Bukau, 2005). Increased expression of *hsp70* is induced by a variety of insults, such as environmental, chemical and physiological stress, and have been proposed as indicators of cellular stress in fish (Iwama et al., 2004). The results from the gene expression analysis indicate that in many cases, different physiological pathways in pre-smolt as compared to post-smolt salmon are affected in a high SBM diet. A broad-scale transcriptomic study could reveal important changes in gene expression, which could help to characterize the effects of high SBM diets and thus help in understanding the fundamental differences in gut related processes of pre-smolt salmon.

The present study also investigated different inclusion levels of *C. utilis* in combination with SBM as a potential strategy to alleviate effects of SBM. Feeding inactive dry yeast has been shown to mitigate the adverse health effect of high dietary SBM levels on the DI of saltwater adapted salmon (Grammes et al., 2013). For comparative purposes, it is important to point out that the levels of crude protein and amino acids in the *C. utilis* meal used in this study were somewhat lower than those measured in yeast strains used in previous experiments (reviewed by Øverland and Skrede, 2017). In the current study, *C. utilis* did not maintain the morphological features of the DI when combined with SBM. However, our results indicate that there are some response on the molecular level in genes related to stress response (*sod1*, *hsp70*, *gsta3*) in diets where *C. utilis* is included at a low level (SBM5CU and SBM20CU). Previous studies reported decreased hepatic *sod1* expression in Atlantic salmon when fed plant-based diets. Olsvik et al. (2011) concluded that the defense against oxygen radicals may be decreased in fish fed plant-based diets. Our results indicate that *C. utilis* could mitigate those effects in combination with SBM as it resulted in no change in *sod1* expression at low inclusion levels. However, the down-regulation of *gsta3* indicated that intestinal cells were exposed to an effect of SBM diet as GSTs detoxify oxygen radicals (Hayes et al., 1999). If oxidative stress was increased and oxidative stress response reduced, an increase in cellular damage and decreased health could be expected. The lack of negative effects on other parameters, such as morphology in this study suggest that the down-regulation could also be seen as an indication for a decreasing need for oxidative protection due to protective effects of *C. utilis* and its compounds. Earlier studies on GST activity revealed conflicting results as to whether an increase or decrease in activity may be good or bad (reviewed by Van der Oost et al., 2003). Expression levels of the acute-phase chaperon protein *hsp70* were increased in SBM fed fish. The addition of 5% or 10% *C. utilis* to the SBM diet seemed to counteract the effects of SBM on cell homeostasis as the expression levels of *gsta3* were similar to the FM control group. Thus, more information is needed to interpret these findings.

5. Conclusions

Our results indicate that the *C. utilis* yeast can be a suitable protein source in diets for Atlantic salmon parr, as it supports high growth performance without any obvious adverse effects on DI health. Feeding diets with 40% SBM gave similar growth performance as the FM control, but induced mild changes in histology and significant changes in gene expression in the DI of salmon parr. The addition of increased dietary level of *C. utilis* did not counteract the mild changes in histology.

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