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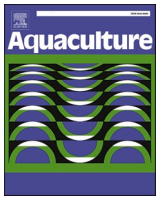

Marine yeast (*Candida sake*) cultured on herring brine side streams is a promising feed ingredient and omega-3 source for rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

A major challenge for the aquaculture industry is the supply of sustainable feeds. A promising model to achieve this is to utilize circular flows where feed ingredients, such as single cell protein, are cultivated using side streams of the food industry. The aim of this study was to evaluate the marine yeast *Candida sake*, produced on herring brine side streams, as a source of protein and immune stimulant in feed for salmonid fish. The dry *C. sake* product contained 54% protein (3.3% lysine and 0.8% methionine) and 13% lipids (1.1% eicosapentaenoic, EPA, and 1% docosahexaenoic acid, DHA). Four experimental diets were designed and tested in a 9-week feeding trial using juvenile rainbow trout (*Oncorhynchus mykiss*). A control diet containing both fish and plant-based ingredients constituted the base feed to which 20% (to evaluate effects on digestibility, growth and intestinal physiology), 20% heat-treated (to evaluate effects of downstream processing) and 3% (to evaluate immune stimulatory properties, replacing 3% soy protein concentrate) *C. sake* was added. The apparent digestibility coefficient of *C. sake* for protein, fat and gross energy was above 80%, and for amino acids above 90% regardless of treatment, suggesting a high bioavailability of *C. sake*. All three yeast containing diets performed equally to the control regarding specific growth rate, feed conversion ratio and functional intestinal health. These results suggest that *C. sake* is a promising alternative protein source for circular feeds in the salmonid industry. The presence of EPA and DHA represents an added value. The heat treatment increased the apparent digestibility coefficient of dry matter by 8% but decreased amino acid digestibility by on average 3%, indicating that heat treatment may not be the optimal downstream processing technique. Furthermore, the inclusion of 3% *C. sake* increased the intestinal lamina propria width and TGF-β transcription, indicating an immune stimulating effect. Future research is needed to understand these immune modulatory effects of *C. sake* supplementation.

1. Introduction

The annual production of finfish aquaculture has more than doubled during the last two decades and is now supplying 57.5 million tons of fish annually (FAO, 2022). This growth has been dependent on a matching increase in the supply of aquafeeds (Tacon and Metian, 2015). Contemporary feeds for salmonid fish contain about 25% fish and 75% plant based meals and oils (Aas et al., 2019). Traditionally, fish meal and oil originating from wild caught fish have been central feed components. However, wild fish stocks are finite and strictly managed to avoid overfishing (FAO, 2022). Therefore, the global supply of fish meal and oil is stable but not expected to increase significantly in the future (Tacon and Metian, 2008). As a result, plant-based ingredients have successively substituted fish meal and oil in salmonid diets (FAO, 2022). While aiding the continuous growth of aquaculture, plant ingredients (such as legumes, oil seeds and grain) demand limited resources like

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arable land and freshwater input, and often directly compete with human consumption (FAO, 2020; Fry et al., 2016). Additionally, vegetable ingredients may display suboptimal nutritional qualities compared to fish meal, and can contain antinutrients which have adverse effects on fish health if not eliminated (Francis et al., 2001; Gatlin et al., 2007; Glencross, 2020). The sustainable development of aquaculture is therefore dependent on the development of alternative and sustainable feed ingredients (Hua et al., 2019; Turchini et al., 2019).

Yeasts are a promising alternative feed ingredient for salmonid aquaculture (Agboola et al., 2021; Mahnken et al., 1980; Rumsey et al., 1990). These unicellular organisms contain 40–65% high quality protein and can convert organic waste and side streams from the food and forest industry into valuable feed ingredients (Jones et al., 2020; Nasseri et al., 2011; Øverland and Skrede, 2017). While yeasts are a highly diverse group of organisms, only a few species, including *Saccharomyces cerevisiae* and *Cyberlindnera jadinii* (formerly *Candida utilis),* have been evaluated for their potential as protein sources for salmonid fish (Agboola et al., 2021). The maximum inclusion levels of the yeasts may vary depending on strain, cultivation substrate, and feed formulation, but inclusion levels of up to 20% have frequently been shown to have no adverse effects on growth (Hansen et al., 2019; Hauptman et al., 2014; Mahnken et al., 1980; Øverland et al., 2013; Vidakovic et al., 2020). The digestibility of yeast products in salmonid fish depends on the type of yeast and the post-harvest down-stream processing technique. Such processing techniques aim to disrupt the yeast's rigid cell wall by inducing autolysis using heat, pressure, or enzymes, thereby increasing digestibility (Hansen et al., 2021; Rumsey et al., 1991). In addition to their macronutrient qualities, yeasts contain bioactive compounds including polysaccharides (β-glucans and mannan oligosaccharides, MOS) and nucleotides. Both β-glucans and MOS interact directly with the immune system via pattern recognition receptors as well as the gut microbiota and have been tested as functional feed supplements to increase growth, stimulate immune responses, and promote intestinal health of farmed fish (Douxfils et al., 2017; Fuller, 1989; Li and Gatlin, 2006; Meena et al., 2013; Torrecillas et al., 2014). In salmonid fish, these purified yeast components have also been shown to positively modulate the morphology of the intestinal mucosa by increasing the absorptive surface and counteracting soybean meal induced enteritis (Dimitroglou et al., 2009; Genc et al., 2007; Refstie et al., 2010).

To utilize side streams efficiently, cultivation substrate and yeast strain need to be compatible. *S. cerevisiae,* for example, can be efficiently grown on substrates containing moderate salt levels but exhibits reduced growth rates at sodium chloride concentrations above 2.5% (Goston et al., 2016; Watson, 1970). This renders many marine side streams suboptimal as substrates. Conversely, marine yeasts tolerate high salt concentrations and thrive on marine biomass as substrates. Additionally, marine yeasts, especially when provided with "marine substrates" may contain essential nutrients and antioxidants such as omega-3 fatty acids, astaxanthin and riboflavin, all of which are highly desirable compounds in salmonid feed (Zaky et al., 2014; Zhenming et al., 2006). With the increase in marine aquaculture, marine side streams are becoming more abundant (FAO, 2022). While many of these side streams can be directly converted into fish meal and fish oil, the use of marine yeast opens a novel avenue to utilize the considerable fraction of so far unused side streams. *Candida sake* (first described as Eutorulopsis sake, Saito and Ota, 1934) is a yeast found in temperate marine environments. In aquaculture, *C. sake* has been tested as immune stimulant, resulting in increased disease resistance of the Indian white prawn (*Fenneropenaeus indicus*, Sajeevan et al., 2006; Sarlin and Philip, 2016). However, to the authors' knowledge, no feeding trials using *C. sake* or any other marine yeast in diets for salmonid fish have been conducted.

The aim of this study was therefore to produce *C. sake* using herring brine side streams and to evaluate *C. sake* as a feed ingredient and additive in diets for rainbow trout.

The following specific objectives were investigated; the nutritional profile of *C. sake* cultivated on herring brine, the potential of *C. sake* as protein replacement in salmonid feed, the effect of downstream processing on digestibility and bioavailability of *C. sake* and the immune modulatory properties with low inclusion levels of *C. sake*.

2. Material and methods

2.1. C. sake cultivation

The *C. sake* strain used in the present study was isolated from the surface of sugar kelp (*Saccharina latissima*) at Tjärnö Marine Laboratory at the Swedish west coast (58.87331092481132, 11.147094395548622, Tjärnö, Sweden,). The isolated strain was identified as *Candida sake* by 26 rRNA sequencing (99.8% sequence identity, National Collection of Yeast Cultures, Norwich, UK). For the biomass production, the yeast was cultivated in a bioreactor (BR 600, Belach Bioteknik, Lund, Sweden) at a volume of 300 L (Industrial Biotechnology, Royal Institute of Technology, Stockholm, Sweden). The substrate for cultivation consisted of 50% pre-salting brine from North Sea herring (*Clupea harengus,* Sweden Pelagic AB, Ellös, Sweden) and 10% molasses (Lantmännen AB, Stockholm, Sweden). The protein content of the brine was 8.5 g/L and the salt content 2.85 g/L NaCl. The cultivation was carried out at 25 °C, pH 6.5, 500 rpm continuous stirring and 100 L/min aeration until growth stagnation. Foaming was inhibited through the addition of Breox B 125 (Cognis Performance Chemicals Ltd., Southampton, UK). Growth stagnation occurred after 22 h at a cell density of $6.7*10^7$ cells/mL. The yeast cells were separated from the culturing medium at 4 °C using a disc stack separator (BTPX 205, AlfaLaval, Lund, Sweden), followed by centrifugation (Thermo Scientific Sorvall Bios 16, Thermo Fischer Scentific, Waltham, MA, USA) to reduce the water content. The *C. sake* product, 22.7 kg in total, was freeze-dried (Medicago AB, Uppsala, Sweden) to a final dry weight of 4.5 kg. The dry product contained 54% protein and 13.3% fat including the highly unsaturated docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). The chemical composition can be found in Table 1.

2.2. Production of experimental diets

The experimental diets were produced at the Feed technology laboratory (FTL), Department of Animal Nutrition and Management, Swedish University of Agricultural Sciences, Uppsala, Sweden. Four experimental diets were formulated (Table 2). The diets included a control diet containing standard fish feed ingredients (C), and three diets where *C. sake* was added. These included 3% *C. sake* (Y3), 20% *C. sake* (Y20) and 20% heat-treated *C. sake* (YH20). Diet Y3 was included to investigate possible immune modulating properties of *C. sake*. In this diet *C. sake* substituted 3% soy protein concentrate. The diets Y20 and YH20 on the other hand, were included to evaluate the suitability of *C. sake* to be used as a source of protein. In these diets the yeast substituted 20% of the total control diet, on weight basis, in order to investigate the apparent digestibility, according to Cho and Slinger (1979). Additionally, diet YH20 was included to investigate possible benefits of downstream processing such as heat treatment. The heat treatment entailed heating the freeze-dried yeast to 105 °C at maximum humidity for 2 min in a convection steam oven (Electrolux, convection oven, 6 GN1/1, Stadshagen, Sweden). All diets were extruded using a twin-screw extruder (maximum temperature 105 °C) with a 2 mm die head (model: Ketse 20/40, Brabender, Duisburg, Germany), and vacuum coated with a mix of fish and vegetable oil (Amandus Khal, Reinbek, Germany). For diet Y3, the lipid absorption during the vacuum coating was incomplete due to a reduced pellet expansion resulting in a lower lipid content (5.8% less lipid compared to diet C and similar to diets Y20 and YH20, Table 2). Due to this reason, we refrain from discussing the effect of 3% yeast inclusion on growth performance, digestibility, and nutrient utilization, but rather focus on possible immune modulating properties, which was the original aim of including diet Y3.

Table 1
Proximate composition, energy, amino acids, fatty acids and mineral profile (g/100 g if not specified differently) of freeze-dried *C. sake*.

	C. sake
Proximate composition	
Crude protein[1]	54.3
Sum of amino acids	44.5
Crude lipid	13.3
Ash	5.43
Gross energy (MJ/kg)	20.7
Indispensable amino acids	
Arginine	3.07
Histidine	1.07
Isoleucine	2.01
Leucine	2.98
Lysine	3.30
Methionine	0.79
Phenylalanine	2.14
Threonine	2.18
Valine	2.30
Dispensable amino acids	
Alanine	3.28
Aspartic acid	4.50
Cysteine	0.51
Glutamic acid	7.41
Glycine	2.08
Ornithine	0.37
Proline	1.57
Serine	2.47
Tyrosine	1.84
Tryptophan	0.63
Fatty acids (% of total fatty acids)	
Saturated fatty acids	19.7
Monounsaturated fatty acids	41.7
Polyunsaturated fatty acids	26.8
Omega n-6 fatty acids	7.80
Total omega n-3 fatty acids	19.0
C 20:5 n-3 (EPA)	8.30
C 22:6 n-3 (DHA)	7.50
Ratio omega n-6/omega n-3 fatty acids	0.41
Minerals and fiber	
Neutral detergent fiber	0.7
Phosphorous	1.2
Calcium Ca (mg/100 g)	78
Iron (mg/100 g)	19
Zinc (mg/100 g)	8.4

[1] According to Dumas (N*6.25).

2.3. Chemical analysis

The *C. sake* product, the experimental diet and the feces where analyzed for their chemical composition. The dry matter content was analyzed after heating at 103 °C for 16 h. To determine the ash content, dried samples were heated to 550 °C until the ash was completely white and then cooled in a desiccator before weighing (AOAC, 1995). Total nitrogen of feed and feces was determined according to the Kjeldahl method using a 2020 digester and a 2400 Kjeltec Analyzer unit (FOSS Analytical A/S, Hilleröd, Denmark), and for the *C. sake* according to the Dumas method (AOAC, 1995). The crude protein content was calculated as N*6.25 (Nordic Committee on Food Analysis, 1976). An extraction unit (ANKOM XT 15 Extractor, Hilleröd, ANKOM Technology, Macedon, NY, USA Denmark) was used to determine the crude lipid content. Neutral detergent fiber was determined as outlined by (Mertens, 2002). Gross energy content was determined using an isoperibol bomb calorimeter (Parr 6300, Parr Instrument Company, Moline, IL, USA). Amino acids analyses were carried out at a certified laboratory (Eurofins Food & Agro Testing Sweden AB, Linköping, Sweden) using high-performance liquid chromatography according to ISO-13903 (2005). Titanium dioxide in feed and feces was determined following the protocol of Short et al. (1996).

Table 2
Feed formulation, proximate composition (g/100 g on dry matter basis, DM) and energy (MJ/kg DM) of the four experimental diets; control (C), 3% yeast (Y3), 20% yeast (Y20) and 20% heat-treated yeast (YH20).

	Diet			
	C	Y3	Y20	YH20
Ingredient				
Fish meal[1]	32.3	32.3	25.8	25.8
Soy protein concentrate[2]	15.0	12.0	12.0	12.0
Wheat gluten[3]	12.0	12.0	9.6	9.6
Wheat meal	15.0	15.0	12.0	12.0
Potato starch	7.0	7.0	5.6	5.6
Fish oil[4]	15.0	15.0	12.0	12.0
Vitamin Premix	1.6	1.6	1.3	1.3
Yeast	0.0	3.0	20.0	0.0
Yeast heat-treated	0.0	0.0	0.0	20.0
L-methionine	0.8	0.8	0.6	0.6
Titanium dioxide	0.5	0.5	0.4	0.4
Monocalcium phosphate	0.8	0.8	0.6	0.6
Proximate composition				
Dry matter (%)	93.5	92.7	92.7	92.7
Crude protein	49.3	49.1	50.0	48.5
Crude fat	21.0	15.2	14.7	16.5
Ash	6.5	7.2	7.6	7.7
Neutral detergent fiber	3.0	3.1	2.6	2.6
Gross energy (MJ/kg DM)	24.9	23.5	23.2	23.2

[1] Low-temperature fish meal, North Atlantic origin.
[2] Hamlet, Horsens, Denmark.
[3] Lantmännen Reppe, Lidköping, Sweden.
[4] North Atlantic origin.

2.4. Fish husbandry and experimental design

One hundred eighty juvenile rainbow trout (~ 60 g) were obtained from Långhults Lax AB (Långhult, Sweden) and acclimatized to a seawater recirculating aquaculture system (RAS) at the Department of Biological and Environmental Sciences, University of Gothenburg, Sweden. During the 6-week acclimatization, the fish were hand-fed a commercial diet (Spirit Trout 4 mm, Skretting, Stavanger, Norway) until visual satiation twice per day, to acclimate the fish to the experimental feeding regime (see below). The fish were kept in cylindrical 600 L holding tanks with an inflow rate of ~3 L/min. The tanks were covered with half opaque Plexiglass, which provided partial shading. The light: dark cycle was 12:12. The temperature was 10.3 ± 0.1 °C, the salinity was 33 ± 1 ppt and the pH was 7.5 ± 0.1 during the course of the experiment. Water quality was assessed weekly by measuring ammonia (0.02 ± 0.01 mg/L), nitrite (0.19 ± 0.05 mg/L) and nitrate (7.89 ± 0.73 mg/L), using commercial photochemical test kits (Hach Lange GmbH, Düsseldorf, Germany).

Ten days before the start of the experiment, the fish were lightly anesthetized (MS-222, Finquel®, Argent Chemical Laboratories, Redmond, Washington, USA, 0.08 g/L) and intraperitoneally tagged using passive transponder (pit) tags (12 mm, Biomark, Boise, Idaho, USA). At the start of the experiment, the fish were sedated and their length and weight were measured. The fish were divided into 12 groups of 15 fish (balanced for weight: 107 ± 4 g) and randomly assigned an experimental tank (180 fish in total, $n = 3$ tanks per diet). During the 9-week feeding trial, the fish were fed twice per day (9:00 and 15:00) in accordance with their estimated daily energy needs (Bailey and Alanärä, 2006), which resulted in a feeding rate of ~1.5% body weight (BW) per day. Excess feed and feces were collected twice per day (feed: 30 min after feeding, feces: just before every feeding) using a pump pre-filter (Neptun, NVF-E 25, iSC GmbH, Landau an der Isar, Germany). Feces

for the determination of the apparent digestibility coefficient (ADC) were collected from the filter during the last 4 weeks of the experiment. All experimental procedures were approved by the Swedish Board of Agriculture (ethical permit number: 5.8.18–15,096/2018). The number of animals and tanks was chosen to minimize the number of animals and mimic aquaculture densities while avoiding hierarchy formation.

2.5. Sampling

All fish were sampled for fork length and initial, intermediate, and final body weight at week 0, 4 and 9 of the feeding trial respectively. At the end of the trial, 4 fish from each tank were randomly netted, anesthetized using 12 mg/L metomidate hydrochloride (Aquacalm, Syndel, Canada) and sacrificed with a sharp blow to the head. Blood samples were drawn from the caudal vein using a heparinized syringe (1 mL) with a 25-gauge needle. After the blood sampling, the fish were opened ventrolaterally. The intestine, between the posterior pyloric ceca and the rectum, was removed and divided into two regions: the proximal intestine (between the last pyloric ceca and ileorectal valve) and the distal intestine (from ileorectal valve to the rectum) as described by Sundell et al. (2003). Both the proximal and distal intestine were divided into two sections for separate analyses. For histology, a 2 mm section of the most anterior part of each region was fixed in buffered formaldehyde (4%) for 24 h before being transferred to 70% ethanol. For the assessment of intestinal health and nutrient transport, the next approximately 2 cm of the each region (proximal and distal) was cut open along the mesenteric border, and carefully rinsed in and transferred to Ringer's solution (mM: NaCl 140, KCl 2.5, $NaHCO_3$ 15, $CaCl_2$ 1.5, KH_2PO_4 1, $MgSO_4$ 0.8, HEPES (4-[2-hydro-xyethyl]-1-piperazineethanesulfonic acid) 5, D-glucose 10, L-lysine 0.5, and L-glutamine 20, pH 7.8; according to Sundell and Sundh, 2012). The liver and intraperitoneal fat were weighed for the calculation of body indices.

2.6. LPS challenge

To examine possible immune stimulating effects of *C. sake* as a supplement, the remaining fish from the Y3 ($n = 30$) and C ($n = 31$) treatments, were used in an immune challenge with lipopolysaccharides (LPS, *E. coli*, Merck, KGaA, Darmstadt, Germany). *C. sake* contains between 20 and 30% carbohydrates, including roughly 10% MOS and β-glucans. Therefore, the inclusion level of 3% was chosen to correspond to roughly 0.2% β-glucan and MOS as commonly used in feeding trials (Torrecillas et al., 2014; Refstie et al., 2010). The fish were anesthetized using 6 mg/L Aquacalm and intraperitoneally injected with 1 μL/g body weight phosphate-buffered saline (PBS) as a control or PBS containing 1 mg LPS/mL). The fish were randomly reassigned to 8 tanks ($n = 7$–8 individuals per tank) and not fed until sampling. Half of the fish (4 tanks) were sampled 12 and 48 h post injection respectively to capture the progression of the immune response. Euthanasia, blood sampling and intestine sampling were carried as described above. The posterior most part of both the proximal and distal intestine (ca. 2 cm) was rinsed and the mucosa was scraped off using two microscope glass slides. The mucosal samples were wrapped in aluminum foil and snap frozen in liquid nitrogen. Liver, head kidney, and spleen were sampled, wrapped in aluminum foil, snap frozen in liquid nitrogen, and stored together with the mucosa samples at −80 °C for qPCR analysis.

2.7. Data analyses

2.7.1. Growth performance and somatic indices

The following indices of growth performance were calculated after the feeding trial as averages for each experimental unit (tank) and fish (when applicable):

$$WG\ (weight\ gain,\ g) = FBW - IBW$$

$$SGR\ (specific\ growth\ rate,\ \%BW/day) = 100 \times ((\ln FBW - \ln IBW)/d))$$

$$K\ (condition\ factor) = 100 \times \left(FBW/FL^3\right)$$

$$FCR\ (feed\ conversion\ ratio) = FC/WG$$

$$HSI\ (Hepatosomatic\ index) = (LW/FBW) \times 100$$

$$VFI\ (Visceral\ fat\ index) = (VFW/FBW) \times 100$$

$$Survival\ (\%) = 100 - (FI/FA) \times 100$$

Using the initial body weight (IBW), final body weight (FBW), fork length (FL), duration of the trial in days (d), feed consumption (FC), total weight gain (WG), liver weight (LW), visceral fat weight (VFW) and the number of fish at the start (FI) and at the end of the 9-week feeding trial (FA).

2.7.2. Apparent digestibility

The ADC of the diets and the test ingredients was calculated using the concentration of titanium dioxide (TiO_2) in the fecal matter according to Cho et al. (1982). In short, using the relative concentration of TiO_2 (I) and the relative concentration of nutrients and (N) in diet (D) and feces (F) the ADC of the diets was calculated. Pooled TiO2 values were used for 2 tanks (one of the C and one of the YH20) due to sample contamination possibly with uneaten pellets. The relationship of the ADC of the different diets and the fraction of yeast meal inclusion (20%) then allowed for the calculation of the ADC for dried and heat-treated *C. sake* according to Bureau et al., 1999.

$$ADC\ (diet,\ \%) = 100 - (ID \times NF)/(IF \times ND) \times 100$$

$$ADC\ (ingredient, \%) = ADC_{diet} + \left(ADC_{diet} - ADC_{reference\ diet}\right) \times \left(N_{reference\ diet} \times 0.8\right)/\left(N_{ingredient.} \times 0.2\right)$$

2.7.3. Blood and plasma analyses

Hematocrit (Hct, %) and hemoglobin (Hb, g/dL) were analyzed directly after blood sampling. For Hct, duplicate subsamples were transferred to 80 μL capillary tubes and centrifuged 5 min at 10,000 rcf using an Hct centrifuge (Haematokrit 210, Hettich, Tuttlingen, Germany). The packed cell volume was read with a Hawksley reader (Hawksley, Sussex, UK) and recorded as percentage of the total blood volume, rounded to the closest 1%. Hb was determined using a handheld Hb 201+ meter (Hemocue® AB, Ängelholm, Sweden). The values were corrected for fish blood according to Clark et al. (2008). Using values of both Hct and Hb, the mean corpuscular hemoglobin content (MCHC) was calculated.

$$MCHC = (Hb/Hct) \times 10$$

The remaining blood was centrifuged at 5000 rpm for 5 min to obtain plasma (Thermo Scientific™ Heraeus™ Pico 17, Thermo Fisher Scientific), which was transferred into separate Eppendorf tubes and stored at −80 °C until further analysis. Plasma cortisol levels were measured using a radioimmunoassay as described by Young (1986) using a cortisol antibody (Code: S020; Lot: 1014–180,182) purchased from Guildhay Ltd. (Guildford, Surrey, UK) and validated by Sundh et al. (2011). The tracer (hydrocortisone-[1,2,6,7-3H(N)]) was bought from NEN Life Sciences Products, Boston, Massachusetts, USA (NET 396). Plasma ion concentrations (K^+, Na^+, Cl^- and Ca^{2+}) were analyzed using an electrolyte analyzer (Convergys® ISE comfort Electrolyte Analyzer, Convergent Technologies, Cölbe, Germany). Plasma osmolality was measured using a cryoscopy osmometer (Advanced Model 3320 Micro-Osmometer4, Advanced Instruments Inc., Norwood, USA).

2.7.4. Histological analyses

The intestinal samples were dehydrated using an ethanol gradient, cleared in Histolab Clear (Histolab Products AB, Askim, Sweden) and embedded in paraffin using a tissue processor (TP 1020, Leica, Mölndal, Sweden). Six 5 μm thick nonconsecutive cross-sections (≥ 50 μm in-between sections) of the intestinal tube were cut using a Microtome (Shandon Scientific; Labex Instrument, Helsingborg, Sweden) and mounted on 3′-aminopropyltriethoxysilane (APES; Merck KGaA, Darmstadt, Germany) coated slides. The sections were then dewaxed using Histolab Clear (Histolab Products AB, Askim, Sweden) and rehydrated using a reverse gradient of ethanol. A combination of hematoxylin (Histolab Products AB, Askim, Sweden), erythrosine (Histolab Products AB, Askim, Sweden) and alcian blue 8 GX (pH 2.5, Merck, KGaA, Darmstadt, Germany) was used to stain the tissue. Two pictures of each section were taken using a DXM1200 camera (Nikon, Tokyo, Japan) mounted to an Eclipse E1000 microscope (Nikon, Tokyo, Japan, 10× magnification), resulting in 12 images from each fish and intestinal section. Six fish were analyzed from each treatment (2/tank). The pictures were analyzed blind using ImageJ software (Wayne Rasband, NIH USA) for villi length, lamina propria width, submucosa width and number of goblet cells (Baeverfjord and Krogdahl, 1996). Additionally, the supranuclear vacuolization was scored in the distal intestine according to Knudsen et al. (2007).

2.7.5. Intestinal transport and barrier function

Ex vivo measurements assessing intestinal functions were carried out using the Ussing chamber technique as described by Sundell et al. (2003). Immediately after sampling, the serosal layer of the two intestinal region samples was carefully removed under a stereomicroscope using surgical forceps. The remaining tissue was mounted into modified Ussing chambers consisting of two half-chambers connected through a 0.75 cm^2 opening, which was covered by the intestinal preparation (Grass and Sweetana, 1988). At all times the tissues were kept at 10 °C and submerged in aerated (air with 0.3% CO_2) Ringer's solution to preserve normal intestinal function (Sundell and Sundh, 2012).

Analyses of electrical parameters were carried out as described in detail by Sundell and Sundh (2012). Alternating DC voltages were applied across the epithelium using two platinum electrodes inducing currents oscillating between −30 and 30 μA (I) at 5-min intervals. Two KCl electrodes connected to the two half chambers with NaCl and KCl-filled agar bridges were then used to measure the resulting transepithelial potential (TEP, U). Using the U/I relationship a straight line was fitted using the least-square method. The slope of this line represents the transepithelial resistance (TER) according to Ohm's law. TER reflects the resistance across the epithelium. In "leaky" epithelia like the intestine of fish and is mainly comprised of the paracellular shunt resistance. The TEP reflects the net distribution of charged particles between the two chamber compartments and is the result of the passive and active transport occurring across the epithelium. The short circuit current (SCC, estimated through the current voltage intercept, U = 0) reflects the net flow of charged particles across the epithelium. After the mounting, the preparations were given 60 min for the electrical parameters to stabilize. Then the Ringer's solution of each half-chamber renewed to restore iso osmotic conditions, and ^{14}C-mannitol and ^{3}H-lysine were added to the mucosal side of the epithelium. The uncharged, water-soluble molecule ^{14}C-mannitol (0.1 mCi/mL, 58 mCi/mmol; Moravek Biochemicals, Brea, California, USA) was used to determine the apparent permeability (P_{app}, cm/s), a proxy for tight junction permeability (Artursson et al., 1993). Radio labeled ^{3}H-lysine (1 mCi/mL, 25.8 Ci/mmol; Moravek Biochemicals, Brea, California, USA), was used to quantify active amino acid uptake dynamics. Aliquots of 50 μL from the serosal compartment were taken over 90 min (t = 0, 20, 25, 30, 60, 80, 85, and 90 min). Samples were analyzed after the addition of 4 mL scintillation fluid (Ultima Gold, PerkinElmer, Waltham, Massachusetts, USA) using a liquid scintillation counter (Beckman wallac1409 Beckman, Fullerton, California, USA). The P_{app} was expressed as mannitol accumulation in the serosal half-chamber in cm per second and the uptake of L-lysine was calculated as mole per minute and square centimeter.

$$P_{app}\ (cm/s) = dQ/dT \times 1/A \times C_0$$

$$Lysine\ \left(mol/min/cm^2\right) = dQ/dT \times 1/A$$

Where dQ/dt is the serosal appearance rate of ^{14}C-mannitol or ^{3}H-lysine respectively, A is the opening area between the two chambers, and C_0 the initial concentration of C^{14}-mannitol in the serosal chamber compartment.

2.7.6. qPCR analysis

To analyze the transcription of selected immune genes in intestine, spleen and head kidney, the RNA of 20–30 mg of tissue was extracted using QIAGEN's RNeasy Mini Kit (Qiagen NV, Hilden, Germany). Tissues were transferred into tubes containing 600 μL RLT plus buffer and a stainless-steel bead and lysed using a TissueLyser II homogenizer (Qiagen NV, Hilden, Germany). The RNA concentration and purity was determined using a micro volume spectrophotometer (NanoDrop™ One/One^{C}, Thermo Fisher Scientific). Samples were then diluted to obtain equal levels of RNA (1000 ng/sample) using RNase free water. cDNA was synthesized using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories Inc., Richmond, California, USA). qPCR reactions were carried out in duplicates with SYBR Green as fluorescent intercalating agent (SsoAdvanced Universal SYBR® Green Supermix, Bio-rad Laboratories Inc., Richmond, California, USA) and 0.5 μM of forward and reverse primers for the analyzed genes (Table 3). The primers were verified with NCBIs Primer-BLAST tool and each primer pair's efficiency was confirmed using a dilution series of cDNA (0.39-50 ng) from 4 randomly pooled samples. The approved efficiency range was 95–110%. The qPCR was run in duplicates for 40 cycles (10 s denaturation at 95 °C and 30 s annealing and extension at 60 or 61 °C (Table 3) using the CFX Connect Real-time PCR Detection System (Bio-Rad Laboratories Inc., Richmond, California, USA). After the qPCR, the melting curve was analyzed by progressively raising the temperature from 60 °C to 95 °C over 20 min to verify product purity. Gene expression of target genes was compared to the reference genes, β-actin and ELF1α and expressed as relative expression using the $2^{-\Delta CT'}$ method (Livak and Schmittgen, 2001):

$$Relative\ expression = 2_T{}^{-(C_T\ (reference) - C_T\ (target))}$$

Products exhibiting too low expression levels, not passing the efficiency test, or containing excess impurities were excluded from the analysis. This resulted in the exclusion of all samples analyzed for IL-6 mRNA expression in the distal intestine and all genes apart from TGF-β in the proximal intestine. Additionally, between 6 and 0 replicates were removed for IL-6 in the head kidney and IL-10 in the distal intestine. Furthermore, 0–1 replicates per group were excluded for TNF-α3 in the distal intestine and IL-1β in head kidney. The number of replicates is displayed in the respective figure (Figs. 6, 7, A2 and A3).

2.8. Statistical analysis

The statistical analyses were performed using SPSS 26 (SPSS Inc., Chicago, Illinois, USA). The results of the feeding trial were analyzed using a general linear model (GLM) with two fixed factors (diet and tank). The factor tank was nested within the factor diet. The results of the immune challenge were analyzed using a GLM with two fixed factors (diet and immune stimulation) for each sampling point. Normality was tested using a Shapiro-Wilk test and visual inspection of the residual Q-Q plots. Homogeneity was tested using Levene's test. When normality or homogeneity were violated, the data set was transformed using a log transformation to meet the requirements, or, if unsuccessful, a nonparametric Kruskal-Wallis followed by Dunn's test was performed.

Table 3
Primer pair information of two reference genes and target genes related to pro- and anti-inflammatory cytokines in the intestine, spleen and head kidney of rainbow trout.

Gene	Product size (bp)	Direction	Sequence	Annealing temperature	Reference
ELF-1α	327	F	CAAGGATATCCGTCGTGGCA	61	AF498320
		R	ACAGCGAAACGACCAAGAGG		
IL-1β	181	F	ACCGAGTTCAAGGACAAGGA	61	AJ223954
		R	CATTCATCAGGACCCAGCAC		
IL-6	288	F	CCTTGCGGAACCAACAGTTTG	60	DQ866150
		R	CCTCAGCAACCTTCATCTGGTC		
IL-10	119	F	GGATTCTACACCACTTGAAGAGCCC	60	AB118099
		R	GTCGTTGTTGTTCTGTGTTCTGTTGT		
IL-17	212	F	CGTGTCGAAGTACCTGGTTGTGT	60	AJ580842
		R	GGTTCTCCACTGTAGTGCTTTTCCA		
TGF-β	275	F	AGATAAATCGGAGAGTTGCTGTG	61	X99303
		R	CCTGCTCCACCTTGTGTTGT		
TNF-α3	148	F	GCTGCACTCTTCTTTACCAAGAAACAAG	60	HE798544
		R	CCACTGAGGACTTGTAATCACCATAGGT		
β-actin	260	F	ATGGAAGATGAAATCGCC	61	AF157514
		R	TGCCAGATCTTCTCCATG		

IL: interleukin, TNF-α3: tumor necrosis factor-α3, TGF-β: transforming growth factor-β, ELF-1α: elongation factor-1 alpha, F: forward, R: reverse.

When differences were found in the GLM, a pairwise Turkey post-hoc adjusted for multiple comparisons was performed. Significance was assumed at $p < 0.05$. Values are presented as means ± standard error of the mean (SE).

3. Results

3.1. Growth and feed intake

The fish grew from an average starting weight of 106.5 ± 1.3 g to an average final weight of 233.6 ± 4.0 g. No significant differences in WG (g) and SGR (% body weight/day) were observed between the treatment groups (Fig. 1). Similarly, the inclusion of *C. sake* had no effect on any of the biometric indices (CF, VFI, HSI) or on FI and FCR (Table 4). The mortality was 3% (6 out of 180) throughout the experiment. Of these, 4individuals were found dead and 2 were euthanized due to loss of buoyancy control. The mortalities all took place in the beginning of the trial and were possibly delayed effects of poor acclimatization to salt-water in these individuals. No difference in survival rate was found between the groups (Table 4).

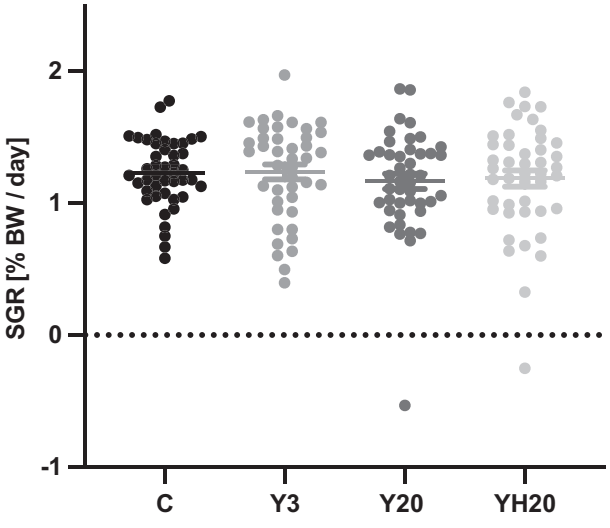

Fig. 1. Specific growth rate (SGR) of juvenile rainbow trout fed one of the experimental diets; control (C), 3% yeast (Y3), 20% yeast (Y20) and 20% heat-treated yeast (YH20). Bars represent mean ± SE (n = 42–44). Circles represent individual fish.

3.2. Apparent digestibility coefficient (ADC)

The ADC of the diets was highest for diet C. The ADCs for gross energy (GE), crude protein (CP) and dry matter (DM) of diet Y20 were significantly lower compared those of diet C. No differences were observed when comparing the ADCs of CP, crude lipid (CL), GE and DM of diet YH20 with those of diet C or Y20. The amino acid (AA) ADCs were lower in diet Y20 compared to diet C except for alanine where higher levels were observed for diet Y20. Diet YH20 displayed the overall lowest AA ADC with a reduction in 15 out of 16 AAs compared to the control diet and in 10 out of 16 AAs compared to diet Y20 (Table 5).

When comparing the ADC for the test ingredients, the heat-treated yeast displayed higher DM ADC compared to the untreated yeast. No differences between the two yeast ingredients were found for the ADC of CP, CL and GE. As observed for the experimental diets, a reduction in the AA ADC was found for the heat-treated yeast compared to the untreated yeast. The reduction was apparent for the AAs: alanine, asparagine, phenylalanine, glycine, isoleucine, leucine, lysine, proline, serine and valine (Table 6).

3.3. Blood and plasma

The inclusion of yeast had a significant effect on Hb, Hct and plasma K^+ concentrations. Fish fed the diet YH20 had lower Hb and Hct levels compared to fish fed the diet Y20. The K^+ concentration of fish fed the YH20 was significantly elevated compared to diets Y3 and Y20. Additionally, fish fed diet C exhibited higher K^+ levels compared to diet Y3. No significant differences were observed for MCHC, pH, plasma ions (Cl^-, Ca^+ and Na^+) or cortisol between any of the experimental groups (Table 7).

3.4. Intestinal barrier function and histology

The intestinal barrier function was unaffected by the dietary treatments. No significant differences were found for the electrophysiological parameters TER (Fig. 2), TEP and SCC in the proximal intestine (Fig. A1). Similarly, no differences were found in the distal intestine (Fig. 2, Fig. A1). The apparent permeability for mannitol and the uptake rate of lysine were also unaffected by the dietary treatments (Fig. 2, Fig. A1).

The histological analysis revealed no morphological changes in the proximal intestine (Fig. 3). However, in the distal intestine the lamina propria width of fish fed diet Y3 was significantly increased compared to diet C (Fig. 4, Fig. 5). No alterations in villi length, submucosa thickness or vacuolization were observed in the distal intestine (Fig. 4).

Table 4
Growth parameters and body indices of rainbow trout fed one of the experimental diets; control (C), 3% yeast (Y3), 20% yeast (Y20) and 20% heat-treated yeast (YH20). The data is shown as mean ± SE.

Parameter	Diet				[1]*p*-value	
	C	Y3	Y20	YH20	Diet	Tank (Diet)
Initial weight, g (n = 15)	105.4 ± 2.8	106.8 ± 2.6	106.7 ± 2.8	107.1 ± 2.7	0.96	0.97
Final weight, g (n = 15)	233.2 ± 7.3	239.4 ± 8.5	229.1 ± 8.2	233.7 ± 8.5	0.83	0.35
WG, g (n = 15)	127.7 ± 5.8	132.6 ± 7.9	122.2 ± 6.3	126.3 ± 6.9	0.77	
Feed intake, g/fish/tank (n = 3)	93.6 ± 3.9	99.1 ± 2.7	93.3 ± 3.6	96.8 ± 1.7	0.51	
FCR (n = 3)	0.74 ± 0.03	0.75 ± 0.01	0.77 ± 0.04	0.77 ± 0.04	0.86	
CF (*n* = 15)	1.46 ± 0.02	1.45 ± 0.02	1.49 ± 0.02	1.48 ± 0.03	0.71	0.35
HSI (n = 4)	2.05 ± 0.08	2.32 ± 0.12	2.01 ± 0.12	2.03 ± 0.13	0.21	0.84
VFI (n = 4)	1.81 ± 0.10	2.06 ± 0.26	1.99 ± 0.30	1.64 ± 0.12	0.80	0.80
Survival, % (*n* = 3)	98 ± 2.3	93 ± 0	98 ± 2.3	98 ± 2.3	0.45	

WG: weight gain, SGR: specific growth rate, FBW: final body weight, FI: feed intake, FCR: feed conversion ratio, VFI: visceral fat index and HSI: hepatosomatic index.
[1] *p*-values obtained from nested GLM for *n* = 15 or 4, and one-way ANOVA for *n* = 3. WG was analyzed using a nonparametric Kruskal-Wallis analysis of variance.

3.5. Immune challenge

In the proximal intestine, *C. sake* had an effect on TGF-β mRNA levels. PBS injected fish fed diet Y3 exhibited higher TGF-β mRNA levels compared to PBS injected control fish (Fig. 6). LPS did not stimulate cytokine expression in the proximal intestine. No effect on the relative gene expression was observed in the distal intestine by neither diet nor LPS (Fig. 7). The intraperitoneal LPS injection resulted in a significant systemic immune stimulation 12 h post injection. In the head kidney, the relative mRNA concentrations of IL-1β and IL-6 and IL-10 increased after LPS stimulation (Fig. A2). In the spleen, the mRNA levels of the pro-inflammatory cytokine TNF-α3 and the anti-inflammatory cytokine IL-10 increased 12 h post LPS injection (Fig. A3). After 48 h, no signs of an immune response were observed in any sampled tissue for both groups.

Table 5
Apparent digestibility coefficient (%) for dry matter, crude protein, gross energy and amino acids of the experimental diets in juvenile rainbow trout; control (C), 20% yeast (Y20) and 20% heat-treated yeast (YH20). The data is shown as mean ± SE. (n = 3).

	Diet			
	C	Y20	YH20	*p*-value
Proximate composition				
Dry matter	83.23^{a} ± 0.76	80.09^{b} ± 0.38	81.72^{ab} ± 0.45	0.020
Crude Protein	95.10^{a} ± 0.23	94.18^{b} ± 0.15	94.37^{ab} ± 0.17	0.028
Crude fat	92.75 ± 1.02	91.04 ± 1.04	91.81 ± 1.47	0.621
Gross energy	91.80^{a} ± 0.47	90.22^{b} ± 0.17	90.72^{ab} ± 0.20	0.028
Indispensable amino acids				
Arginine	97.02^{a} ± 0.20	96.22^{b} ± 0.11	95.99^{b} ± 0.08	0.004
Histidine	96.56^{a} ± 0.20	95.59^{b} ± 0.14	94.79^{c} ± 0.17	0.001
Isoleucine	95.56^{a} ± 0.26	94.41^{b} ± 0.14	92.90^{c} ± 0.17	<0.001
Leucine	96.06^{a} ± 0.21	95.15^{b} ± 0.12	93.99^{c} ± 0.21	0.001
Lysine	96.94^{a} ± 0.20	96.33^{a} ± 0.08	95.53^{b} ± 0.19	0.003
Methionine	98.21^{a} ± 0.12	97.64^{b} ± 0.06	97.33^{b} ± 0.06	0.001
Phenylalanine	96.10^{a} ± 0.23	94.85^{b} ± 0.14	93.80^{c} ± 0.19	<0.001
Threonine	95.29^{a} ± 0.20	93.40^{b} ± 0.19	91.96^{c} ± 0.31	<0.001
Valine	95.56^{a} ± 0.26	94.77^{a} ± 0.10	93.68^{b} ± 0.21	0.002
Dispensable amino acids				
Alanine	95.51^{a} ± 0.25	96.65^{b} ± 0.10	96.04^{ab} ± 0.14	0.011
Aspartic acid	95.90^{a} ± 0.18	95.00^{b} ± 0.14	93.84^{c} ± 0.21	0.001
Cysteine	94.38^{a} ± 0.31	90.74^{b} ± 0.35	90.81^{b} ± 0.50	0.001
Glutamic acid	97.96^{a} ± 0.15	96.76^{ab} ± 0.23	96.18^{b} ± 0.09	0.001
Glycine	95.37^{a} ± 0.25	94.83^{a} ± 0.15	93.55^{b} ± 0.20	0.002
Proline	97.22^{a} ± 0.14	95.97^{b} ± 0.13	94.77^{c} ± 0.14	<0.001
Serine	95.63^{a} ± 0.21	94.63^{b} ± 0.13	93.18^{c} ± 0.27	0.001

Values within rows with different superscripts are significantly different ($p < 0.05$).

4. Discussion

4.1. C. sake production and nutritional profile

The first objective of the present study was the production of *C. sake* on herring brine and the assessment of its nutrient profile. The growth rate and cell density at stagnation after 22 h suggest that *C. sake* can be produced in a commercial setting. With 54 g CP/100 g DM, the protein content of *C. sake* was slightly lower than that of fish meal (55–70 g CP/100 g), but similar to that of many plant protein sources including soybean meal (40–55 CP/100 g; Glencross, 2020; Glencross et al., 2007; Moutinho et al., 2017). Compared to fish meal, the ash content of *C. sake* was lower (5.43 g/100 g vs 11–16 g/100 g) while the lipid content was similar (13.3 vs. 8–15 g/100 g; Glencross, 2020). Compared to other promising yeast species which generally range in crude protein content between 38 and 52 g/100 g, the protein content of *C. sake* was high (Agboola et al., 2021). The amino acid profile of *C. sake* was similar

Table 6
Apparent digestibility coefficient (%) for dry matter, crude protein, gross energy and amino acids of the experimental ingredients in juvenile rainbow trout; freeze dried *C. sake* (Y20) and heat-treated *C. sake* (YH20). The data is shown as mean ± SE. (n = 3).

	Ingredient		
	Y20	YH20	*p*-value
Proximate composition			
Dry matter	67.13 ± 1.93	75.49 ± 2.30	0.049
Crude Protein	91.04 ± 0.67	91.91 ± 0.74	0.432
Crude fat	80.89 ± 7.17	86.25 ± 10.19	0.689
Gross energy	83.11 ± 0.92	85.83 ± 1.08	0.127
Indispensable amino acids			
Arginine	93.74 ± 0.44	93.18 ± 0.30	0.351
Histidine	92.04 ± 0.68	89.72 ± 0.68	0.072
Isoleucine	90.23 ± 0.66	85.69 ± 0.62	0.007
Leucine	91.07 ± 0.68	86.89 ± 0.95	0.023
Lysine	94.26 ± 0.37	91.85 ± 0.70	0.038
Methionine	88.42 ± 1.11	86.62 ± 0.75	0.250
Phenylalanine	90.28 ± 0.65	87.27 ± 0.72	0.036
Threonine	87.13 ± 0.83	83.36 ± 1.10	0.052
Valine	91.72 ± 0.51	87.96 ± 0.83	0.018
Dispensable amino acids			
Alanine	101.24 ± 0.52	97.74 ± 0.58	0.011
Aspartic acid	91.96 ± 0.61	88.55 ± 0.76	0.025
Cysteine	76.39 ± 1.73	77.81 ± 2.34	0.650
Glutamic acid	90.90 ± 1.33	89.64 ± 0.42	0.418
Glycine	92.51 ± 0.81	87.71 ± 0.82	0.014
Proline	88.08 ± 0.94	83.78 ± 0.75	0.023
Serine	91.36 ± 0.57	87.13 ± 0.94	0.018

Table 7
Plasma chemistry and pH of rainbow trout fed one of the experimental diets; control (C), 3% yeast (Y3), 20% yeast (Y20) and 20% heat-treated yeast (YH20). The data is shown as mean ± SE (n = 4).

Parameter	Diet				*p*-value[1]	
	C	Y3	Y20	YH20	Diet	Tank (Diet)
Hct (%)	33.28^{ab} ± 1.49	33.33^{ab} ± 1.11	33.82^{a} ± 1.41	28.90^{b} ± 1.08	0.03	0.61
Hb (g/dL)	6.72^{ab} ± 0.27	6.79^{ab} ± 0.26	7.08^{a} ± 0.25	5.97^{b} ± 0.28	0.04	0.76
MCHC (g/dL)	20.25 ± 0.37	20.42 ± 0.62	21.02 ± 0.50	20.63 ± 0.40	0.74	0.02
Na^+ (mM)	163.0 ± 1.67	161.9 ± 1.55	162.4 ± 1.74	160.9 ± 2.16	0.83	0.13
K^+ (mM)[2]	5.93^{ac} ± 0.41	4.91^{b} ± 0.13	5.32^{bc} ± 0.18	7.14^{a} ± 0.46	<0.001	
Cl^-(mM)[2]	138.6 ± 1.80	137.4 ± 2.78	137.4 ± 1.41	134.3 ± 1.40	0.20	
Ca^{2+}(mM)	1.09 ± 0.03	1.12 ± 0.04	1.10 ± 0.03	1.00 ± 0.05	0.13	0.22
Cortisol (ng/mL)[2]	3.69 ± 0.67	5.82 ± 1.06	2.78 ± 0.64	6.00 ± 1.61	0.09	
pH	7.19 ± 0.02	7.19 ± 0.02	7.24 ± 0.01	7.19 ± 0.01	0.08	0.004

Hct: hematocrit, Hb: hemoglobin, MCHC: mean corpuscular hemoglobin concentration, K^+: potassium, Na^+: sodium, Cl^-: chloride, Ca^{2+}: calcium.
Values within rows with different superscripts are significantly different ($p < 0.05$).
[1] *p*-values obtained from nested GLM except for [2]
[2] K^+, Cl^- and cortisol were analyzed using a nonparametric Kruskal-Wallis analysis of variance (no nested model).

compared to other yeast strains like *C. jadinii* and *S. cerevisiae*, as well as soybean meal (Agboola et al., 2021; Glencross et al., 2007). In fish nutrition, lysine and methionine have received increasing attention as limiting amino acids due to their low availability in plant protein sources (Gatlin et al., 2007). In yeasts, including *C. jadinii*, *S. cerevisiae* and *C. sake,* the first limiting amino acids may be methionine (Agboola et al., 2021). In the present study, all diets were supplemented with methionine.

The lipid content of *C. sake* (13 g/100 g DM) was similar to that of fish meal and high compared to other yeasts explored as fish feed ingredients (2–9 g/100 g DM, Agboola et al., 2021). Additionally, 27% of these fatty acids were polyunsaturated including 7.5% DHA and 8.3% EPA, suggesting that *C. sake* is able to bioaccumulate or synthesize DHA and EPA. Marine yeast strains have been shown to incorporate EPA and DHA when provided with it through the media (Fabiszewska et al., 2021; Gou and Ota, 2000; Kang et al., 2006). However, in these studies the media was enriched with fish oil. In the present experiment, *C. sake* was cultured on herring brine without any additional supplementation. The supply of highly unsaturated n-3 fatty acids is a major bottleneck for the replacement of fish oil and meal in feeds for marine, high trophic fish such as salmonids (Aas et al., 2019; Løvmo et al., 2021; Turchini et al., 2009). Therefore, *C. sake* may be a highly attractive microorganism and feed ingredient for salmonid aquaculture.

4.2. *Effects of yeast inclusion on growth physiology and health*

The second objective was to evaluate *C. sake* as a protein source. Both diets with 20% *C. sake* performed well, and no differences in weight gain, SGR, VFI, HSI and CF were observed compared to the control diet. Interestingly, this was despite the fact that the diets were not isocaloric (by virtue of the experimental design to assess apparent digestibility;

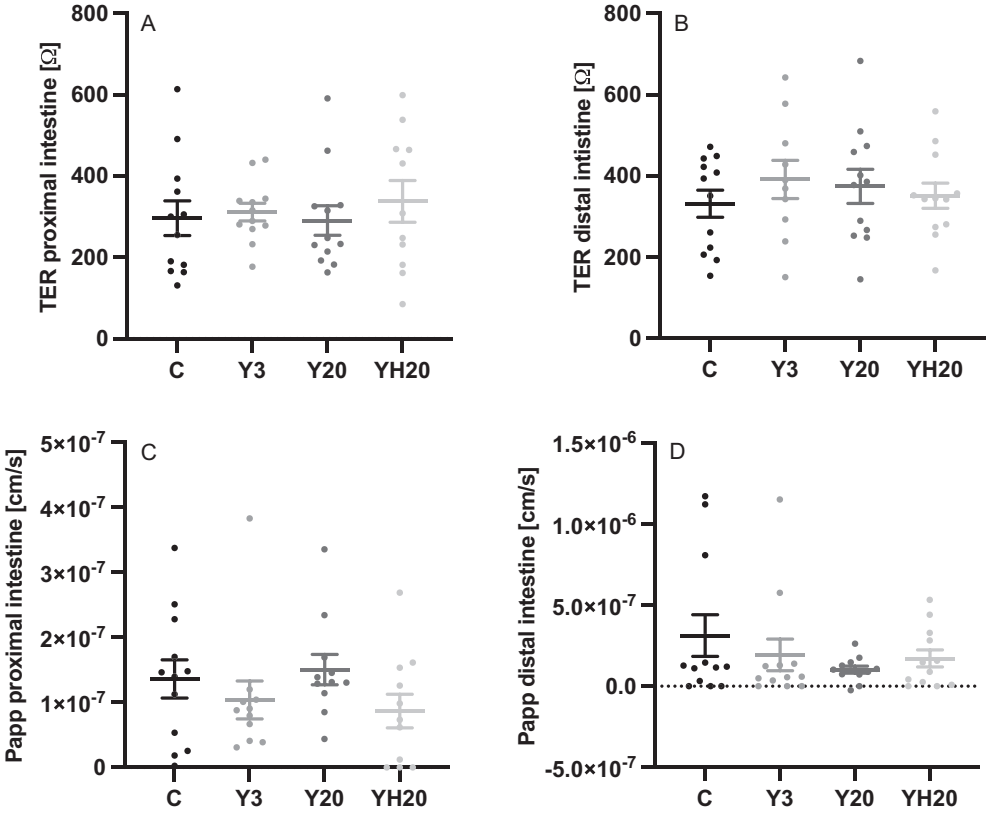

Fig. 2. Ussing chamber measurements of transepithelial resistance (TER) of proximal (A) and distal (B) intestine as well as apparent permeability (Papp) for 14C-labeled mannitol in the proximal (C) and distal (D) intestine in rainbow trout fed one of the experimental diets; control (C), 3% yeast (Y3), 20% yeast (Y20) and 20% heat-treated yeast (YH20). Bars represent mean ± SE (n = 11–12). Circles represent individual fish.

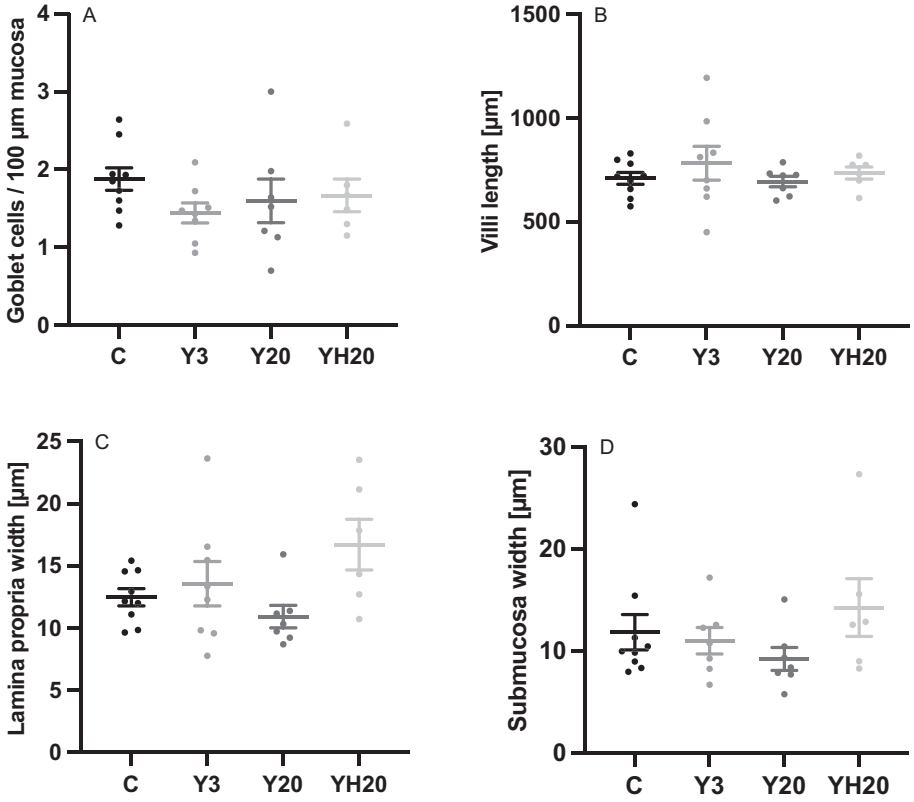

Fig. 3. Histological analysis in the proximal intestine of rainbow trout for number of goblet cells per 100 µm mucosa (A), villi length (B), lamina propria width (C) and submucosa width (D). Bars represent mean ± SE (n = 6–9). Circles represent individual fish.

Cho and Slinger, 1979). The most likely explanation for the equal growth rates is a compensatory increase in feed intake. While not significant, feed intake and FCR were higher in the diets containing 20% *C. sake* and SGR was highest in the control. This suggests that the difference in gross energy of 1.8 MJ/kg was likely not enough to significantly affect growth or feed consumption during the feeding trial. The similar growth rates also indicate a high bioavailability and digestibility of *C. sake*. The apparent digestibility for protein and amino acids was high (> 80%) for both the heat-treated and untreated yeast. Reported protein ADC values for yeast vary between ~50–100% (Agboola et al., 2022; Hauptman et al., 2014). However, direct comparisons may be misleading as ADC values vary depending on feces collection technique, innate digestibility markers, feed composition, and feed production (Gomes et al., 1995; Vandenberg and De La Noüe, 2001). The two most common feces collection methods in studies evaluating yeast are stripping and settling columns (Austreng, 1978; Cho et al., 1982). Settling columns are mounted to the outflow of each tank and feces accumulate in the base of the column due to their high density compared to water. This method has a tendency to overestimate ADC due to the leaching of nutrients in the water column while stripping may slightly underestimate ADC as feces are removed before natural evacuation (Blyth et al., 2015; Glencross et al., 2005). Our results are similar to ADC values obtained using the settling column in feeding trials with Arctic charr and Eurasian perch (*Perca fluviatilis*) fed extruded diets containing 30% *S. cerevisae* (Langeland et al., 2016). In the present study, a custom fitted filter system was used, where feces naturally settled at the center of a conical tank bottom before they were collected in a filter connected to the outflow of each tank. While no direct comparison has been performed, this approach is based on the settling column principle and may result in comparable measurements. Thus, the ADC of *C. sake* may be similar to that of *S. cerevisae* as well as plant-based meals like soy or lupin protein concentrates (Glencross et al., 2005; Langeland et al., 2016).

4.3. Effect of the heat treatment on digestibility

The additional heat treatment of *C. sake* improved dry matter ADC compared to the untreated yeast. The cell wall of yeast makes up ca. 30% of the yeast's dry weight and is composed of complex polysaccharides that are difficult for carnivorous fish to digest (Glencross, 2009; Nguyen et al., 1998). Additional downstream processing of yeast products has been used to disrupt complex carbohydrates and to increase digestibility for *S. cerevisae*, *C. jadinii, Blastobotrys adeninivorans* and *Wickerhamomyces anomalus* salmonid diets (Agboola et al., 2022; Hansen et al., 2021; Langeland et al., 2016; Rumsey et al., 1991). Thus, the increase in digestibility of the heat-treated *C. sake* may be the result of a rupture in the yeast's cell wall, leading to an increased carbohydrate bioavailability. However, the present study also revealed a possible undesired effect of the heat treatment as it lowered amino acid ADC by on average 3%. Excessive heat can lead to endothermic decomposition of amino acids (Weiss et al., 2018). However, amino acids have been shown to be relatively stable at temperatures below 180 °C and the temperature we applied to the heat-treated yeast (105 °C) is comparatively mild and in range of commonly used extrusion temperatures (Weiss et al., 2018). Another possible explanation for the reduction in amino acid ADC may be the Maillard reaction, which can destroy and reduce the bioavailability of amino acids at temperatures around 100 °C (Seiquer et al., 2006). While this process is generally slow, it is possible that the additional heat treatment contributed to the formation of Maillard reaction products and/or protein cross links, which have been shown to reduce nutrient digestibility in rainbow trout (Hurrell and Carpenter, 1977; Plakas et al., 1985). To avoid this trade-off of increasing dry matter and gross energy ADC, and a decreasing amino acid ADC, alternative downstream processing techniques such as autolysis may be preferable to heat in future studies (Agboola et al., 2022). Alternatively, considering the generally high digestibility (>80%), the high protein content and good growth rates of fish fed the

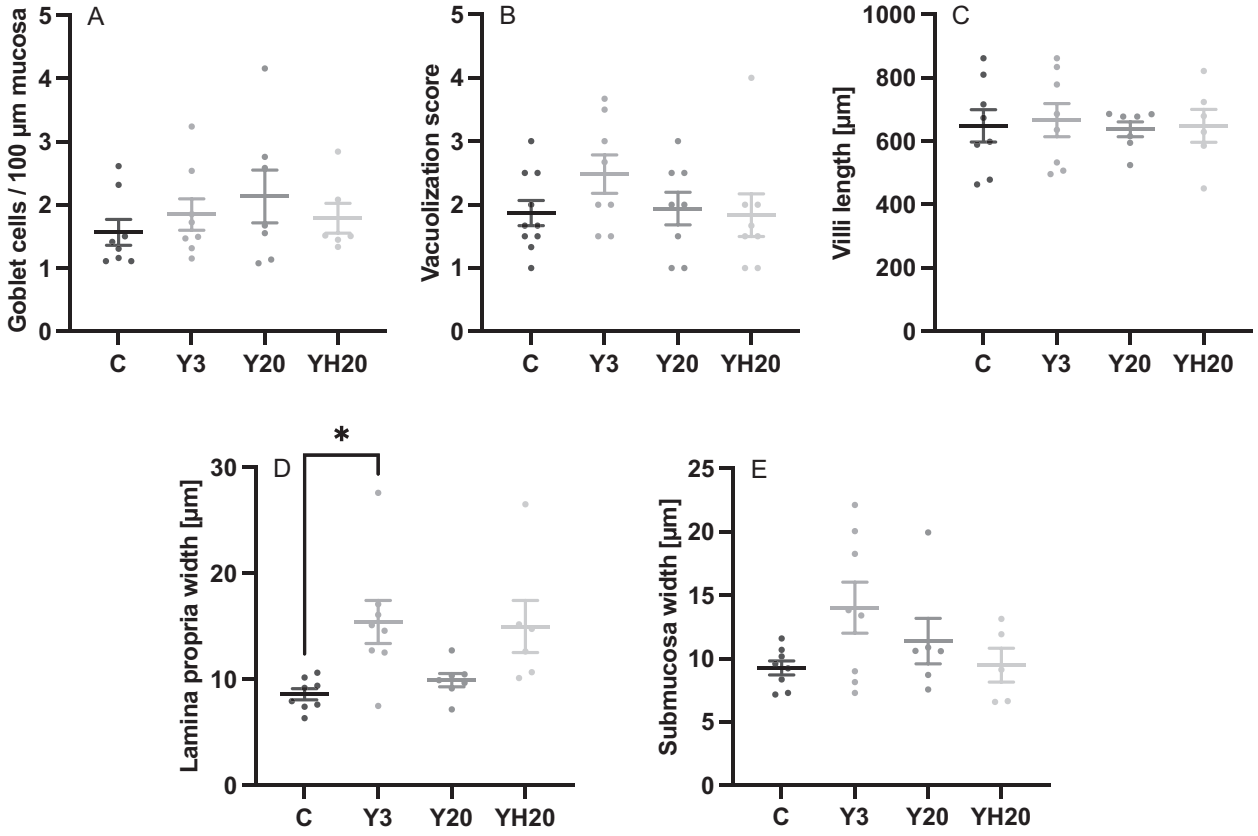

Fig. 4. Histological analysis in the distal intestine of rainbow trout for number of goblet cells per 100 µm mucosa (A, n = 8 (C, Y3), 7 (Y20) and 6 (YH20)), vacuolization score (B, n = 10 (C), 8 (Y3, Y20, YH20)), villi length (C, n= 8 (C, Y3), 7 (Y20) and 6 (YH20)), lamina propria width (D, 8 (C, Y3), 7 (Y20) and 6 (YH20)), and submucosa width (E, 8 (C, Y3), 6 (Y20) and 5 (YH20)). The vacuolization scoring system was adopted from Knudsen et al. (2007) where: 1 = large vacuoles occupy almost the entire apical part of the enterocytes, 2 = medium-sized vacuoles, 3 = small-sized vacuoles are near the apical membrane in most enterocytes, 4 = scattered small vacuoles are still present in some enterocytes and 5 = no supranuclear vacuoles are present. The asterisk (*) indicates significant differences between groups ($p \leq 0.05$). Bars represent mean ± SE. Circles represent individual fish.

untreated yeast, additional downstream processing may not be required for *C. sake*. Minimal processing benefits the scalability and production costs of potential commercial *C. sake* products. Additionally, 20% yeast inclusion, irrespective of treatment, had no negative effect on the growth and intestinal health suggesting that *C. sake* may be safely used as a protein source in diets for rainbow trout.

4.4. Immune modulating properties of low C.sake inclusion

The last objective of the present study was to explore possible immune modulating effects of *C. sake*. Yeasts contain indigestible components, such as β-glucans and MOS, which can promote the growth of beneficial microorganisms in the digestive tract of the host as well as interact directly with the host's immune system. While *C. sake* has been used to improve disease resistance and phenoloxidase activity (equivalent to the complement system in vertebrates) in the Indian white prawn (*Fenneropenaeus indicus*), this is the first evaluation of the immune modulating properties of *C. sake* in fish (Sajeevan et al., 2009; Sarlin and Philip, 2016). Three percent *C. sake* inclusion resulted in an increase in the lamina propria width in the distal intestine, and increased gene transcription of TGF-β in the proximal intestine. Dietary β-glucans and MOS have been shown to alter villi and microvilli morphology in rainbow trout and red drum (*Sciaenops ocellatus*; Dimitroglou et al., 2009; Genc et al., 2007; Sweetman and Davies, 2006; Zhou et al., 2010). An explanation for the increase in lamina propria width may be a higher concentration of leukocytes in the mucosa, which has been observed in response to β-glucans and probiotics (Gisbert et al., 2013; Standen et al., 2015; Tsukada et al., 2003). In mice (*Mus musculus*), the increased lymphocyte concentration coincided with an increase in cytokine production (Tsukada et al., 2003). TGF-β is a multifunctional cytokine, involved in both pro and anti-inflammatory pathways and has been shown to inhibit the LPS induced upregulation of TNF-α (Zou and Secombes, 2016; Smythies et al., 2005). Furthermore, TGF-β has been shown to inhibit the production of several proinflammatory cytokines including IL-1β and IL-10 (Fadok et al., 1998). While a systemic proinflammatory response was observed in spleen and head kidney, the oral administration of *C. sake* may have led to an increased TGF-β transcription in the intestine which in turn may counteract a proinflammatory response to LPS. This mechanism may be especially important considering the increase in lamina propria width, possibly due to an increased number of lymphocytes. While located in different sections of the intestine, both the increase in lamina propria width and the altered TGF-β transcription indicate an immune stimulating effect. However, the observed effects were mild and further research including an assessment of the microbiota and specific staining for leukocytes is needed.

The lamina propria width was originally included as a histopathological indicator to identify enteritis (Baeverfjord and Krogdahl, 1996; Knudsen et al., 2007). In the present study however, no changes were observed for the other inflammatory indicators, which included villi length, submucosa width, vacuolization and goblet cell count. As such, no developed enteritis was observed. Enteritis may also affect the physiological functions of the intestine including epithelial barrier function and nutrient uptake efficacy (Halpern and Denning, 2015; Knudsen et al., 2008; Sundh et al., 2011; Venkatakrishnan et al., 2019). In the present study, no effects of yeast inclusion were observed on

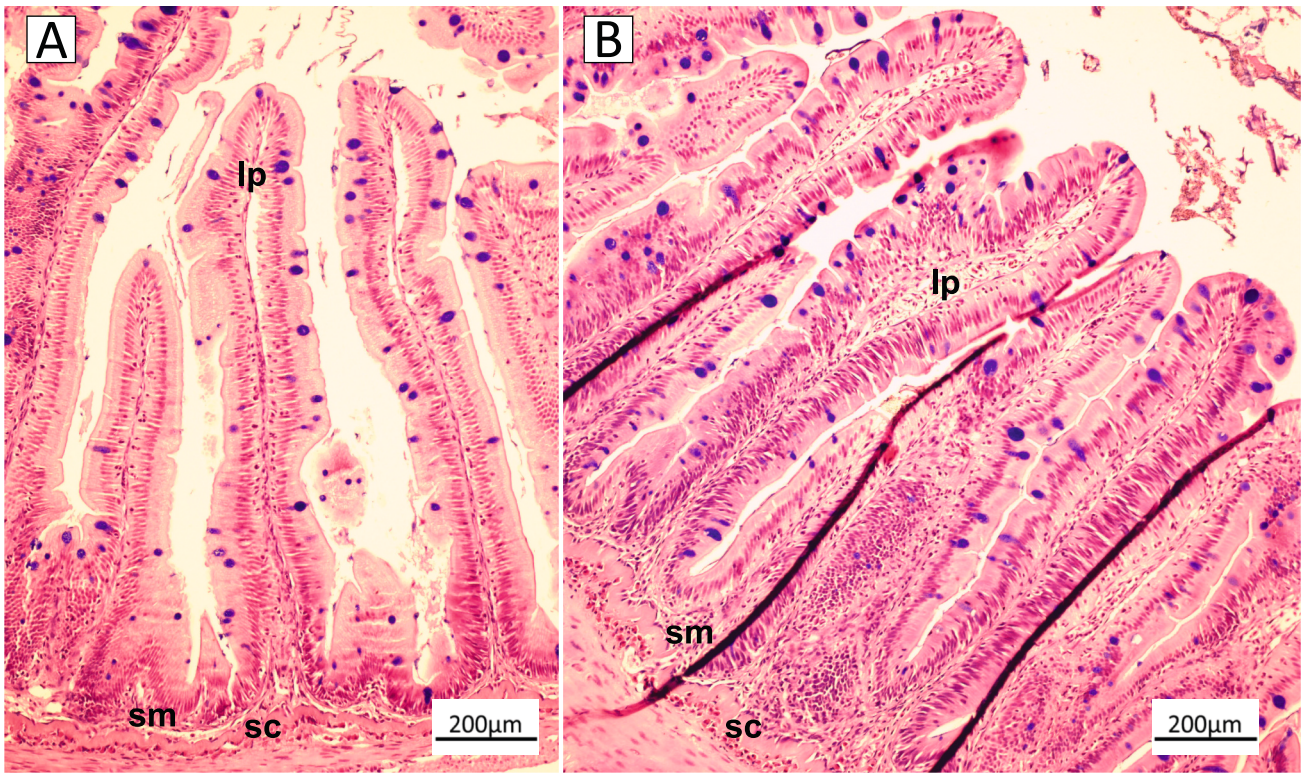

Fig. 5. Histological section of the distal intestine of juvenile rainbow trout fed the control feed (A), and diet Y3 containing 3% *C. sake* (B). Sections were stained using hematoxylin, erythrosine and alcian blue. Magnification: 10×. lp: lamina propria, sc: stratum compactum, sm: submucosa. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

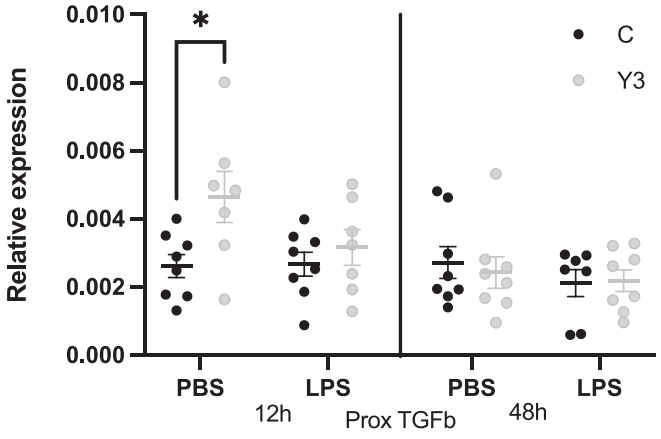

Fig. 6. Gene expression relative to ELF-1α and β-actin mRNA in proximal intestine of TGFβ for rainbow trout injected with either PBS or LBS and fed either a control feed (C) or feed containing 3% *C. sake* (Y3), 12 and 48 after injection. The asterisk (*) indicates significant differences between subgroups ($p \leq 0.05$). Bars represent mean ± SE ($n = 7–8$). Circles represent individual fish.

electrophysiology (SCC, TEP and TER), amino acid uptake, or mannitol permeability in either the proximal or distal intestine. This further supports the notion of good intestinal health in all treatments. Additionally, no change in morphology was observed in either treatment containing 20% yeast (Y20, YH20), thus the observed effect of 3% yeast inclusion is unlikely due to the presence of antinutritional factors contained within the yeast. The reason for why the increase in lamina propria width was restricted to the 3% inclusion treatment remains unclear.

4.5. Effects of *C. sake* on blood chemistry and hematology

Dietary *C. sake* has also been shown to alter the hemocyte count of the Indian white shrimp, promoting immune function (Sajeevan et al., 2009). In the present study, heat-treating *C. sake* resulted in a decrease in Hb and Hct coinciding with an increase in plasma potassium compared to the untreated yeast, which suggests hemolysis (Santos and Hall, 1990). The high content of purine nucleotides of yeast may lead to the production of hydrogen peroxide that can damage red blood cells and yeast inclusion has been shown to induce hemolytic anemia in rainbow trout (Bontemps et al., 1986; Huyben et al., 2017). However, the nucleotide content was likely similar in both heat-treated and untreated yeast. Furthermore, salmonid fish have been shown to tolerate high levels of purine nucleotides through an upregulation of the enzyme urate oxidase (Andersen et al., 2006). Dietary nucleotide exposures equal to 500 g/kg *S. cerevisiae* have been shown to have no negative effect on growth, feed intake and nutrient retention (Rumsey et al., 1992). While mainly applicable to low concentrations, dietary nucleotide supplementation has even been shown to improve health and disease resistance in fish (Li and Gatlin, 2006). Alternatively, the heat treatment could have affected the bioavailability and uptake of nucleotides. However, the nitrogen (crude protein) ADC remained unaffected by the heat treatment indicating no increase in nucleotide bioavailability and thus exposure for the animal. Thus, the observed effect may be a result of the heat treatment independent of the nucleotides.

5. Conclusion

This is the first study evaluating a marine yeast as a feed ingredient for salmonid fish. *C. sake* contained above 50% protein as well as the essential fatty acids DHA and EPA. The presence of long chained n-3 fatty acids such as EPA and DHA represent an added value compared to terrestrial yeast, especially in feed for carnivorous fish. In the 9-week

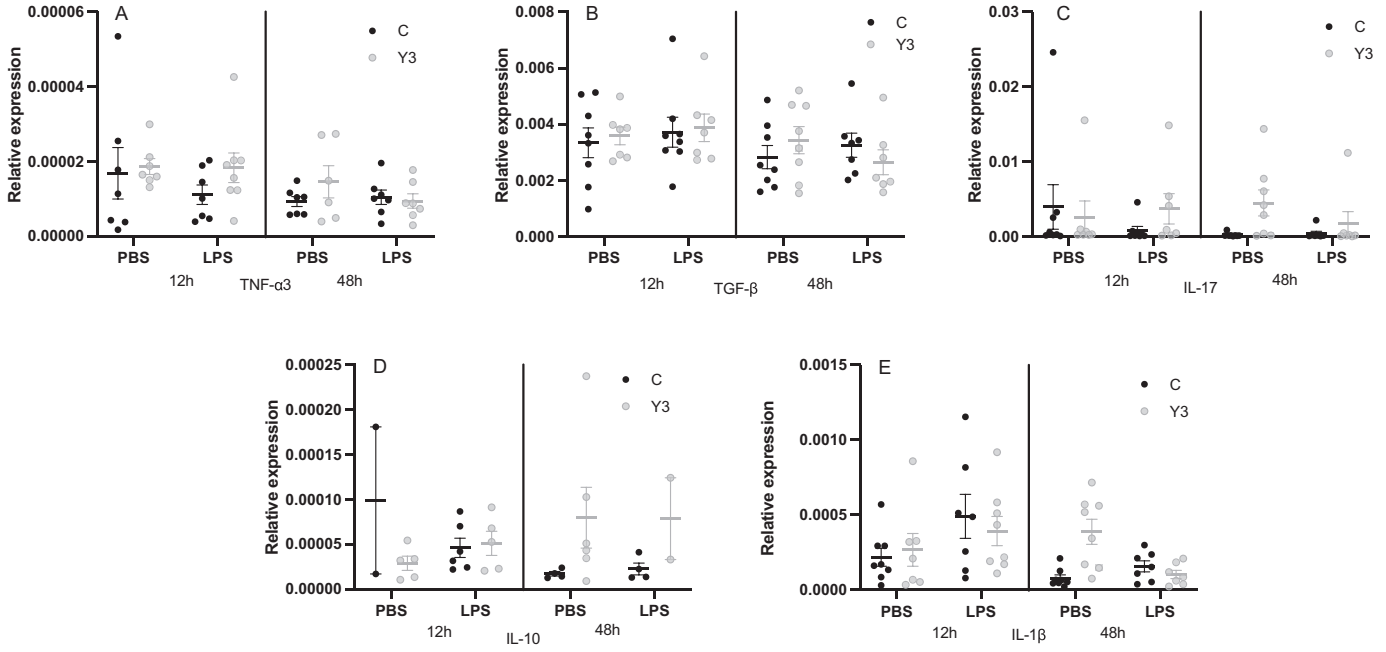

Fig. 7. Gene expression relative to ELF-1α and β-actin mRNA in the distal intestine of TNF-α3 (A), TGF-β (B), IL-17α (C) (n = 6–8),IL-10 (D) (*n* = 2–6) and IL-1β (E) (n = 7–8) for rainbow trout injected with either PBS or LBS and fed either a control feed (C) or feed containing 3% *C. sake* (Y3), 12 and 48 after injection. Bars represent mean ± SE. Circles represent individual fish.

feeding trial using rainbow trout, 20% *C. sake* inclusion resulted in digestibility values comparable to soy protein concentrate and had no negative effects on animal welfare or growth, suggesting that *C. sake* is well suited as a protein source in diets for rainbow trout. Mild downstream processing may further improve the digestibility, but heat treatment may not be the most suitable method. Low *C. sake* supplementation can modulate the intestinal morphology and cytokine transcription indicating additional potentially beneficial properties of *C. sake*. However, the observed immune modulating effects were mild and future research is needed to understand the effect of *C. sake* supplementation. The results of this experiment show that herring brine side streams can be transformed into a sustainable protein source using marine yeast as a vector and underline the potential of *C. sake* as an attractive feed ingredient for salmonid fish.

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CRediT authorship contribution statement

Niklas Warwas: Conceptualization, Methodology, Formal analysis, Investigation, Visualization, Writing – original draft. **Jenny Veide Vilg:** Conceptualization, Project administration, Resources, Writing – review & editing. **Markus Langeland:** Supervision, Project administration, Writing – review & editing. **Jonathan A.C. Roques:** Investigation, Methodology, Writing – review & editing. **James Hinchcliffe:** Investigation, Writing – review & editing. **Henrik Sundh:** Supervision, Writing – review & editing. **Ingrid Undeland:** Conceptualization, Funding acquisition, Writing – review & editing. **Kristina Sundell:** Conceptualization, Supervision, Funding acquisition, Project administration, Writing – review & editing.

Declaration of competing interest

The authors declared no competing interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2023.739448.

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