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Haematological and intestinal health parameters of rainbow trout are influenced by dietary live yeast and increased water temperature

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Abstract

Live yeast may be a sustainable protein source in salmonid diets while exhibiting a probiotic effect to counteract environmental stressors, such as increased water temperature that is being exacerbated by climate change. The objective of this study was to evaluate the effects of feeding a high dietary inclusion of live yeast and increased water temperature on growth, haematological and intestinal physiology of rainbow trout. For six weeks, 129 g fish in 16 tanks (n=4) were fed either a diet based on fishmeal or based on live yeast (214 g kg^{-1} of diet or 7.6 log CFU g^{-1} of *Saccharomyces cerevisiae*) that replaced 40% of fishmeal protein while fish were reared in water temperatures of either 11 °C (cold) or 18 °C (warm). Fish weights, caudal blood and proximal and distal intestines were collected and analysed. Fish fed live yeast resulted in reduced growth (SGR and WG) and higher FCR, while growth in cold and warm water was similar despite differences in TGC. However, increased mortality, plasma cortisol, and intestinal oedema and villous damage indicated fish reared in warm water were subjected to chronic stress. Temperature had a significant effect on haematocrit and red blood cell counts that resulted in significantly higher haemoglobin levels in fish kept in warm water attributed to an elevated oxygen demand. In the proximal intestine, increased temperature resulted in reduced expression of pro-inflammatory cytokines, e.g. TNFα and IL8, that were further reduced in fish fed live yeast. In addition, feeding live yeast reduced gene expression of CLD6 involved in gut barrier function, which suggests that the level of yeast was too high and masked any beneficial effects on fish health. In conclusion, feeding a high inclusion of live yeast reduced fish growth and expression of intestinal genes, while increasing the temperature from 11 to 18 °C subjected fish to chronic stress that restricted growth, suppressed innate immunity and induced intestinal damage. Replacing 40% of fishmeal protein with live yeast did not counteract negative effects caused by increased temperature, thus alternative strategies need to be explored and implemented to protect the growth and health of rainbow trout from seasonal and long-term rises in water temperature.

Keywords

Blood biochemistry; Gene expression; Heat stress; Histology; qPCR; Salmonids; Saccharomyces cerevisiae

1. Introduction

1.1 Yeast as protein or probiotic

Alternative protein sources are needed to replace the unsustainable use of fishmeal while maintaining the health of farmed fish that need to cope with a variety of stressors. Yeast is a sustainable protein source since it is a by-product from ethanol and brewing industries and can be grown on organic waste substrates. Yeasts, such as *Saccharomyces cerevisiae*, have successfully replaced up to 40-50% (112-289 g kg^{-1}) of fishmeal protein in salmonid diets without reducing growth performance of rainbow trout (*Oncorhynchus mykiss*) [1-4], Atlantic salmon (*Salmo salar*) [5], Arctic charr (*Salvelinus alpinus*) [6] and lake trout (*Salvelinus namaycush*) [7]. However, most of these studies produced diets with heat-extrusion that inactivates high counts of live yeast in the diet [1], thus reducing the production of secretory metabolites that benefit the intestine as a live probiotic [8]. Compounds in the cell walls of yeast, such as β-glucans and mannan-oligosaccharides, can improve growth performance and immune response of salmonids [9-15]. However, live yeast is mainly supplemented at low inclusions in fish diets as a probiotic, but not at high inclusions as both a probiotic and protein source.

The intestine of fish is a multifunctional organ important in osmoregulation, nutrient uptake and as an immunological and physical barrier towards the environment. The intestine is composed of three main barriers. Firstly, a mucus barrier that consists of secreted mucins with anti-microbial compounds and residual (autochthonous) microbiota [16-19]. Secondly, the physical barrier consisting of enterocytes that consists mainly of the absorptive epithelial cells connected at the apical membrane through tight junctions, such as claudin, occludin and zonula occludens-1, important for selectivity and integrity of the paracellular pathway [20]. Lastly, an immunological barrier is underneath the epithelium that consists of the gut-associated-lymphoid-tissue (GALT) that responds to immune active substances and intestinal microbes [16, 21, 22]. Low inclusions (e.g. 1-10 g kg^{-1}) of live yeasts, such as *S. cerevisiae* and *Candida utilis*, as probiotics have been found to influence one or more of these three intestinal barriers and improve growth performance and innate immune response of rainbow trout [23-26], Nile tilapia [27, 28], gilthead seabream [29], hybrid striped bass [30, 31] and olive flounder [32]. A recent study on Nile tilapia found that only live yeast rather than heat-inactivated yeast reduced intestinal inflammation and theorized, along with other studies, that live yeasts may improve growth performance by producing metabolites or immune substances directly, or indirectly through shifts in intestinal microbes, that contribute energy more efficiently to the host [8, 22, 33-35].

1.2 Temperature and dietary stressors

Rainbow trout and Atlantic salmon are commonly reared in open-water cages in lakes and ocean sites that are vulnerable to seasonal increases in water temperature that are expected to increase due to climate change [36]. Any region with water temperatures above 18 °C will be above the optimal temperature for growth of juvenile rainbow trout (i.e. 13-17 °C) [37, 38] and Atlantic salmon (i.e. 12-16 °C) [39-41] and may induce a stress response. The stress response in fish is mediated by the hypothalamic-pituitary-interrenal axis, as reviewed by Perry and Bernier [42], Iwama, Pickering and Sumpter [43]. A stress response results in increased reactive oxygen species that impair red blood cells [44, 45], modulated expression of both pro- and anti-inflammatory cytokines involved in the innate immune response [46, 47], increased expression of heat shock proteins involved in cellular repair [48] and impaired intestinal barrier function [49]. Salmonids are increasingly being fed plant proteins, such as

soy, that may contain anti-nutritional factors that result in intestinal inflammation, loss of appetite and reduced growth [50], often enhanced in warmer water, a condition called "summer gut syndrome" [51, 52]. In contrast, live yeast and yeast cell wall compounds can reduce soybean meal induced enteritis (SBMIE) in fish. The *C. utilis* yeast has been shown to counteract SBMIE based on reduced expression of amino acid, fat and drug pathways as well as reduced signs of oedema in the distal intestine of Atlantic salmon [33]. Yeast derived mannan-oligosaccharides have been shown to reduce SBMIE in rainbow trout [53] and Atlantic salmon [54]. Inclusion of plant protein sources and their anti-nutritional factors in salmonid diets will most likely continue to increase, thus non-plant alternatives or counter-measures are needed.

The main objective of the present study was to determine the effects of a high dietary inclusion of live yeast as a protein source and increased water temperature on growth performance, blood physiology and intestinal health parameters of rainbow trout, as well as any diet-temperature interactions. A secondary objective was to investigate whether the inclusion of live yeast as a probiotic can counteract negative effects of stress caused by increased water temperature. These effects were assessed by examining fish growth, feed conversion, haematology, plasma biochemistry, intestinal histology and intestinal gene expression in rainbow trout fed live yeast and reared at 11 and 18 °C.

2. Materials and Methods

2.1 Fish and facilities

The experiment was carried out in the Aquatic Facility of the Centre for Veterinary Medicine and Animal Science at the Swedish University of Agricultural Sciences (SLU; Uppsala, Sweden). Rainbow trout of approximately 110 g were acquired from a commercial producer, Vilstena fiskodling AB (Fjärdhundra, Sweden), and 15 fish were distributed into each of the 16 tanks (240 fish in total) to represent four replicates for each of the four treatment groups. Each 200 L oval tank was equipped with a partial shade, LED light and water was supplied at a flow rate of approximately 5-10 L min^{-1}. Eight tanks were supplied with "cold" water of 11 °C while the water temperature supplied to the other eight tanks was increased gradually over one week until "warm" water of 18 °C was achieved. The tank system was flow-through and sourced with municipal freshwater. Cold and warm water fish tanks were analysed daily for temperature (11.4 ± 0.3 and 18.0 ± 0.3 °C) and weekly for dissolved oxygen solubility (9.7 ± 0.3 and 8.6 ± 0.5 mg L^{-1}), dissolved oxygen saturation (88.7 ± 2.6 and 90.4 ± 4.9%) and pH (8.1 ± 0.2 and 8.0 ± 0.1) using handheld probes (HACH, Sköndal, Sweden). Over one week, fish were acclimated to their assigned water temperature at a 12 hr light cycle and fed a commercial diet (3mm Nutra, Skretting AS, Norway). Fish weight (128.5 ± 8.4 g; mean ± SD) and length (22.0 ± 0.5 cm) were recorded and the experiment started the following day. The present study was performed in compliance with laws and regulations on the use of animals for research purposes in Sweden, which is overseen by the Swedish Board of Agriculture.

Fish were fed either a control diet of 30% fishmeal or a yeast diet that replaced 40% of the fishmeal protein with *S. cerevisiae*. Quantity of dietary live yeast was determined to be 7.6 log CFU g^{-1} via plate counts on yeast–peptone–dextrose agar (BD Co, Frankin Lakes, NJ, USA) supplemented with chloramphenicol (Sigma-Aldrich Co, St. Louis, MO) and incubated at 25°C for 2–3 days, as according to Huyben, Sun, Moccia, Kiessling, Dicksved and Lundh [35]. Fishmeal was replaced on a digestible protein basis of 380 g kg^{-1} dry matter (DM) based on 95% and 86% digestibility coefficients for fishmeal and yeast [55-57]. Both diets were formulated to be iso-energetic (Table 1) and were produced at the SLU Feed Science Laboratory (Uppsala, Sweden). Dry ingredients were first mixed in a horizontal drum-mixer, oil was added to the mixer and then portioned into smaller batches. Gelatin dissolved in hot water was added as a binder to each batch in a bench-top mixer. The wet mash was pressed through a single-screw meat grinder with a 3 mm die (Nima Maskinteknik AB, Örebro, Sweden) and air-dried for 12 hr at 50 °C. Pellets were cut to 3-5 mm length using a blender, manually sieved to remove pellets smaller than 3 mm and stored at 4 °C until distribution. For proximate analysis, crude protein (% N x 6.25) was analysed

according to the Kjeldahl method [58], crude lipid was analysed according to the analytic equipment manufacturer's manual (ANKOM Technology, Macedon, NY, USA), neutral detergent fibre was analysed according to the Amylase Neutral Detergent method [59] and gross energy and ash content were analysed according to standard methods [60].

Fish were fed rations equivalent to 1.5% body weight (BW) via automatic belt feeders (Hølland teknologi, Sandnes, Norway) twice per day (i.e. 10:00 and 14:00) over a period of six weeks. Beforehand, diets were randomly assigned to the tanks and distributed for the duration of the experiment. Each diet was distributed to four tanks supplied with cold water and four tanks supplied with warm water. Each week, feed rations were increased based on temperature growth coefficients of rainbow trout held at 11 and 18 °C according to Cho [61]. Feed waste from each tank was collected continuously using belt collectors (Hølland teknologi, Sandnes, Norway), weighed daily and pooled weekly. Feed and feed waste were analysed for DM content after drying at 103 °C for 16 hr and these values were used to calculate feed intake based on the feed recovery method according to Helland, Grisdale-Helland and Nerland [62].

2.2 Growth performance analyses

After 42 days (D) at different temperatures (T), final body weight (FBW) was recorded and together with initial weight (IBW) were used to calculate fish growth parameters as according to Cho [61]:

- Weight Gain (WG; %) = 100 x [(FBW – IBW) x IBW^{-1}]
- Specific Growth Rate (SGR; g BW day^{-1}) = 100 x [(ln FBW – ln IBW) x D^{-1}
- Thermal Growth Coefficient (TGC) = 100 x [($FBW^{1/3}$ – $IBW^{1/3}$) x $(T \times D)^{-1}$]

Values of total feed intake (FI) were used to calculate feed conversion ratio (FCR = FI x WG^{-1}). Viscera weight (VW) and liver weight (LW) of three fish per tank (n=12) were recorded and used to calculate Viscerosomatic Index (VSI % = 100 x (VW x FBW^{-1}) and Hepatosomatic Index (HSI % = 100 x (LW x FBW^{-1})).

Blood and plasma analyses

Three fish from each tank were heavily sedated with 200 mg L^{-1} tricaine methane sulphonate (MS222; Finquel, Scan Aqua AS, Årnes, Norway) buffered with sodium bicarbonate to prevent pH changes. From three fish per tank (n=12), approximately 2 mL of blood was collected from the caudal vein/artery using a 150 IU Na-heparinised syringe. Micro-capillary tubes were filled with blood, centrifuged at 12,000 *g* for 5 min and measured for haematocrit (Hct). To determine haemoglobin (Hb), blood was diluted 1:1000 with Drabkin's solution of ferric cyanide and Brij® L23 solution (Sigma-Aldrich Co, St. Louis, MO, USA) and measured by a UV-spectrophotometer (540nm wavelength) [63]. Blood was diluted 1:100 in Natt-Herrick's solution and pipetted, in duplicate, in a Neubauer improved haemocytometer (Sigma-Aldrich Co), according to Stoskopf [63]. Red blood cells (RBC) were counted at 400x magnification in five 0.2 mm^2 secondary squares within the large central square of the haemocytometer using NIS Elements software (Nikon Instruments Europe BV, Amsterdam, Netherlands) and calculated per µL 10^6 of blood (RBC = [total count x 5000]/10^6) according to Stoskopf [63]. In addition, RBC area, elongation and other size parameters were measured using semi-automated thresholding criteria via NIS Elements software (Nikon Instruments Europe BV). Lastly, blood values of Hct (%), Hb (g dL^{-1}) and RBC counts (million cells μL^{-1}) were used to calculate RBC indices of mean corpuscular volume (MCV (fL) = 10 x (Hct x RBC^{-1})), mean corpuscular haemoglobin (MCH (pg) = 10 x (Hb x RBC^{-1})) and mean corpuscular haemoglobin concentration (MCHC (g dL^{-1}) = 100 x (Hb x Hct^{-1}) [63].

Remaining blood was centrifuged at 500 *g* for 3 min and plasma was collected and stored at -80 °C. Plasma cortisol was analysed using 96-well, multi-species ELISA kits (DetectX®, Arbor Assays, Ann Arbor, MI, USA) according to the manufacturer's manual, except plasma was diluted 1:25 with assay buffer. Before the experiment, 12 fish reared at 11 °C were sampled for blood that was analysed for plasma cortisol to compare stress levels before and after the experiment. Plasma glucose was analysed using the D-glucose UV method (340nm wavelength) that includes initial reactions with hexokinase and G6P-dehydrogenase, as according to the manufacturer's manual (R-Biopharm AG, Darmstadt, Germany).

After blood collection, the branchial arches were cut, the abdomen was opened and the distal intestine was cut and squeezed with forceps to remove intestinal content (faeces and mucus). The pH of both blood and intestinal content were measured by an Orion ROSS® micro-electrode and Orion Star® pH meter (Thermo Fisher Scientific Inc, Waltham, MA, USA) and these values measured at 25 °C were corrected to the temperature of the fish (i.e. 11 or 18 °C) according to Ashwood, Kost and Kenny [64].

2.3 Intestinal histology

Proximal intestines were dehydrated through an alcohol gradient, washed with Histolab-clear (Histolab Products AB, Gothenburg, Sweden) and embedded in paraffin wax using standard protocols at the University of Gothenburg. Longitudinal sections (5 µm) were cut with a Shandon Finesse Microtome (Shandon Scientific; Thermo Fisher Scientific) and mounted on 3′-aminopropyltriethoxysilane (APES; Sigma-Aldrich)-coated slides, dried at 37 °C for 24 h. Slides were stained with a combination of haematoxylin- eosin and alcian blue 8 GX (pH 2.5). The slides were scanned using a slide scanner (Axio Scan.Z1, Carl Zeiss AG, Oberkochen, Germany) at 200x magnification and evaluated using ZEN imaging software version 2.3 (Blue Edition, Carl Zeiss AG). Intestinal sections (n=9) were randomised and blindly scored from 0 (healthy) to 5 (unhealthy) based on the inflammatory criteria lamina propria thickness, villi oedema and overall villi morphology described by Knudsen, Jutfelt, Sundh, Sundell, Koppe and Frokiaer [65].

2.4 Gene expression by two-step qPCR

From three fish per tank (n=12), proximal and distal intestines were removed and squeezed with forceps to exclude the intestinal content. Intestines were cut open and a scalpel was used to scrape 100-200 mg of mucosal tissue into tubes that contained 1 mL of RNAlater® (Sigma-Aldrich Co), which was stored at -80 °C. Later, mucosa samples were separated from RNAlater® and lysed in 600 µL RLT Plus buffer with 5 mm steel beads using a TissueLyser II homogeniser (Qiagen NV, Hilden, Germany) for 2 cycles of 3 min at 25 rotations sec^{-1}. Samples were centrifuged for 3 min at 17,000 *g* and supernatant was pipetted into spin columns for mRNA extraction using RNeasy© Plus Mini kits (Qiagen NV), according to the manufacture's manual. RNAse-free water was used to elute the mRNA and quantity was determined by Nanodrop (Thermo Fisher Scientific Inc). Samples were diluted twice to obtain 1000 ng of mRNA. The cDNA was synthesized by reverse transcriptase using iScript™ Synthesis kits (Bio-Rad Laboratories Inc, Copenhagen, Denmark) with random primers in 20 µL reactions in one cycle of 5 min at 25 °C, 30 sec at 42 °C and 5 min at 85 °C in a thermocycler (Bio-Rad Lab Inc).

Primers were verified using NCBIs Primer-BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast) and obtained from Eurofins MWG operon (Ebersberg, Germany), for more information see Table 2. Efficiencies of each primer pair were confirmed to be between 90-105% using a dilution series of 2-50 ng cDNA pooled from six random samples. For each sample, duplicate 10 µL reactions of 10 ng cDNA, 0.5 µM of each primer pair (0.3 µM for β-actin) and SYBRGreen Supermix (Bio-Rad Lab Inc) were pipetted into a reaction plate. Plates were analysed for 40 cycles with initial denaturation at 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 sec and 57-61 °C (see Table 2) for 30 sec using a CFX Connect Real-time PCR Detection System (Bio-Rad Lab Inc). The

ratio of relative expression between the target and reference gene was calculated based on the threshold cycle (C_T): relative expression= $2^{-(C_T(target)-C_T(reference))}$ using the '$2^{-\Delta C_T}$' method [66]. Only β-actin was used as a reference gene since the primer pair efficiency for elongation factor 1α (ELF1α; Accession No. AF321836) was insufficient.

2.5 Statistical Analyses

Significant effects between treatments and overall effects of diet, temperature and diet-temperature interactions on fish growth performance, body organ indices, blood physiology, intestinal histology and gene expression were determined using Linear Mixed Effects (Lme4 package) models in R® statistical software version 3.22 [67, 68]. The LME models included diet and temperature as fixed effects as well as diet-temperature interaction and the random effect of tank. For gene expression, data were log-transformed and fish weight was included as an additional fixed effect in the LME model. In addition, differences in gene expression between the proximal and distal intestine were also determined. A two-way ANOVA was performed on the LME model for each growth, haematological and intestinal health parameter to determine the significance of each factor (diet*temp). A value of $p<0.05$ was considered significant and $p<0.10$ was considered a tendency.

3. Results

3.1 Fish growth performance and relative body indices

For growth performance, main effects of diet and/or temperature were found, whereas no diet-temperature interactions existed (Table 3). Compared with the fishmeal diet, fish fed the yeast diet resulted in reduced TGC ($p=0.033$), SGR ($p=0.015$) and WG ($p=0.013$) along with increase FCR ($p<0.001$) after six weeks of feeding. Compared with cold water, fish in warm water resulted in reduced TGC ($p<0.001$) and survival ($p=0.016$) as well as elevated FI ($p<0.001$) and FCR ($p=0.003$) while no significant effects on SGR and WG were found ($p>0.05$). For relative body indices, warm water resulted in lower VSI ($p<0.001$) and HSI ($p=0.015$) compared with fish in cold water, while no effect of diet was found ($p>0.05$).

3.2 Blood biochemistry and haematology

Only main effects of temperature were found regarding blood physiology (Table 4). Warm water resulted in decreased the blood pH ($p<0.001$), but elevated plasma cortisol levels ($p=0.002$), Hct ($p=0.017$), Hb ($p<0.001$), RBC ($p<0.021$) and MCHC ($p<0.001$). No significant effects of diet were found, although feeding yeast resulted in tendencies of decreased blood pH ($p=0.059$) and MCH ($p=0.088$) and increased RBC elongation ($p=0.071$) compared with fish fed fishmeal. For intestinal pH, warm water resulted in reduced values ($p<0.001$) and a diet-temperature interaction was found ($p=0.018$).

3.3 Intestinal histology

In the proximal intestine, warm water resulted in increased occurrence and severity of oedema ($p<0.001$) and villi damage ($p=0.021$; Table 3 and Fig. 1). In several intestines from both fishmeal and yeast groups, lamina propria was inflamed and retracted from the epithelium (Fig. 1B). The villi tips were also be severely damaged (Fig. 1D). No significant effects of diet (Fig. 1A & 1C) or diet-temp interaction were found ($p>0.05$) but a clear tendency towards increased lamina propria inflammation was observed in fish fed yeast ($p=0.056$).

3.4 Intestinal gene expression

Main effects of diet and/or temperature were found regarding gene expression of several pro- and anti-inflammatory cytokines and tight junction proteins in the proximal intestine compared with limited effects in the distal intestine (Table 5, Fig. 2 and Fig. 3). Fish fed yeast resulted in reduced gene expression of pro-inflammatory cytokines (i.e. TNFα, IL1β and IL8) and a tight junction protein (i.e. CLD6) in the proximal intestine. Warm water resulted in reduced gene expression of pro-inflammatory cytokines (i.e. IFNγ, TNFα, IL8 and IL17), an anti-inflammatory cytokine (i.e. TGFβ) and a tight junction protein (i.e. CLD6), except for increased expression of TRIC. No diet-temperature interactions were found in the proximal intestine, but an interaction on IL1β expression was found in the distal intestine ($p=0.036$). No significant effects were found for expression of heat shock proteins in the proximal intestine, whereas warm water resulted in increased expression of HSP90 ($p=0.005$) in the distal intestine. In comparison to intestinal regions (i.e. proximal and distal), significant differences in expression of IL1β, IL17, TGFβ and TRIC were found ($p<0.05$).

4. Discussion

4.1 Limited growth performance for fish fed yeast and kept in warm water

Fish fed yeast resulted in negative effects on fish growth performance, which suggests that replacing 40% of fishmeal protein with live yeast is not viable for rainbow trout, at either water temperature. In contrast, previous studies have successfully replaced up to 40-50% of fishmeal protein with yeast in diets for rainbow trout [1-4]. However, these studies fed heat-extruded diets (commercial grade) whereas diets in the present study were cold-pelleted to provide high loads of live yeast to act as a probiotic. Previous studies have shown that cold-pelleted diets have lower digestibility than steam and heat-extruded diets [69, 70]. In addition, extruded diets of yeast have lower protein digestibility than fishmeal when fed to salmonid fishes [4, 6, 71]. Therefore, lower inclusion levels of live yeast in cold-pelleted or extruded diets are recommended in future studies to improve diet digestibility and maintain fish growth performance.

Fish were expected to have higher growth performance at 18 than 11 °C since increased water temperature typically results in increased metabolic activity, nutrient absorption and weight gain [72, 73], whereas SGR and WG were similar in the present study (Table 3). Reduced Thermal Growth Coefficient (TGC) in fish kept in warm water indicated that fish underperformed as higher growth was expected at the higher temperature. Previous studies have found that growth rates and weight gain of rainbow trout are highest between temperatures of 16 and 19 °C [38, 74, 75]. However, fish in these studies were smaller than those used in the present study and larger rainbow trout have been found to have lower thermal tolerances [37, 76]. Recently, studies have found that growth rates of larger rainbow trout were highest at 14 °C [37, 77]. Therefore, a water temperature of 18 °C may have been outside the optimal range for fish growth in the present study, thus resulting in limited growth performance.

4.2 Haematological response to increase metabolism in warm water

The present study showed that increased temperature resulted in increased Hb levels in the blood of rainbow trout (Table 4), which has been shown previously in fish due to increased metabolic rate [78]. A rise in temperature directly decreases oxygen affinity of Hb to increase oxygen unloading to the tissues while decreasing oxygen loading in the gills, which leads to increased cellular oxygen demand [79]. In the present study, elevated levels of Hct, Hb, RBC count and cortisol in fish reared in warm water (Table 4) indicates that RBC production in the

spleen and release into circulation may have been triggered via higher cortisol levels in order to increase oxygen transport and carrying capacity [72, 79-81]. Previous studies have found that Hb increased in rainbow trout when temperature increased from 15 to 21 °C [78], 10 to 18 °C [82] and 5 to 22 °C [83]. Martinez, Garcia-Riera, Ganteras, De Costa and Zamora [78] found that Hb levels were more influenced by temperature than fish weight, stocking density or dissolved oxygen. These results support previous findings that increased water temperature results in increased Hb levels by releasing more RBC as a physiological response to an elevated metabolic rate, possibly mediated by higher cortisol levels and lower oxygen availability.

Increased levels of plasma cortisol, Hct, RBC counts and HSI are primary and secondary indicators of stress in fish [84], thus fish in warm water may have been under stress (Table 4). The duration of the experiment was six weeks after an additional week acclimation period, thus the shock and acute stress from the initial temperature increase should have returned cortisol to baseline levels. Fish in the present study were not expected to be under stress in 18 °C water since rainbow trout are reported to have long-term survival between 8-18 °C with an ultimate upper incipient lethal temperature (UUILT) of approximately 26 °C [37, 38, 85]. In contrast, fish reared at 18 °C in the present study had reduced survival (i.e. 100 to 90-93%) and 2-3 fold increase in plasma cortisol (i.e. 12-14 to 33-36 ng mL^{-1}), which suggests that the temperature increase subjected these fish to a chronic stressor. Slight cortisol increases (e.g. 5-10 to 15 ng mL^{-1}) have been found in chronically stressed salmonids that result in reduced growth, suppressed immunity and disease resistance [49, 86-88]. Onset of chronic stress is also supported by the unchanged level of plasma glucose in fish reared in warm water (Table 4) since increases are commonly observed in rainbow trout subjected to acute stress [89, 90]. Glucose levels may have been similar due to diminishing energy stores (i.e. reduced HSI and VSI) and increased feed intake for fish reared in warm water. In addition, HSI was affected by temperature and this decrease has been shown in stressed rainbow trout along with increased cortisol levels [91]. Therefore, increased levels of Hct, RBC counts, HSI and plasma cortisol in fish reared in warm water indicated a potential chronic stress at 18 °C compared with 11 °C.

Increased temperature and release of glucocorticoids have also been shown to inhibit exchange of hydrogen ions and decrease plasma pH in order to increase /oxygen affinity of Hb [79, 81], which explains the decrease in blood pH in the present study (Table 4). On the other hand, feeding live yeast had a tendency ($p=0.059$) to alter blood pH while there was a significant diet-temperature interaction on intestinal pH. Blood can counteract increased acidity in the stomach following a meal (alkaline tide), which can be influenced by the level of cations or ash in the diet [92, 93]. Huyben, Vidakovic, Nyman, Langeland, Lundh and Kiessling [89] found that blood pH increased in rainbow trout fed inactivated yeast and reared at 15 °C and suggested that this was due to reduced buffering capacity and ash content of yeast compared with fishmeal. In contrast, blood pH was similar between dietary treatments in the present study although the yeast diet only had 10 g kg^{-1} lower ash content than the fishmeal diet (Table 1). Alternatively, Huyben, Sun, Moccia, Kiessling, Dicksved and Lundh [35] suggested that fermentation products from yeast or other microbes could alter the intestinal pH. Therefore, differences in blood and intestinal pH between fish fed fishmeal and yeast may be due to differences in buffering capacity or microbial fermentation, but more research is required.

Previous studies have found that feeding high inclusions of yeast (321-812 g kg^{-1}) to rainbow trout can result in blood anaemia attributed to the high content of nucleic acid in yeast cells that are not adequately metabolised and lead to Hb damage, irregular shape and dysfunction of red blood cells [89, 94, 95]. No significant effects on haematology were found for fish fed live yeast in the present study, although tendencies existed for reduced MCH and increased elongation of red blood cells (Table 4). Compared with the above studies, the present study fed lower levels of yeast (214 g kg^{-1}) to fish and in a parallel study high counts of yeast were found in the faeces, suggesting that yeast bypassed metabolic pathways [35]. Therefore, levels of yeast-derived nucleic acids would be lower and sufficiently metabolised without the production of harmful by-products that cause red blood cell dysfunction and anaemia. The live form and 214 g kg^{-1} inclusion level of yeast did not induce signs of anaemia in rainbow trout, although we recommend not to exceed this inclusion level in future studies.

4.3 Impact of live yeast and warm water on intestinal gene expression and histology

The decreased expression of pro-inflammatory cytokines (TNFα, IL1β and IL8) in the proximal intestine of fish fed live yeast rather than fishmeal (Table 5 and Fig. 2) is in agreement with previous findings [8]. Previous studies suggest that yeast can reduce intestinal inflammation by secreting metabolites that improve the efficiency of metabolic pathways in the intestine and potentially lead to the secretion of antimicrobial peptides that down-regulate NOD- and Toll-like receptors (NLR and TLR) that are involved in the innate immune response [33, 34]. In previous studies, feeding live yeast *S. cerevisiae* to rainbow trout reared at a low density suppressed expression of immune related genes in the intestine and counteracted deleterious effects when fish were reared at a high density [96]. Feeding live yeast or β-glucans to Nile tilapia and common carp suppressed the expression of pro-inflammatory cytokines, such as TNFα, and counteracted SBMIE [8, 97]. In contrast, feeding live yeasts *Candida utilis* and *Kluyveromyces marxianus* to Atlantic salmon counteracted villous damage and oedema caused by SBMIE, while feeding *S. cerevisiae* did not [33]. In the present study, feeding live *S. cerevisiae* yeast did not counteract intestinal damage caused by elevated water temperature. It can be debated that the suppression of pro-inflammatory pathways may have a negative effect on fish health as suppression of the gut immune system can result in increased disease susceptibility. Replacing 40% of fishmeal protein with yeast has been shown to reduce intestinal barrier function in Arctic charr [6], a state which can be correlated to intestinal inflammation and increased disease susceptibility of Atlantic salmon [47, 49, 65, 98]. More research is needed to determine whether or not the down-regulation of innate immune genes is beneficial to fish health and the influence of live yeast.

A potential improvement in intestinal health parameters may have been negated by the high inclusion of yeast that instead resulted in an increased tendency in lamina propria inflammation and reduced expression of CLD6 (Table 3, Table 5 and Fig. 2). In mirror carp, feeding yeast-derived β-glucans (10-20 g kg^{-1}) increased infiltration of leucocytes from the lamina propria into the epithelial layer in the proximal intestine that indicated a localized immune response with no detrimental effects on gut morphology [99]. This may be the case in the present study, albeit the high level of dietary yeast may have intensified this effect resulting in inflammation. Another theory is that the high amount of prebiotic fibres, such as mannan-oligosaccharides, derived from yeast caused excessive fermentation that irritated the intestine, which has been shown previously in Arctic charr fed high inclusions of the prebiotic inulin [100]. On the other hand, it can not be excluded that the yeast inclusion made the tissue more vulnerable to handling and that part of the intestinal damage was created during tissue processing and sectioning. Nevertheless, more damage at high temperature is in agreement with the suppression of CLD6 in fish fed yeast in warm water in our study that points toward a potentially impaired intestinal barrier, thus providing an explaination for the tendency of diet induced lamina propria inflammation. In support, mice deficient of CLD7, but expressing intact tight junctions composed of six other CLDs, develop lethal colitis caused by loss of intestinal barrier function towards small molecules (~400 Da), but not macromolecules (~4 kDa) or Na^+ and Cl^- [101]. These findings evidences that dysregulation of one single claudin is enough to cause severe intestinal damage and/or disease. In fish, a lack of knowledge exists for the association between gene expression of tight junction proteins and intestinal health parameters, such as intestine inflammation, thus more research is required.

In support of the beneficial effects of yeast, a decreased tendency in gene expression of HSP90 in the proximal intestine was observed for diet ($p=0.055$) and indicates a positive effect as this gene is associated with cellular repair and nutrient transport [20, 48]. Previous studies have found that feeding probiotics, such as *Lactobacillus*, to fish altered the gene expression of heat shock proteins [102, 103]. In terms of increased temperature, increased expression of heat shock proteins as well as villous damage and oedema in the intestine have be found previously in salmonids [47, 81, 104, 105]. Expression of HSP90 did increase with temperature in the present study, but only in the distal intestine (Fig. 3). The conflicting results of histology and gene expression imply that live yeast may have a beneficial effect on the intestine, although the high inclusion of yeast in the diet may be masking these effects.

In the current study, the suppression of pro- (IFNγ, TNFα, IL8 and IL17) inflammatory cytokines observed by diet and temperature in the proximal intestine (Table 5 and Fig. 2) is in contrast with reported signs of intestinal inflammation. These apparent discrepancies could be due to time differences in the manifestation of the different

inflammatory responses evoked. In mammals, Crohn's disease is characterized by high expression of pro-inflammatory cytokines (TNFα, IFNγ) while ulcerative colitis is driven by IL13 and IL4, known to inhibit innate pro-inflammatory cytokines, while stimulating adaptive immunity [106]. Thus, the tendency towards inflammation observed in our study could be mediated by cells stimulated by an adaptive immune response rather than an innate. This is supported by findings in Atlantic salmon intestinal inflammation was reported in concert with impaired barrier function after chronic stress concurrent with suppression of pro-inflammatory cytokines [47, 49]. A shift towards adaptive immunity is a possible consequence of the 2-3 fold higher plasma cortisol levels in fish reared in warm water (Table 4) indicating chronic stress. In fish, cortisol can suppress pro-inflammatory cytokines, innate immune responses and disease resistance [86, 87, 107, 108]. In vitro, glucocorticoids have been shown to act via cytoplasmic/nuclear receptors on antigen presenting cells to suppress the production of IL12, which is the main inducer of the T helper 1 cell pathway for mammals [109]. In Atlantic salmon, Niklasson, Sundh, Olsen, Jutfelt, Skjodt, Nilsen and Sundell [110] found that cortisol implants reduced CD8α lymphocytes and suppressed IFN type 1 expression in the proximal intestine. Houston, Dobric and Kahurananga [111] found that lymphocyte counts and lymphocyte:heterophil ratio decreased in rainbow trout in temperatures from 5 to 18 °C. In vitro, Saeij, Verburg-van Kemenade, van Muiswinkel and Wiegertjes [112] found that cortisol can down-regulate expression of immune genes, such as IL1β and TNFα, and induce apoptosis of lymphocytes in common carp (*Cyprinus carpio*). Interestingly, the expressions of most cytokines were affected by temperature in the proximal intestine, but no affects were found in the distal intestine (Table 5). Nevertheless, increased water temperature reduced the expression of inflammatory cytokines, possibly mediated by higher plasma cortisol levels.

In conclusion, we have demonstrated that replacing 40% of fishmeal protein with live yeast and increasing water temperature from 11 to 18 °C has negative effects on growth performance and intestinal health parameters of rainbow trout, while haematology was not adversely affected. We concluded that fish reared at 18 °C were subjected to chronic stress based on limited growth and increased levels of mortalities, plasma cortisol, HSP expression, intestinal oedema and intestinal villous damage. The results indicated that feeding live yeast did not counteract the negative effects caused by the temperature stressor, despite lower expression of pro-inflammatory cytokines in the proximal intestine. Feeding live yeast may benefit fish by modulating the innate immune response, although the high inclusion of yeast may have masked these effects by irritating the intestine. However, more research is needed to associate the expression of immune and tight junction genes with intestinal health. In summary, these results suggest that rainbow trout are vulnerable to water temperature elevations that are expected to increase due to climate change and that feeding a high inclusion of live yeast will not counteract these negative effects.

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747

748

Tables

Table 1

Diet formulation and proximate analysis of fishmeal and yeast diets (g kg^{-1}).

	Diet	
Ingredients (as-fed basis)	Fishmeal	Yeast
Fish meal[1]	300	180
S. cerevisiae yeast[2]	-	214
Soy protein concentrate	130	130
Wheat gluten	110	110
Corn starch	100	16
Wheat meal	60	60
Fish oil	100	115
Rapeseed oil	40	40
Gelatin	60	60
α-Cellulose	65	40
Carboxymethyl cellulose	10	10
Mineral-vitamin premix	15	15
Monocalcium phosphate	10	10
Proximate analysis (DM basis)		
Dry matter	977	974
Crude protein[3]	468	484
Crude lipid	158	161
Neutral detergent fibre	95	70
Ash	86	76
Gross energy (MJ kg^{-1})	22	23

[1]Low-temperature dried blue whiting meal (Pelagia AS, Bergen, Norway); 607 g kg^{-1} crude protein.

[2]Dried yeast meal (Jästbolaget AB, Uppsala, Sweden); 466g kg^{-1} crude protein.

[3]Diets balanced at a digestible protein content of 380g kg^{-1}, based on 95 and 86% ADC for rainbow trout fed fishmeal and yeast [6, 55, 56].

759 Table 2

760 Primer pair information of a reference gene and target genes related to pro- and anti-inflammatory cytokines and
761 heat shock proteins in the intestine of rainbow trout.

Gene	Amplicon size (bp)	Primer	Sequence (5'-3')	Anneal temperature (°C)	Accession number
β-actin	155	F	GGAAGATGAAATCGCCGCAC	60	AB196465
		R	AGCTGTCTTTCTGGCCCATC		
IFNγ	210	F	CAAACTGAAAGTCCACTATAAGATCTCCA	60	AJ616215
		R	TCCTGAATTTTCCCCTTGACATATTT		
TNFα	208	F	CAAGAGTTTGAACCTCATTCAG	61	AJ401377
		R	GCTGCTGCCGCACATAAAG		
IL1β	181	F	ACCGAGTTCAAGGACAAGGA	61	AJ223954
		R	CATTCATCAGGACCCAGCAC		
IL8	162	F	CACAGACAGAGAAGGAAGGAAAG	57	AJ279069
		R	TGCTCATCTTGGGGTTACAGA		
IL17	212	F	CGTGTCGAAGTACCTGGTTGTGT	60	AJ580842
		R	GGTTCTCCACTGTAGTGCTTTTCCA		
TGFβ	275	F	AGATAAATCGGAGAGTTGCTGTG	61	AJ007836
		R	CCTGCTCCACCTTGTGTTGT		
IL10	119	F	GGATTCTACACCACTTGAAGAGCCC	61	AB118099
		R	GTCGTTGTTGTTCTGTGTTCTGTTGT		
HSP70	67	F	CCACTTCATCGCAGAGTTCAAA	60	AB196460
		R	GCGAACAGCCCTCTTGTTGT		
HSP90	63	F	AGGGTCAAGGAGGTGGTCAA	60	AB196457
		R	AACGAAGAGGGTGATGGGATATC		
CLD6	245	F	TGAAACCACGGGACAGATG	60	KF445436
		R	TGAAACCACGGGACAGATG		
OCLN	341	F	CAGCCCAGTTCCTCCAGTAG	61	GQ476574
		R	GCTCATCCAGCTCTCTGTCC		
TRIC	170	F	GTCACATCCCCAAACCAGTC	60	KC603902
		R	GTCCAGCTCGTCAAACTTCC		
ZO1	291	F	AAGGAAGGTCTGGAGGAAGG	60	HQ656020
		R	CAGCTTGCCGTTGTAGAGG		

762 IFNγ; interferron-γ, IL; interleukin, TNFα; tumor necrosis factor-α, TGFβ; transforming growth factor-β, HSP;
763 heat shock protein, OCLN; occludin, CLD6; claudin-6, ZO1; zonula occludens-1, TRIC; tricellulin, F; forward,
764 and R; reverse.

765

766 Table 3

767 Growth performance, relative body indices and intestinal histology of rainbow trout fed yeast or fishmeal diets
768 kept in cold (11 °C) or warm (18 °C) water (n=4, pooled per tank for each treatment).

Variable	Cold water		Warm water			p-value[1]		
	Fish meal	Yeast	Fish meal	Yeast	SE[2]	diet	temp	diet x temp
Growth performance								
TGC	0.19	0.15	0.12	0.11	0.01	**0.033**	**<0.001**	0.205
SGR (% BW day^{-1})	1.16	0.91	1.18	1.07	0.06	**0.015**	0.211	0.328
WG (%)	63.36	46.90	64.31	57.02	4.16	**0.013**	0.246	0.337
FI (% BW day^{-1})	1.16	1.10	1.36	1.39	0.04	0.834	**<0.001**	0.451
FCR	1.02	1.23	1.17	1.31	0.04	**<0.001**	**0.003**	0.416
Survival (%)	100.0	100.0	90.0	93.3	2.3	0.631	**0.016**	0.631
Body indices								
VSI	12.04	12.23	10.49	11.34	0.31	0.109	**<0.001**	0.308
HSI	1.33	1.47	1.22	1.27	0.06	0.117	**0.015**	0.494
Intestinal histology[3]								
Oedema	2.22	2.28	3.61	3.11	0.28	0.424	**<0.001**	0.317
Villous damage	2.83	2.78	3.89	3.56	0.40	0.624	**0.021**	0.726
Lamina propria inflammation	2.00	3.44	2.50	2.78	0.45	0.056	0.853	0.195

769 TGC; Thermal growth coefficient, SGR; specific growth rate, WG; weight gain, FBW; final body weight, FI;
770 feed intake, FCR; feed conversion ratio, VSI; viscerosomatic index and HSI; hepatosomatic index.
771 [1]P-values from linear mixed effects models with fixed effects of diet, temperature and diet-temp plus random
772 tank effect. Bold numbers indicate significant effect ($p<0.05$).

773 [2]SE; pooled standard error of the mean.

774 [3]N=9 for histological scoring (0=healthy, 5=unhealthy) of the proximal intestine.

775

776 Table 4

777 Blood biochemistry, haematology and intestinal pH of rainbow trout fed yeast or fishmeal diets kept in cold (11
778 °C) or warm (18 °C) water (n=12, fish per treatment).

	Cold water		Warm water			p-value[1]		
Variable	Fishmeal	Yeast	Fishmeal	Yeast	SE[2]	diet	temp	diet x temp
Blood pH	7.50	7.43	7.35	7.38	0.03	0.059	**<0.001**	0.202
Intestinal pH	8.51	8.17	7.63	7.93	0.12	0.631	**<0.001**	**0.018**
Plasma cortisol (ng mL^{-1})[3]	13.84	12.03	32.51	36.46	5.48	0.880	**0.002**	0.683
Plasma glucose (mmol L^{-1})	6.91	8.29	8.07	8.15	0.57	0.227	0.403	0.288
Hct (%)	34.73	35.79	38.37	38.26	1.22	0.712	**0.017**	0.645
Hb (g dL^{-1})	6.66	6.32	9.13	8.52	0.47	0.333	**<0.001**	0.785
RBC count (10^6 μL^{-1})	0.86	1.02	1.33	1.25	0.14	0.798	**0.021**	0.423
MCV (fL)	467.37	376.35	337.48	325.04	52.04	0.342	0.096	0.471
MCH (pg)	86.94	66.17	75.65	71.37	6.79	0.088	0.678	0.261
MCHC (g dL^{-1})	19.39	17.75	24.12	22.37	1.20	0.208	**<0.001**	0.969
RBC area (μm^2)	518.95	505.35	501.67	507.73	21.47	0.869	0.744	0.667
RBC elongation	1.33	1.38	1.33	1.35	0.02	0.071	0.373	0.373

779 Hct; haematocrit, Hb; haemoglobin, RBC; red blood cell; MCV; mean corpuscular volume; MCH; mean
780 corpuscular haemoglobin and MCHC; mean corpuscular haemoglobin concentration.

781 [1]P-values from linear mixed effects models with fixed effects of diet, temperature and diet-temp plus random
782 tank effect. Bold numbers indicate significant effect ($p<0.05$).

783 [2]SE; pooled standard error of the mean.

784 [3]Cortisol levels before the experiment were 19.8 ± 2.0 ng mL^{-1}.

785

Table 5

P-values for the effects of diet, temperature, diet-temperature interaction on the relative gene expression in the proximal and distal intestine of rainbow trout.

	Proximal Intestine[1]			Distal Intestine[1]			Intestinal
	diet	temp	diet x temp	diet	temp	diet x temp	region
Pro-inflammatory cytokines							
IFNγ	0.978	**<0.001**	0.163	0.622	0.086	0.711	0.667
TNFα	**0.004**	**<0.001**	0.675	0.523	0.109	0.610	0.501
IL1β	**0.002**	0.267	0.903	0.383	0.218	**0.036**	**<0.001**
IL8	**0.001**	**<0.001**	0.999	0.621	0.086	0.635	0.549
IL17	0.209	**<0.001**	0.908	0.747	0.416	0.137	**<0.001**
Anti-inflammatory cytokines							
TGFβ	0.532	**<0.001**	0.108	0.288	0.058	0.753	**0.017**
IL10	0.846	0.163	0.072	0.941	0.132	0.871	0.770
Heat shock proteins							
HSP70	0.342	0.344	0.463	0.213	0.606	0.368	0.760
HSP90	0.055	0.384	0.270	0.261	**0.005**	0.582	0.076
Tight junction proteins							
CLD6	**0.002**	**<0.001**	0.969	0.370	0.110	0.644	0.579
OCLN	0.611	0.390	0.779	0.343	0.339	0.498	0.877
TRIC	0.876	**0.007**	0.327	0.484	0.098	0.136	**0.005**
ZO1	0.496	0.237	0.734	0.553	0.818	0.166	0.957

IFNγ; interferron-γ, IL; interleukin, TNFα; tumor necrosis factor-α, TGFβ; transforming growth factor-β, HSP; heat shock protein, OCLN; occludin, CLD6; claudin-6, ZO1; zonula occludens-1, and TRIC; tricellulin.

[1]P-values from linear mixed effects models with fixed effects of diet, temperature and diet-temp plus random tank effect. Bold numbers indicate significant effect ($p<0.05$).

Figures

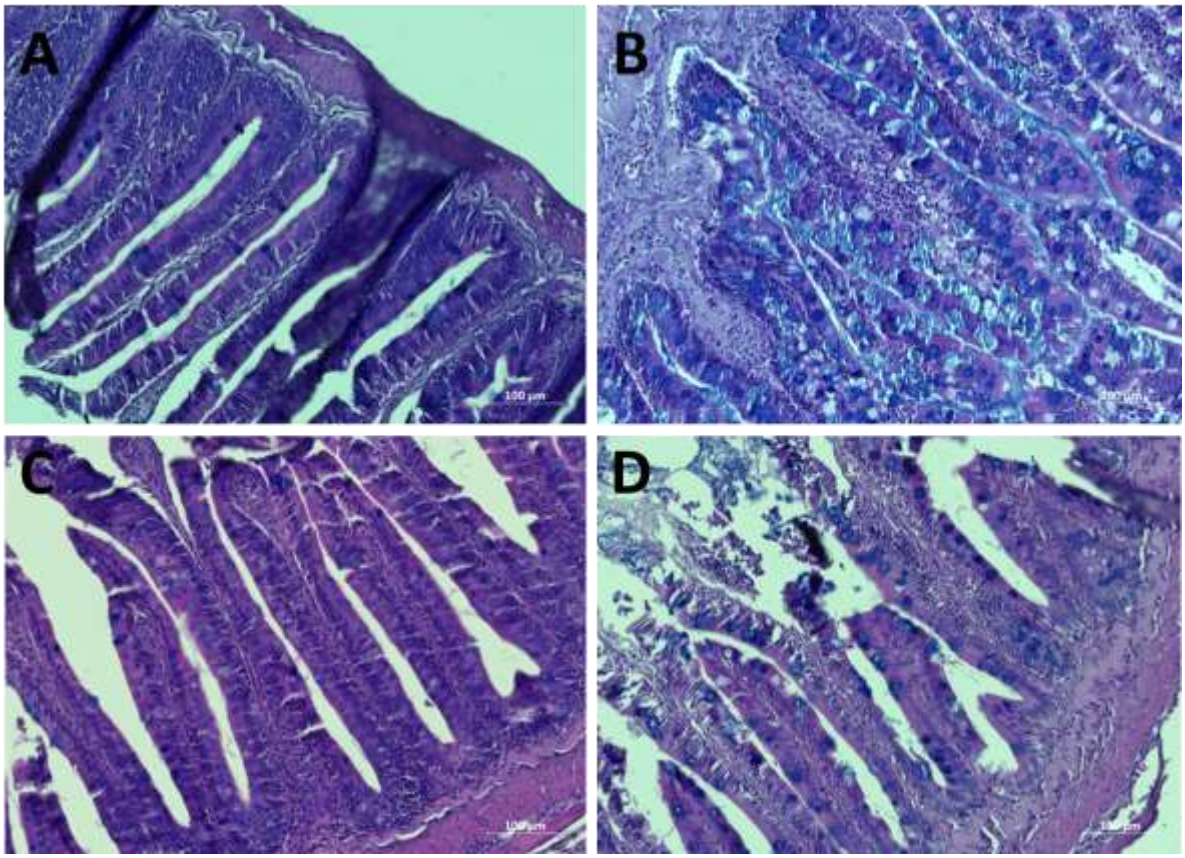
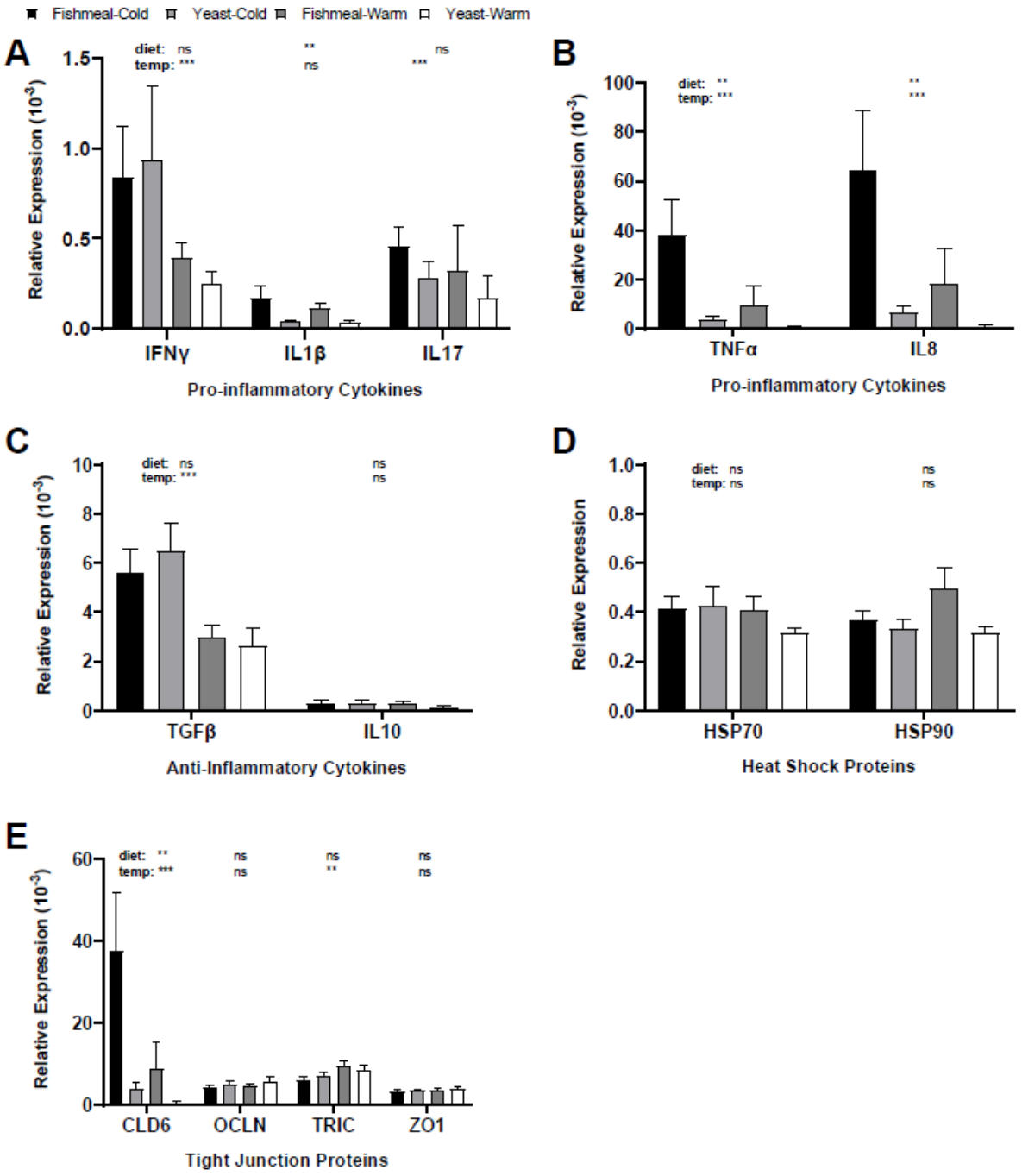


Fig. 1. Histological images (200x magnification) of the proximal intestine of rainbow trout fed: (A) fishmeal in cold water, (B) yeast in cold water, (C) fishmeal in warm water, and (D) yeast in warm water. For fish kept in warm water, image B indicates severe oedema and image D indicates villi damage compared with fish kept in cold water (A and C).



804

805 Fig. 2. Gene expression relative to β-actin (mean ± SE) for (A & B) pro-inflammatory cytokines, (C) anti-
806 inflammatory cytokines, (D) heat shock proteins, and (E) tight junction proteins in the proximal intestine of
807 rainbow trout fed yeast or fishmeal kept in cold or warm water (n=12). Above each parameter, symbols ***, **,
808 * and ns refer to p-values <0.001, <0.01, <0.05 and not significant for the effect of diet and temperature. IFNγ;
809 interferron-γ, IL; interleukin, TNFα; tumor necrosis factor-α, TGFβ; transforming growth factor-β, HSP; heat
810 shock protein, CLD6; claudin-6, OCLN; occludin, TRIC; tricellulin, and ZO1; zonula occludens-1.

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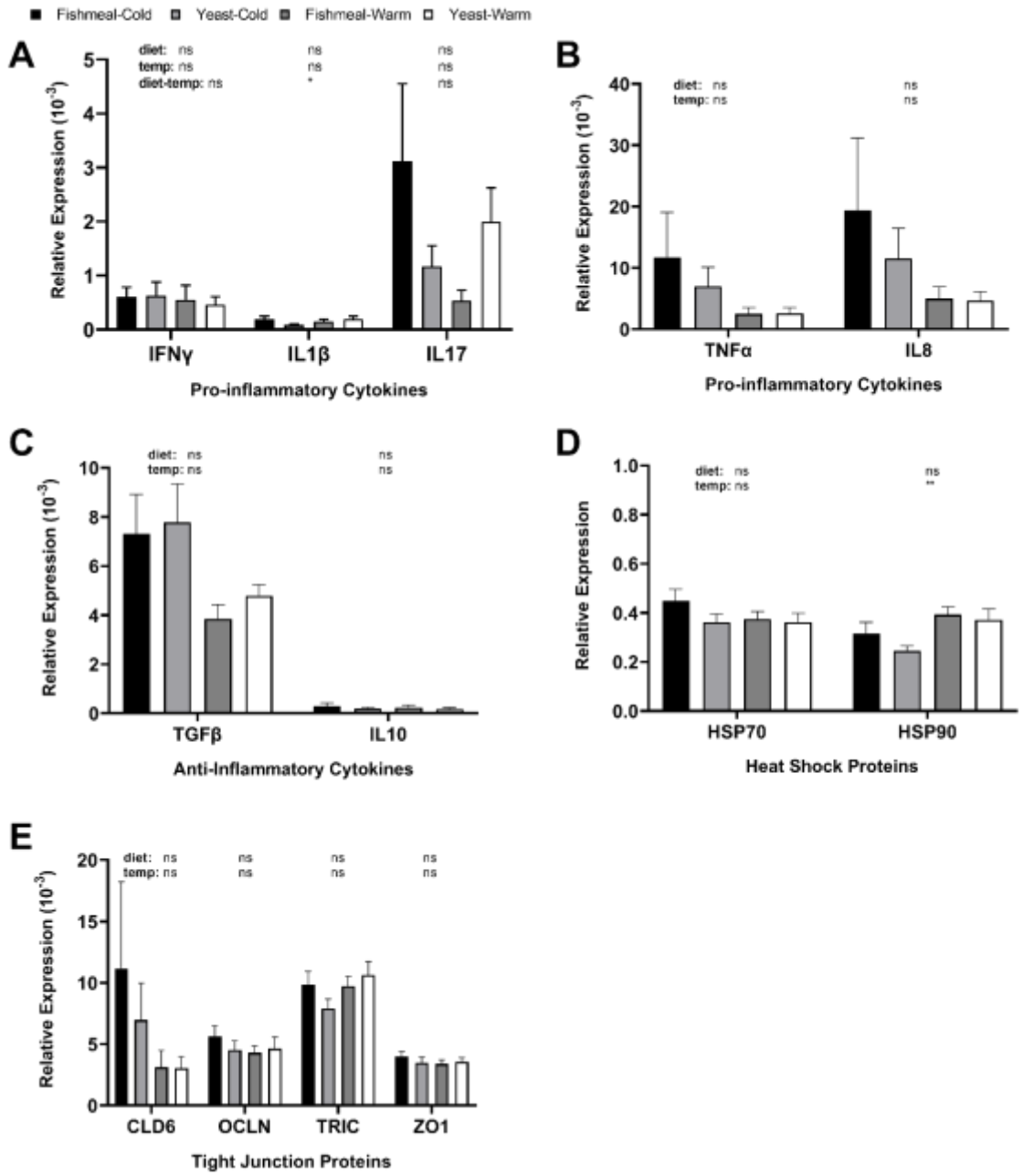

Fig. 3. Gene expression relative to β-actin (mean ± SE) for (A & B) pro-inflammatory cytokines, (C) anti-inflammatory cytokines, (D) heat shock proteins, and (E) tight junction proteins in the distal intestine of rainbow trout fed yeast or fishmeal kept in cold or warm water (n=12). Above each parameter, symbols ***, **, * and ns refer to p-values <0.001, <0.01, <0.05 and not significant for the effect of diet and temperature. IFNγ; interferron-γ, IL; interleukin, TNFα; tumor necrosis factor-α, TGFβ; transforming growth factor-β, HSP; heat shock protein, CLD6; claudin-6, OCLN; occludin, TRIC; tricellulin, and ZO1; zonula occludens-1.