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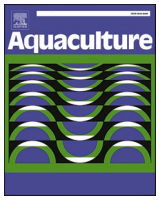

Yarrowia lipolytica yeast as a dietary supplement for rainbow trout (*Oncorhynchus mykiss*): Effects on gut microbiota, health and immunity

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ABSTRACT

This study evaluated the effect of whole and autolysed *Yarrowia lipolytica* CBS 7504 strain on growth performance, gut immunity and gut microbiota composition in juvenile rainbow trout (*Oncorhynchus mykiss*). Fish were fed diets containing 2% and 5% whole or autolysed *Y. lipolytica* (WY and AY) in a 45-day trial, with a no-yeast diet as control. Log2 fold change analysis of *Y. lipolytica* diets showed relative change in abundance and association of certain microbial taxa with respect to control such as a correlation between the relative abundance of *Desulfovibrionaceae* in diets with WY. Moreover, diets at 5% and 2% levels demonstrated a synergistic association with relative levels of *Sphingobacteriaceae* and *Rhodobacteraceae,* respectively. However, the overall analyses showed little/no impact of *Y. lipolytica* supplementation on overall intestinal microbial composition, growth parameters and body indices. Gene expression analysis of the intestine revealed significant elevation in expression of immune-related genes of the complement pathway (*c3* and *c-type lectin*), membrane receptor pathway (*tlr2* and *tlr5*), mucosal innate immune pathway (*muc-2*), cytokines (*tnf-α*) and adaptive immune pathway (*igt* and *cd4*) in 5% WY. In conclusion, it can be inferred that dietary *Y. lipolytica* can be a useful functional feed supplement for rainbow trout when used as whole yeast at a 5% inclusion level since it can modulate gut microbial communities and act as a potential immunostimulant for the host.

1. Introduction

Global interest in the use of unconventional feed resources deriving from under-utilised, low-cost agricultural, forest or food waste with bioconversion and nutrient capture ability has been growing rapidly in recent years (Øverland and Skrede, 2017; Cooney et al., 2023). Incorporation of these resources into fish diets can increase the biocirculation of waste material to aquaculture production, thereby making the industry more resilient, sustainable and eco-friendly (Binati et al., 2021). Among them, yeast and fungi have shown potential as unconventional feed resources for aqua diets in the recent past (Huyben et al., 2017; Vidakovic et al., 2020; Singh et al., 2021). The ascomycetous yeast *Yarrowia lipolytica* is a potentially valuable microbial aquafeed resource since it grows in diverse environments, including municipal waste streams, contaminated soil sediments, coastal habitats, food waste hydrolysates and industrial and forest by-products, and has a high fat/hydrocarbon content (Barth and Gaillardin, 1997; Beopoulos et al., 2009; Zinjarde et al., 2014). Owing to its suitable nutrient profile and ability to alter its composition according to the culture substrate or through genetic manipulation, *Y. lipolytica* already has many useful biotechnological applications in the food and feed industry (Bankar et al., 2009; Liu et al., 2015; Licona-Jain et al., 2020; Guardiola et al., 2021). The GRAS (generally regarded as safe) recognition of *Y. lipolytica* biomass and its derivatives by European and American agencies has further accelerated its use in the feed and food industry (Turck et al., 2019).

As a rich source of essential fatty acids, *Y. lipolytica* is used to produce polyunsaturated fatty acids (PUFAs) such as docosahexaenoic acid (DHA; 22:6n-3) and eicosapentaenoic acid (EPA; 20:5n-3) (Liu et al., 2015; Carsanba et al., 2018; Seternes et al., 2020). It is also used for the production of diverse groups of enzymes, such as lipases, esterases, proteases and phosphatases, and other important metabolites, such as

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polyamines and citric acid, by *de novo* synthesis or by absorption from the growth substrate (Ong et al., 2019; Liu et al., 2019). A high protein content (30–70% of total biomass) and high levels of essential amino acids (lysine, phenylalanine, valine, tryptophan, isoleucine), vitamins (B1, B2, B6, pantothenic acid, niacin, folic acid, biotin), minerals and other immunostimulants such as β-glucans, mannan-oligosaccharides and chitin make *Y. lipolytica* a potentially valuable functional feed ingredient (Morgunov et al., 2018; Gálvez-López et al., 2019; De Marco et al., 2021). Owing to these properties, studies have reported positive impacts of dietary *Y. lipolytica* in higher animals such as mammals (Michalik et al., 2013; Czech et al., 2016; Stefańska et al., 2018; Cheng et al., 2022) and birds (Czech et al., 2014, 2016, 2020; Merska et al., 2015) with respect to growth and productivity, gut health and microbiota, and immunological and physiological parameters.

In fish, *Y. lipolytica* has been shown to have the potential to replace fishmeal in the diet of Atlantic salmon (*Salmo salar*), improving the essential fatty acid profile and enhancing digestibility and bioavailability of EPA and DHA when fed as inactivated whole or disrupted yeast biomass, respectively (Hatlen et al., 2012; Berge et al., 2013). Dietary *Y. lipolytica* has also been demonstrated to have profound immunomodulatory effects in Pacific red snapper (*Lutjanus peru*) (Alamillo et al., 2017) and Nile tilapia (*Oreochromis niloticus*) (Neuls et al., 2021) in terms of nitric oxide production, peroxidase, lysozyme and myeloperoxidase activity and immune gene expression. In addition, *Y. lipolytica* is reported to secrete antibacterial molecules that can inhibit the growth of fish pathogenic bacteria, such as *Aeromonas hydrophila*, *Vibrio parahaemolyticus*, *Vibrio vulnificus* and *Photobacterium damselae* (Reyes-Becerril et al., 2021). A probiotic effect of *Y. lipolytica* in zebrafish (*Danio rerio*) fed live yeast, through modulation of gut mucosal genes and immune protection against *Vibrio anguillarum*, has also been demonstrated (Caruffo et al., 2015, 2016). Taking into account these multidimensional functionalities, it is relevant to investigate the effect of *Y. lipolytica* also in other farmed fish species, such as rainbow trout (*Oncorhynchus mykiss*). Such knowledge could enable a shift from conventional fishmeal and contribute towards bio-circular aquaculture.

Moreover, for better utilization of the yeast-derived nutrients and high palatability in fish, several authors have recommended the use of yeast extract or autolysis of the yeast cells before dietary inclusion (Berto et al., 2016; Sönmez, 2017; Hoshino et al., 2020; Rimoldi et al., 2020). The downstream processing of the yeast leads to the breakdown of the insoluble macromolecules like proteins and nucleic acids into soluble peptides, amino acids and nucleotides which in turn helps in better modulation of the gut microbial activity in the host fish. However, contrarian views also exist where it is reported that heat treatment could change the configuration of the immunogenic compounds (e.g., β-glucans) and thereby alter their bioactivity (Kaur et al., 2019; Zheng and Huang, 2022). Therefore, before commercial use of *Y. lipolytica* as a feed ingredient, the effect of its downstream processing during inactivation or autolysis by heat treatment, needs to be evaluated so that it can maintain its structural and functional properties and improve its bioactivity in the host fish (Hansen et al., 2021; Agboola et al., 2021).

The aim of this study was thus to evaluate the functional properties of *Y. lipolytica* in rainbow trout by determining its effects on fish growth, modulation of gut microbiota, mucosal health and immune-related gene expression.

2. Materials and methods

2.1. Ethical statement

Experiments on fish were carried out in the Aquatic Facility, Department of Animal Nutrition and Management, Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden. Experiment was carried out in full compliance with laws and regulations on procedures and experiments on live animals in Sweden are overseen by the Swedish Board of Agriculture (Registration number: 5.8.18–16,347/2017).

2.2. Fish rearing and maintenance

A total of 270 juvenile rainbow trout (35.1 ± 5.3 g mean body weight) were procured from Vilstena Fiskodling AB, Fjärdhundra, Sweden, and randomly distributed between 15 experimental tanks (water capacity 200 L) with $n = 18$ fish per tank. Each tank was fitted with waste feed and faeces belt collectors (Hølland teknologi, Sandnes, Norway) and a partial water recirculation system, where tank water was replaced by fresh municipal water at a rate of 3 L min^{-1}. Water temperature was maintained at 12 ± 1 °C with a 12 h dark/12 h light photoperiod (08:00–20:00 h), and dissolved oxygen level was 9–10 mg L^{-1} (measured by HQ40D Portable Multi Meter, Hach, Loveland, CO, USA) during the whole experiment. Before the start of the experiment the fish were acclimatised for 14 days, during which they were fed a commercial diet (INICIO 917, Biomar, Denmark), twice per day at 2% of tank biomass.

2.3. Yeast culture and molecular identification

The yeast strain used in experiments was *Y. lipolytica* CBS 7504, originally isolated from sewage from a wastewater treatment plant in Uppsala, Sweden. The yeast was taken from an *in-house* culture collection at the Department of Molecular Sciences, SLU, and cultured at 30 °C in yeast peptone dextrose (YPD; Sigma, Missouri, USA) agar medium containing 50 mg mL^{-1} chloramphenicol. The taxonomy and purity of strain CBS 7504 were confirmed by genetic characterisation of the D1/D2 domains of the large subunit rRNA gene (26S) by PCR amplification, using the primers 18S forward (5′-TACCTGGTTGATCCTGCCAGT-3′) and 18S reverse (5′-TTGATCCTTCTGCAGGTTCACCTAC-3′) (Alamillo et al., 2017), followed by analysis of homology using the BLASTN program against nucleotide (NT) database with default parameters (http://www.ncbi.nlm.nih.gov/blast).

2.4. Biomass production of Y. lipolytica

The *Y. lipolytica* CBS 7504 strain was inoculated in YPD broth medium (maintained at pH 6.2) containing 27 g L^{-1} sodium acetate, 20 g L^{-1} sodium DL-lactate, 2.6 g L^{-1} sodium propionate, 1.7 g L^{-1} yeast nitrogen base (without amino acids and ammonium sulphate), and 5 g L^{-1} ammonium sulphate, and incubated for 24 h at 30 °C in an orbital shaker. Pre-cultured yeast was harvested from the medium on reaching the exponential growth phase (24 h post-inoculation) by centrifugation at 1000 × *g* for 10 min at 4 °C and washed twice with saline (0.9% NaCl). The yeast pellet was collected by decanting the saline medium and inoculated in 1.5 L YPD broth medium containing 20 g L^{-1} acetic acid, 16 g L^{-1} DL-lactic acid, 2 g L^{-1} propionic acid, 1.7 g L^{-1} yeast nitrogen base and 5 g L^{-1} ammonium sulphate, in a Dolly bioreactor (6 L working volume; Belach Bioteknik AB, Huddinge, Sweden) for continuous cultivation. Continuous culture was maintained at pH 3.5 using the pH-stat method, by connecting the feed medium with acid titrant (pH set at 6.2 with a 0.10 deadband) fed through a 0.2 μm sterile filter (Sartolab P20 plus, Sartorius Stedim). The culture was aerated using compressed air, with a pO_2 set point at 20%, maintained by stirring speed. To compensate for the acidification of the medium due to the use of ammonium sulphate, aliquots of 5 M NaOH were added manually throughout cultivation. A maximum working volume of 5 L was maintained by pumping out the liquid using a tube positioned inside the bioreactor at the corresponding height. The yeast biomass was harvested when the culture reached the target optical density of 1.3, by centrifugation at 1000 × *g* for 30 min at 4 °C. After decanting the supernatant, the yeast biomass was washed twice with deionised water by centrifugation at the same settings.

2.5. Whole and autolysed yeast biomass

The harvested yeast cell biomass (2.29 kg wet weight, corresponding

to 588 g dry weight) was freeze-dried and divided into two equal parts. One part was kept as whole yeast and the other part was subjected to autolysis by heating to 50 °C for 16 h under continuous stirring (200 rpm). Successful autolysis of the yeast (no cell growth) was confirmed by plating the cell on YPD agar medium and incubating at 30 °C for 24 h. The autolysed cells were freeze-dried and stored at −20 °C.

2.6. Diet preparation, experimental design and feeding

Feed preparation was performed in SLU's Feed Technology Laboratory, using a twin-screw extruder, Brabender KETSE 20/40 (Brabender GmbH & Co. KG, Duisburg, Germany) equipped with five heating zones and a 2 mm die head (extrusion parameters are listed in Table S1 in Supplementary Material). Five iso-nitrogenous and isoenergetic experimental diets were prepared with 2% and 5% (as is) inclusion of whole yeast (WY) or autolysed yeast (AY) and a control diet with no yeast. Feed ingredient composition is shown in Table 2. All diets were formulated to slightly exceed the minimal nutritional requirements of rainbow trout (NRC, 2011). Feed waste was collected using the belts, stored at −20 °C and later analysed for dry matter content to calculate the actual feed intake and subsequent feed conversion ratio.

Three tanks, with 18 fish (35.1 ± 5.3 g mean body weight) per tank, were randomly assigned to each diet. Fish in each tank were fed twice a day for 45 days with the assigned diet, at a rate of 2% of body weight, by an automatic belt feeder (Hølland teknologi, Sandnes, Norway). The feed ration was adjusted every week based on the thermal growth coefficient (Cho, 1992).

2.7. Proximate composition analysis

For analysis of proximate composition, the experimental diet samples were dried in a hot-air oven for 16 h at 103 °C, cooled in a desiccator and weighed, followed by analysis of dry matter and ash content according to standard AOAC A (1995) methods. Experimental feeds were analysed for crude protein content (nitrogen, N × 6.25) by the Kjeldahl method (Nordic Committee on Food Analysis, 1976) using a 2020 Digester (with Cu as a catalyst) and 2400 Kjeltec Analyser unit (FOSS Analytical A/S, Hilleröd, Denmark). Crude lipid content was analysed according to the Official journal of the European Communities (1984), using a Soxhlet extraction unit (1047 Hydrolysing Unit, Soxtec System HT 1043, FOSS Analytical A/S). Neutral detergent fibre (NDF) was measured based on the method described by Chai and Udén (1998), using a 100% neutral detergent solution, with amylase and sulphite used for the reduction of starch and protein, respectively. Gross energy (GE) content was determined with an isoperibol bomb calorimeter (Parr 6300, Parr Instrument Company, Moline, IL, USA). The proximate composition of the whole and autolysed yeast in Table 1 and that of the experimental diets are shown in Table 2.

The glucan content of the whole and autolysed yeast was also analysed, using a β-glucan Assay Kit (Megazyme, Michigan, USA) according to the manufacturer's protocol. In addition, amino acid profiling was carried out using liquid chromatography coupled with tandem mass spectrometry (HPLC) method, by Eurofins Biopharma Product Testing Sweden AB (Uppsala, Sweden). The data obtained are shown in Table 1.

2.8. Sampling

For the determination of growth parameters and Fulton's condition factor, the initial and final body weight and length of fish from each treatment were measured at the start and end of the feeding trial, respectively. Following measurement of body indices at the end of the 45-day experimental period, nine fish per treatment (3 fish/tank) were randomly selected and anesthetised using tricaine methane sulphonate (MS-222; 300 mg L^{-1}, Western Chemical Inc., Ferdale, WA, USA). After swabbing with ethanol under a fume hood, all nine euthanised fish from each treatment were aseptically dissected out from the ventral side. The liver and intestine were dissected out from six fish per treatment (2 fish/tank/treatment) and their weight and length were recorded for calculation of hepato-somatic index and viscero-somatic index, respectively. The hindgut of each fish was then dissected from the ileocaecal valve up to 0.5 cm above the anus and digesta were collected in cryotubes, snap-frozen in liquid nitrogen and stored at −80 °C until DNA extraction for analysis of microbiota. After removal of the intestinal content, six distal intestine samples per treatment (2 fish/tank/treatment) were collected and stored in RNAprotect Tissue Reagent (Qiagen, Hilden, Germany) for 24 h at 4 °C and then at −20 °C until RNA extraction for gene expression analysis.

Table 1
Proximate composition (g/100 g), amino acid profile (g/100 g) and glucan content (g/100 g) expressed on as is basis of whole and autolysed *Yarrowia lipolytica* yeast.

	Yarrowia lipolytica	
	Whole yeast (WY)	Autolysed yeast (AY)
Proximate composition (%)		
Dry matter (DM)	94.5	88.8
Crude protein	42.20	32.70
Crude lipid	6.81	9.96
Neutral detergent fibre (NDF)	–	–
Ash	7.26	5.80
Gross energy (MJ/kg)	19.50	18.77
Amino acid profile (g/100 g)		
Essential amino acids		
Histidine	0.95	1.05
Isoleucine	1.26	1.64
Leucine	2.01	2.69
Lysine	2.06	2.68
Metionine	0.46	0.61
Phenylalanine	1.20	1.55
Threonine	1.66	2.26
Valine	1.60	2.01
Tryptophan	ND	ND
Non-essential amino acids		
Arginine	1.30	1.78
Asparagine	3.07	3.98
Cysteine	0.27	0.44
Glutamine	3.12	4.21
Glycine	1.43	1.98
Tyrosine	ND	ND
Proline	1.78	1.93
Serine	1.67	2.19
Alanine	2.63	3.05
Glucan content (% w/w)		
Total glucan	10.0	10.1
α-glucan	2.9	1.0
β-glucan	7.1	9.1

ND = not detected.

2.9. Growth parameter analysis

Four growth parameters [weight gain (WG %), feed conversion ratio (FCR), specific growth rate (SGR), survival percentage (Survival %)], two body indices [hepato-somatic index (HSI), viscero-somatic index (VSI)] and condition factor (CF) were calculated using the following equations:

$$\text{WG (\%)} = [\text{Final weight (g)} - \text{Initial weight (g)}]/\text{Initial weight (g)} \times 100$$

$$\text{FCR} = \text{Feed intake (g)}/\text{Weight gain (g)}$$

Table 2
Formulation (g kg^{-1}) and proximate composition (%) of the experimental diets expressed on 'as is' basis.

	Experimental diet				
	Control	2%-WY	5%-WY	2%-AY	5%-AY
Ingredient (g kg^{-1})					
Fish meal	330.0	330.0	330.0	330.0	330.0
Soy protein concentrate	**180.0**	**170.0**	**155.0**	**170.0**	**155.0**
Wheat gluten	100.0	100.0	100.0	100.0	100.0
Wheat meal	135.0	135.0	135.0	135.0	135.0
Fish oil	110.0	110.0	110.0	110.0	110.0
Rapeseed oil	70.0	70.0	70.0	70.0	70.0
Pot starch	10.0	10.0	10.0	10.0	10.0
Vitamin mineral premix	10.0	10.0	10.0	10.0	10.0
Monocalcium phosphate	10.0	10.0	10.0	10.0	10.0
Methyl cellulose	**42.0**	**32.0**	**17.0**	**32.0**	**17.0**
DL-methionine	3.0	3.0	3.0	3.0	3.0
Whole *Yarrowia lipolytica* (WY)	–	**20.0**	**50.0**	–	–
Autolysed *Yarrowia lipolytica* (AY)	–	–	–	**20.0**	**50.0**
Proximate composition (%)					
Dry matter (DM)	92.3	92.2	92.3	92.8	92.5
Crude protein	43.8	42.6	44.5	42.7	44.9
Crude lipid	19.9	18.0	18.9	18.5	18.3
Neutral detergent fibre (NDF)	2.8	3.2	3.4	3.2	3.0
Ash	7.1	7.1	7.2	7.0	7.3
Gross energy (MJ/kg)	22.0	22.0	22.2	22.0	22.0

$$\text{SGR}\,(\%/\text{day}) = [\mathit{log}\,(\text{final weight}) – \mathit{log}\,(\text{initial weight})]/\text{time}\,(\text{days}) \times 100$$

$$\text{Survival}\,(\%) = [\text{Number of fish at the end}/\text{Number of fish at the start}] \times 100$$

$$\text{HSI}\,(\%) = \text{Weight of liver of fish}\,(\text{g})/\text{Weight of fish}\,(\text{g}) \times 100$$

$$\text{VSI}\,(\%) = \text{Weight of fish gut}\,(\text{g})/\text{Weight of fish}\,(\text{g}) \times 100$$

$$\text{CF}\,(\%) = \text{Weight of fish}\,(\text{g}) \Big/ [\text{Total length of fish}\,(\text{cm})]^3 \times 100$$

2.10. Gut microbiota analysis

2.10.1. Extraction of DNA

Intestinal content samples (10–100 mg) were transferred to sterile cryotubes containing 1 mL of InhibitEX buffer (Qiagen, Germany) and 0.5 g silica beads (0.1 mm diameter). The samples were homogenised in a Precellys Evolution homogeniser (Bertin Technologies, Montigny-le-Bretonneux, France) at 6000 rpm for 1 min in two runs, with a 5 min interval on ice. DNA was then isolated from the homogenised samples using a QIAamp Fast DNA Stool Mini Kit (Qiagen) according to the manufacturer's protocol.

2.10.2. Amplicon library preparation, sequencing and data analysis

The barcoded 16S rRNA gene amplicon library was constructed for Illumina sequencing of the V3-V4 region using primers 341F (5'-CCTACGGGNGGCWGCAG-3′) and 805R (5'-GGACTACHVGGGTWTCTAAT-3′) (Hugerth et al., 2014), according to the method described by Müller et al. (2016). The correct length of the amplified PCR product was confirmed gel electrophoresis (2% agarose). Same amount of PCR products from each sample was pooled, end-repaired, A-tailed and further ligated with Illumina adapters. Libraries were sequenced on a paired-end Illumina platform to generate 250 bp paired-end raw reads. The library was checked with Qubit and real-time PCR for quantification and bioanalyzer for size distribution detection. Quantified libraries were pooled and sequenced on NovaSeq6000 PE250 (Novogene, Cambridge, United Kingdom), according to effective library concentration and data amount required.

Raw sequencing data were quality-controlled (Q-score > 20) and adapters/primers were trimmed with Cutadapt (v3.5) (Martin, 2011), and the paired end reads were merged using VSEARCH v2.3.4 software (Rognes et al., 2016). The merged reads per sample were used for sequence analysis with the package *dada2* (v1.22.0) (Callahan et al., 2016), using R (v4.1.3) (R Core Team. R: A language and environment for statistical computing, 2021) in RStudio (v 2021.09.0 + 351) (http://www.rstudio.com) (RStudio RT, PBC B, RStudio MA, 2020). Taxonomic annotations were performed using the 16S rRNA database formatted for *dada2* with Genome Taxonomy Database taxonomies (v207) (Alishum, 2020). The taxonomic profile of the microbial community was visualised with the packages *phylosEq.* (v1.38.0) (McMurdie and Holmes, 2013) and *ggplot2* (v3.3.6) (Wickham, 2016). The sequences obtained have been submitted to Sequence Read Archive under Bioproject accession number PRJNA939018.

2.11. Quantification of immune genes in the intestinal tissue samples

2.11.1. RNA isolation and cDNA synthesis

Intestinal tissue samples (30 mg each) were added to RNase-free bead beating tubes containing 1–3 mm corundum and 3 mm steel beads and 600 μL of Buffer RLT Plus (Qiagen). The samples were homogenised twice at 6000 rpm for 30 s in a Precellys Evolution homogeniser (Bertin Technologies). RNA extraction was carried out using the RNeasy Plus Mini kit (Qiagen) according to the manufacturer's protocol. The concentration and purity of the extracted RNA were measured using NanoDrop ND-1000 (NanoDrop Technologies Montchanin, USA). RNA quality (RIN) was determined using Agilent Tapestation 4150 (Agilent Technologies, Germany). Genomic DNA contamination was removed by treating 1.2 μg of each RNA sample with RQ1 RNase-Free DNase (Promega, USA). cDNA was synthesised using the GoScript™ Reverse Transcriptase (Promega), following the manufacturer's protocol. The cDNA reaction was split so that 0.2 μg RNA was used for control without reverse transcriptase (−RT control). The cDNA samples were diluted at 1:5 ratio using nuclease-free water and stored at −20 °C until use.

2.11.2. Gene expression analysis by qPCR

All specific primers for qPCR of the targeted and reference genes were selected from previously published studies (Tsujita et al., 2004; Løvoll et al., 2011; Ballesteros et al., 2012, 2014; Tacchi et al., 2015; Johansson et al., 2016; Dupuy et al., 2019; Huyben et al., 2019; Mehrabi et al., 2019; Rawling et al., 2021) [for details of product length, annealing temperature and accession number, see Table S2 in Supplementary Material]. For all experimental samples, qPCR amplification of two reference genes [beta actin (*β-actin*), and ribosomal protein S20 (*rsp20*)] and 11 mucosal immune responsive genes [interleukin-1beta (*il-1β*), tumour necrosis factor-alpha (*tnf-α*), interleukin-10 (*il-10*), transforming growth factor-beta (*tgf-β*), mucin-2 (*muc-2*), immunoglobulin tau heavy chain (*igt*), cluster of differentiation 4 (*cd4*), complement factor 3 (*c3*), *c-type lectin*, toll-like receptor 2 (*tlr2*) and toll-like receptor 5 (*tlr5*)] was carried out in a CFX96Touch PCR machine (Bio-Rad, California, USA), using Quantitect SYBR Green (Qiagen, Germany). Each reaction was prepared in duplicate to a total volume of 25 μL per reaction, with the reaction mixture consisting of 12.5 μL Quantitect SYBR Green (2×), 1.25 μL forward primer, 1.25 μL reverse primer, 8 μL nuclease-free water and 2 μL cDNA samples as a template. The thermal profile used for qPCR amplification consisted of an initial cycle of denaturation at 95 °C for 15 min, followed by 39 cycles of 95 °C for 15 s, annealing at gene-specific annealing temperature (T_a) for 30 s and extension at 72 °C for 30 s. The thermal cycle ended with melt curve analysis to verify the PCR product. Relative expression of each stress-mediated and immune-response gene was normalised with the two

selected reference genes and calibrated with respect to the control samples. The efficiency of *β-actin* (M value: 0.17, Stability: 1.76) and *rsp20* (M value: 0.17, Stability: 1.76) was calculated using the CFX software. The ΔΔCt value of each sample was determined by subtracting the average ΔCt value of the control from the ΔCt of the test sample. Relative quantification, or fold change in expression for each gene compared with the control, was thus expressed as $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001).

2.12. Statistical analysis

The data obtained on growth parameters and gene expression were subjected to one-way ANOVA, followed by Tukey's multiple comparison test using GraphPad PRISM 9.3.1 for pairwise comparison of the different dietary groups. The significance level was set at $p < 0.05$. The 16S rRNA gene differential abundance analysis was performed using normalised mean of control versus treatment in pairwise analysis with the package *DESeq2* (v1.34.0) (Love et al., 2014). For estimation of alpha diversity, the Observed, Shannon and Inverse Simpson indices were calculated using the *estimate_richness* function in *phyloseq*. For estimation of beta diversity, multivariate analysis (non-metric multidimensional scaling (NMDS) with Bray-Curtis dissimilarity matrix) was performed using the packages *phyloseq* and *vegan* (v2.5.7) (Oksanen et al., 2015).

3. Results and discussion

3.1. Effect of Y. lipolytica on fish performance

The fish growth was similar for fish fed the yeast-supplemented diets, irrespective of inclusion level and pretreatment, and there were no significant differences in WG, SGR and FCR compared with control fish after the 45-day feeding period (Table 3). This is in accordance with several previous studies examining diet supplementation of up to 30% of *Y. lipolytica* (previously *Candida lipolytica*), wherein, no negative or positive effects on growth parameters in sea bass (Alliot et al., 1979), rainbow trout (Atack and Matty, 1979) and Atlantic salmon (Hatlen et al., 2012) were found. The HSI, VSI and CF values ranged between 1 and 1.6%, demonstrating good overall health status of the fish, with no differences between fish fed the different experimental diets. Hence, our data demonstrated that up to 5% *Y. lipolytica* supplementation does not influence growth performance and relative body indices in rainbow trout.

3.2. Modulation of gut microbiota

Initial processing for quality control and adapter/priming of raw 16S rRNA gene amplicon sequencing data resulted in on average ~ 96,600 (±10,700) reads per samples, which were merged and used for sequence analysis with the package *dada2*. Approximately 78,500 (±8800) reads per sample were identified as non-chimeric and used for calling amplicon sequence variants (ASVs). A total of 21,630 ASVs were identified and ASVs classified as unknown at kingdom level and kingdom *Archaea* were discarded. Filtering was performed for ASVs which were singletons and ASVs not present in at least two samples. After the filtering steps, 3448 ASVs remained for visualisation and statistical analyses.

It was expected that autolysis of yeast would provide different nutritional properties and cell wall composition, due to enzymatic degradation of cell walls and leakages of other cellular components (Wang et al., 2018). Thus, there was the possibility of a differential impact of the autolyzed and whole yeast on gut microbiota composition. However, the lack of effect on growth parameters and alpha diversity estimation (Shannon and Simpson index), for analysed taxa, between whole or autolyzed yeast and the control diet (Fig. 1A), could therefore be explained by the low inclusion levels of the yeast biomass in the experimental diets. Likewise, the NMDS analysis (stress value = 0.22), showing the spatial distribution of individual samples in the multidimensional space, did not demonstrate any clear differences in overall gut microbiota composition linked to individual diets (Fig. 1B). Similar results have been reported for Arctic charr (*Salvelinus alpinus*), with no differences in bacterial richness and diversity in gut microbiota in fish when 40% of fishmeal in the diet was substituted by intact or extracted yeast cells (Nyman et al., 2017). A study on gilthead sea bream (*Sparus aurata*) also found that the inclusion of 5% autolysed yeast did not affect the Simpsons index (Rimoldi et al., 2020). Hence, it can be inferred that the amount of inclusion of microbial biomass in diets does not affect the gut microbiota in fish irrespective of the autolysis process.

Taxonomic analysis of microbial communities based on mean relative abundance at the family level (>2%) showed the dominance of *Brevinemataceae* (43.4%), *Rhizobiaceae* (7.5%), *Desulfovibrionaceae* (6.3%), *Lactobacillaceae* (5%) and *Ruminococcaceae* (3.5%) for all diet groups (Fig. 2A). At genus level (>2%), *Brevinema* (45.5%), *Aureimonas* (6.9%) and *Desulfovibrio* (6.7%) were found to be the dominant taxa, irrespective of diet (Fig. 2B). Previous studies also reported high abundance of *Brevinema* in prebiotic-fed Atlantic salmon with no changes in the alpha diversity (Merrifield et al., 2010; Gupta et al., 2019; Li et al., 2021). Therefore, it indicates that *Brevinema* is associated with the core microbiome of salmonid fish independent of any feed supplement.

3.2.1. Association of microbial taxa with diets

Our analysis revealed that despite different inclusion levels and autolysis of *Y. lipolytica*, there was no significant change in the overall composition of the gut microbiota (beta diversity). However, certain taxonomic families exhibited notable shifts in abundance, as evidenced by significant log2 fold change analysis (LFCa). LFCa was performed to observe the relative change in abundance and to compare positive and

Table 3
Growth parameters and performance index values in rainbow trout fed the control diet and experimental diets containing two and 5 % whole (WY) and autolysed (AY) *Yarrowia lipolytica* yeast for 45 days.

Parameter/index	Experimental diet					*p*–value
	Control	2% -WY	5%-WY	2%-AY	5%-AY	
Initial weight (g)	35.3 ± 5.5	34.9 ± 5.4	34.7 ± 5.3	35.0 ± 5.4	34.6 ± 5.3	–
Final weight (g)	68.3 ± 16.7	67.1 ± 16.9	75.0 ± 14.5	71.1 ± 17.0	72.6 ± 18.0	–
WG (%)	93.0 ± 10.5	92.9 ± 21.9	116.3 ± 2.9	103.5 ± 11.6	110.0 ± 18.7	0.460
SGR (%/day)	1.22 ± 0.01	1.20 ± 0.20	1.43 ± 0.06	1.32 ± 0.11	1.37 ± 0.17	0.045
FCR	0.83 ± 0.11	0.85 ± 0.16	0.64 ± 0.03	0.74 ± 0.01	0.70 ± 0.11	0.298
Survival (%)	100	100	100	100	100	–
HSI (%)	1.33 ± 0.04	1.51 ± 0.05	1.46 ± 0.14	1.41 ± 0.05	1.61 ± 0.09	0.197
VSI (%)	1.05 ± 0.07	1.21 ± 0.06	1.16 ± 0.04	1.13 ± 0.03	1.16 ± 0.07	0.389
CF (%)	1.22 ± 0.04	1.21 ± 0.00	1.24 ± 0.01	1.19 ± 0.02	1.22 ± 0.04	0.899

Values shown are mean ± SE.
Initial weight (g); Final weight (g); WG (%) = Weight gain percentage; SGR (%/day) = Specific growth rate; FCR = Feed conversion ratio; HIS (%) = Hepato-somatic index; VSI (%) = Viscero-somatic index; CF (%) = Condition factor.

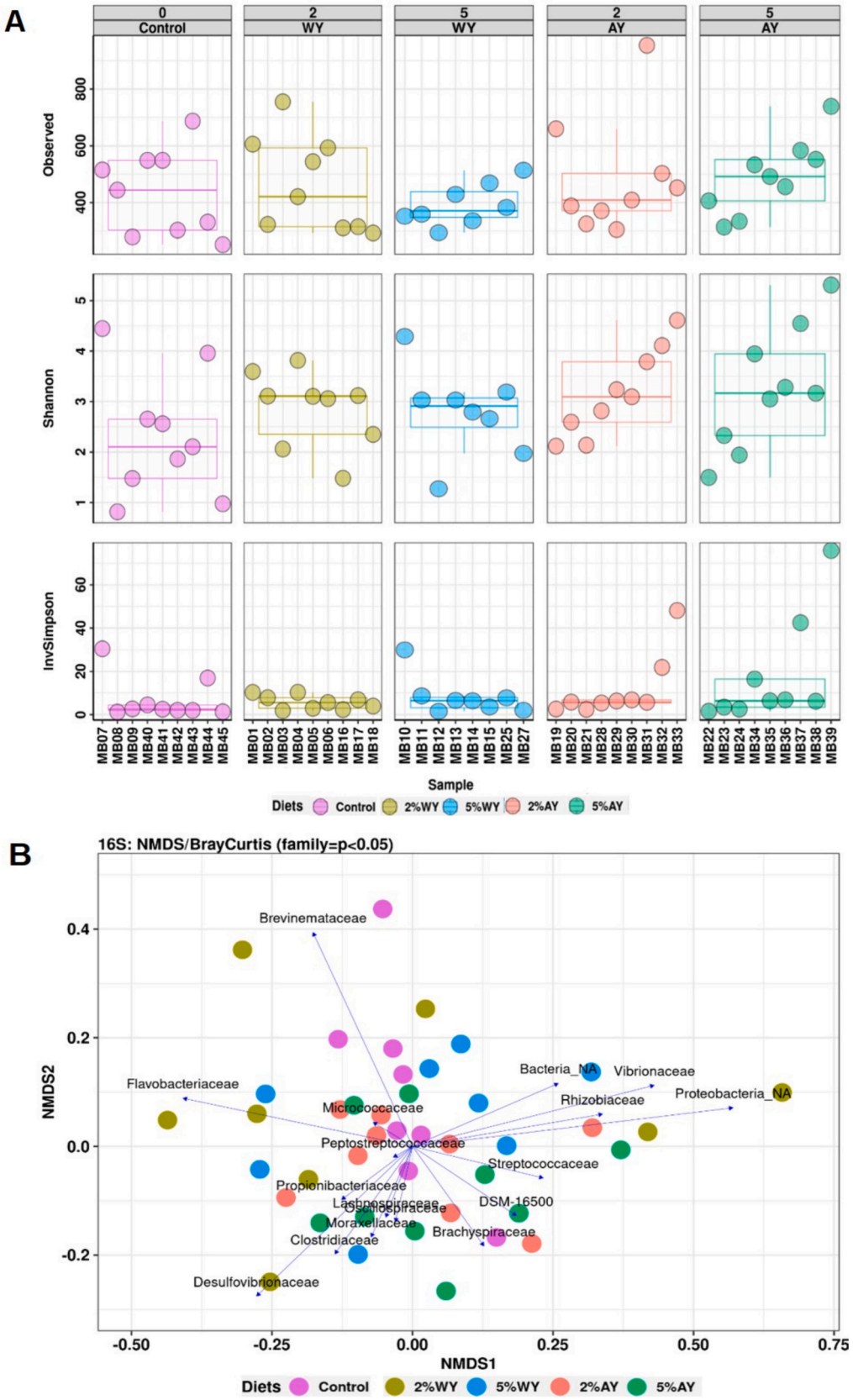

Fig. 1. (A) Alpha diversity (Shannon and Simpson indices) and (B) beta diversity (Bray-Curtis analysis) of gut microbial communities in nine rainbow trout per diet fed the control, 2% WY, 5% WY, 2% AY and 5% AY diets (WY and AY = whole and autolysed *Yarrowia lipolytica* yeast, respectively).

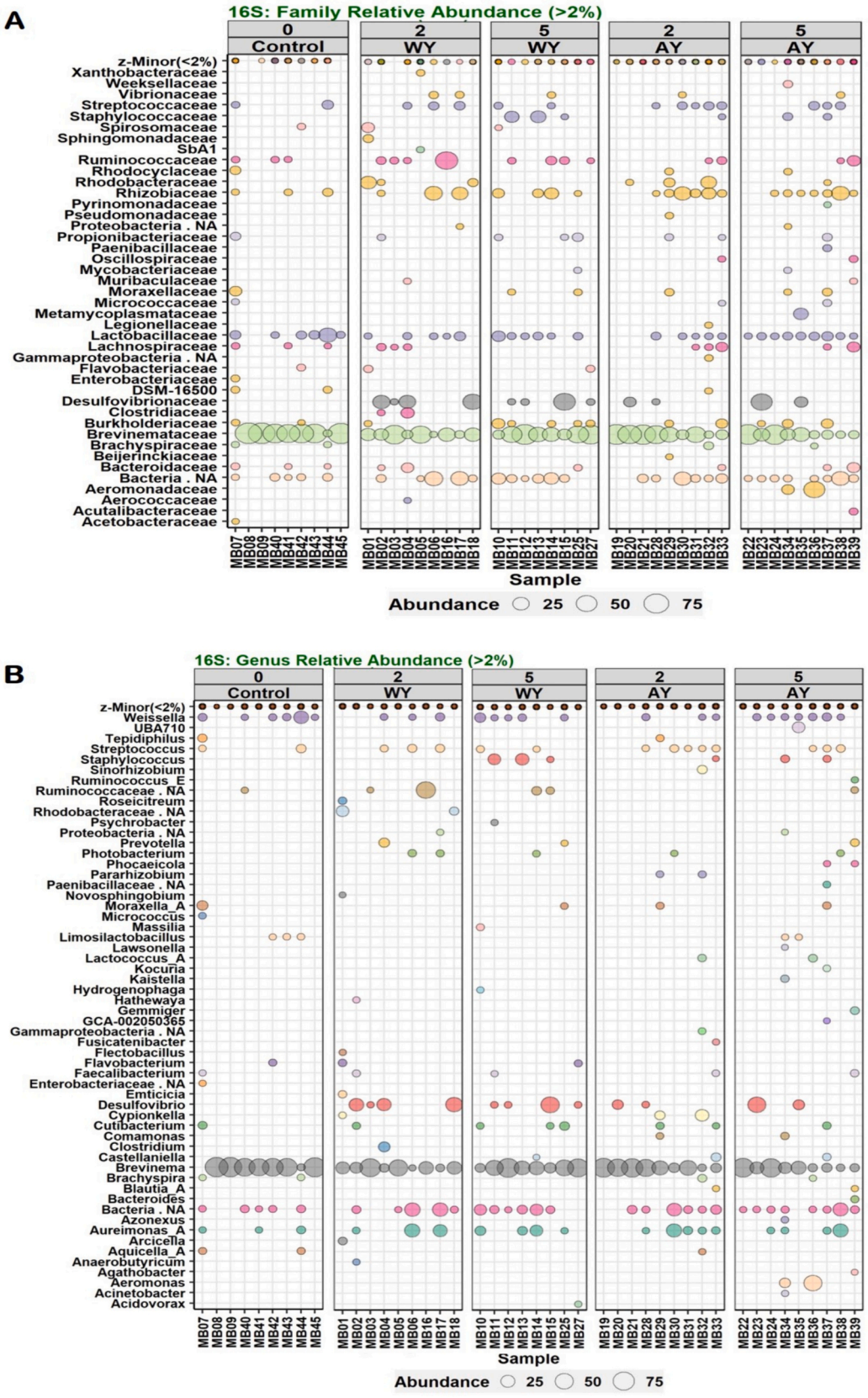

Fig. 2. Relative abundance at (A) family level and (B) genus level (with relative abundance <2% merged as 'minor family' and 'minor genus', respectively) after 45 days of the gut microbial community (identified using 16S rRNA gene amplicon sequencing) in nine rainbow trout per diet fed the control, 2% WY, 5% WY, 2% AY and 5% AY diets (WY and AY = whole and autolysed *Yarrowia lipolytica* yeast, respectively). The relative abundance >1% is represented as the bubbles in the plot.

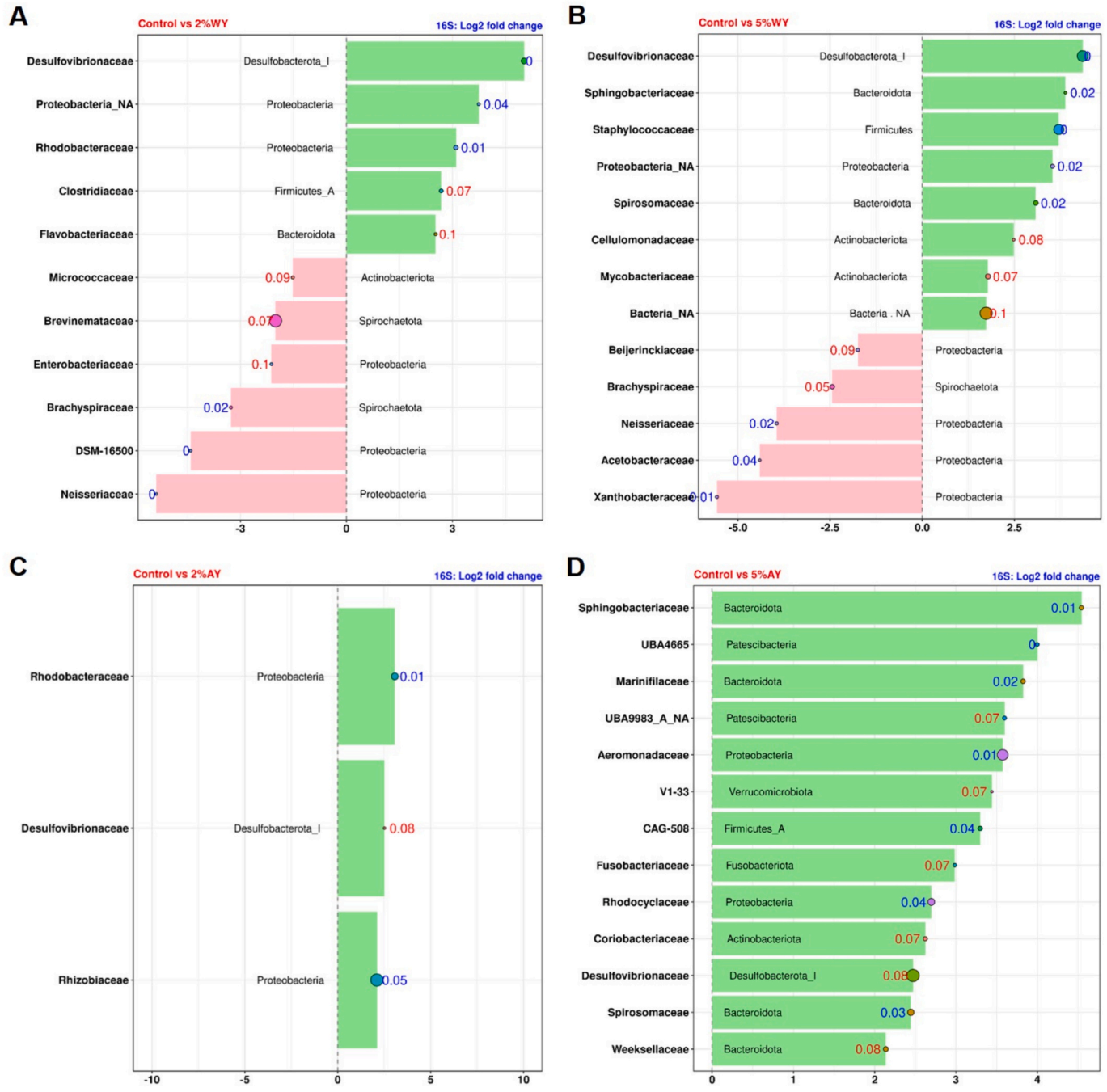

Fig. 3. Log2 fold change analysis (LFCa) plot showing differential abundance in microbial families. Positive and negative Log2 fold change represents a synergistic and inhibitory effect, respectively, on the microbial community at family level due to different experimental diets. (A) Control vs. 2% WY, (B) control vs. 5% WY, (C) control vs. 2% AY and (D) control vs. 5% AY (WY and AY = whole and autolysed *Yarrowia lipolytica* yeast, respectively). Bubble colour indicates phylum, bubble size indicates differential abundance. Numerical values beside bubbles indicate *p*-value (blue $p < 0.05$, red $p > 0.05$ to $p < 0.1$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

negative associations of microbial communities between *Y. lipolytica* diets and the control diet (Fig. 3). *Desulphovibrionaceae* exhibited higher abundance and a synergistic association with 2% WY (LFCa ~5, $p < 0.05$) and 5% WY (LFCa ~4.75, $p < 0.05$) diets compared to the control (Fig. 3A, B). *Sphingobacteriaceae* (LFCa ~4.5, $p < 0.05$) showed positive associations with 5% inclusion of both WY and AY diets (Fig. 3A, C), while *Rhodobacteraceae* (LFCa ~3, $p < 0.05$) correlated at the lower inclusion level (2%) of these diets, (Fig. 3B, D). Although *Desulfovibrionaceae* and *Rhodobacteraceae* have been proposed to be associated with certain metabolic activities in other animal species, their presence in fish guts remains relatively unexplored (Karpe et al., 2023). The positive association of *Sphingobacteriaceae* with 5% inclusion of *Yarrowia* might be related to yeast cell lipid accumulation, although, crude fat content was higher in autolysed yeast (9.96 g kg^{-1} dry weight) as compared to the whole yeast (6.81 g kg^{-1} dry weight). These *Sphingobacteriaceae* are reported to metabolise yeast lipid substrate and are capable of producing sphingolipid and inositol in the host animal, which has regulatory functions in cell differentiation, immunomodulation and induction of the metabolic pathway genes (El Alwani et al., 2006; Hannun and Obeid, 2018; Johnson et al., 2020; Heaver et al., 2022). The synergistic association of *Staphylococcaceae* (LFCa ~4, $p < 0.05$) with the 5% WY diet warrants further investigations on its effect on gut-

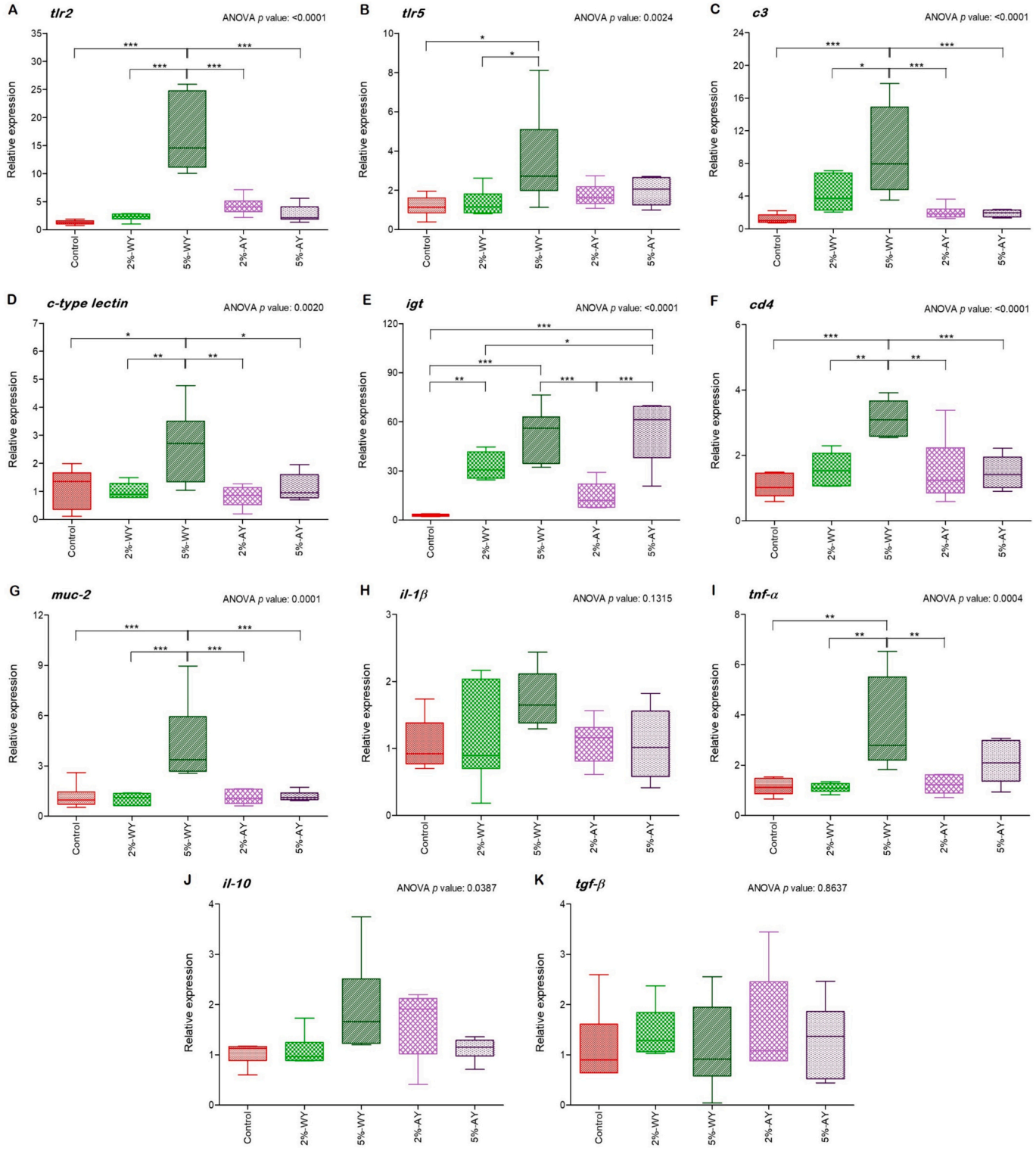

Fig. 4. Relative expression after 45 days of immune-related genes in intestine tissue of rainbow trout fed the control, 2% WY, 5% WY, 2% AY and 5% AY diets (WY and AY = whole and autolysed *Yarrowia lipolytica* yeast, respectively). Expression level of each gene was compared between the experimental groups and the control. Relative expression level of (A) *tlr2*, (B) *tlr5*, (C) *c3*, (D) *c-type lectin*, (E) *igt*, (F) *cd4*, (G) *muc-2*, (H) *il-1β*, (I) *tnf-α*, (J) *il-10*, and (K) *tgf-β* plotted as individual and mean ($n = 6$) fold change in gene transcript level. Asterisks indicate significant differences between all diets in one-way ANOVA (*$p < 0.05$) and in pairwise Tukey's multiple comparison tests (*$p < 0.05$; **$p < 0.01$, ***$p < 0.001$).

associated microbiota. Previous studies have shown augmentation of *Staphylococcaceae* in the gut microbiota of Atlantic salmon fed different dietary prebiotics or synbiotics (Bakke-McKellep et al., 2007; Askarian et al., 2012; Abid et al., 2013).

The lack of change in beta diversity as discussed above suggests that the microbial community structure remained relatively stable despite individual taxa experiencing shifts in abundance. This may indicate a degree of functional redundancy within the gut microbiota, where

compensatory changes occur to maintain ecosystem stability. Hence, these findings highlight the complexity of microbial community responses to the *Y. lipolytica* diets and underscore the importance of investigating individual taxonomic groups in addition to overall community metrics. Future studies could explore the functional implications of the observed changes in specific taxonomic families and their potential impact on gut dynamics.

3.3. Modulation of mucosal immune genes

Yeast-derived β-glucan and α-mannans, and other molecules (proteins, vitamins and nucleotides), can activate microbe-associated molecular patterns (MAMPs) (Hassan, 2011; Shurson, 2018; Guerreiro et al., 2018; Rawling et al., 2021). These MAMPs are used for recognition by the host's immunocompetent cells (macrophages, neutrophils and dendritic cells), and are reported to activate different pattern-recognition receptors (PRRs) present on innate immune cells effectively, allow the host to determine the immune fate of localised gut-associated lymphoid tissues (Erwig and Gow, 2016; Ji et al., 2020). Upon interaction and binding, the MAMPs-PRRs complex activates multiple signalling pathways and thereby takes part in immunomodulation in the host (Dalmo and Bøgwald, 2008). Thus, in the present study, immunological responses at the molecular level by mRNA expression were examined by analysis of 11 immune-relevant genes in the distal intestine (Fig. 4).

The *tlrs* and *c-type lectins* families are important in the recognition of yeasts and its derivatives such as mannans and β-glucans (Patin et al., 2018; Petit et al., 2019). Thus, the significantly higher ($p < 0.05$) upregulation of *tlr2* (~13-fold), *tlr5* (~3-fold), *c3* (~4-fold) and *c-type lectin* (~2-fold) gene transcripts (Fig. 4A-D) in the 5% WY, signifies the structural stability and antigenicity of whole yeast derived MAMPs as compared to the autolysed or lower inclusion level diets. Similarly, the higher level of *cd4* (~3-fold) gene transcript (Fig. 4F) in the 5% WY diet would also suggest that, the whole yeast was efficient in presenting the antigenic motifs to the CD4+ T-cells for recognition, binding and processing (Salinas, 2015; Hoare et al., 2022). However, the present results also showed a positive association between the *igt* (~15-to-54-fold change) gene transcript (Fig. 4E) and the level of *Y. lipolytica*. This can be explained by the ability of the yeast-derived β-glucan/mannans, regardless of its structural conformation due to autolysis, to act as a potent antigen to stimulate B-cells for the production of immunoglobulins. Similar upregulation of immunoglobulin gene transcripts was noticed in rainbow trout's intestine, post dietary supplementation of β–glucan (Porter et al., 2023). Thus, it can be ascertained that the yeast-derived molecules can directly act upon the gut immune cells and alter the gene expression in intestinal tissue as indicated by the enhancement of the innate immune response.

The expression of the *muc-2* gene, a precursor for mucus production in the gut mucosa (Hoare et al., 2022), was also analysed. The *muc-2* gene showed significantly higher ($p < 0.05$) upregulation in fish fed the 5% WY diet (~3-fold change) (Fig. 4G). Although the reason for the high expression of *muc-2* in the 5% WY diet remains unknown, it has been shown that both Th1 and Th2 cytokines are reported to induce MUC2 mucin transcription through nuclear factor kappa-B (NF-κB) activation (Liu et al., 2020).

Among the cytokines, the *il-1β* (Fig. 4H) and *tgf-β* (Fig. 4K) genes showed wide variation in expression in individual fish in each diet group, with no clear trend. In contrast, the *tnf-α* (Fig. 4I) gene showed a significantly higher ($p < 0.05$) transcript level in fish fed the 5% WY diet compared with all other diets, including the control, while the *il-10* (Fig. 4J) gene transcript displayed a distinct trend for higher expression in fish fed the 5% WY diet, followed by fish fed the 2% AY diet, but no pairwise significant difference was found between the diets. These increased levels of *il-10* and *tnf-α* support homeostasis and protection against inflammation (Liu et al., 2020).

Moreover, the present study demonstrated an overall higher immune response for the whole yeast diet (β–glucan: 9.1%, *w*/w) as compared to the autolyzed yeast diet (β–glucan: 7.1%, w/w). These discrepancies in the immunomodulation concerning the β–glucans concentration can be explained by the structural and functional variation of the β-–glucans originating from their sources and the processing methods used for their extraction and purification (De Marco Castro et al., 2021). Consequently, these structural diversities lead to distinct interactions with the host. It has also been observed that highly purified β-1,3-1,6-glucans possessing extensive branching along the β-1,3-glucan backbone and a substantial molecular weight exhibited enhanced immunity in the host (De Marco Castro et al., 2021). In this context, further research is warranted to elucidate the structural and molecular changes upon autolysis leading to specific immune stimulation and understanding the mechanisms orchestrated by the *Y. lipolytica* along with the optimised dietary supplementation required to maximize the health benefit in the aquaculture species.

4. Conclusion

This study provides insights into *Y. lipolytica*'s bioactivity in fish feed, affecting gut microbiota and immune responses. The results showed that *Y. lipolytica* affected innate and adaptive immune responses in rainbow trout. Expression of immune-related genes increased significantly in 5% of WY-fed fish, indicating higher immunomodulatory effects compared to autolysed yeast with the same inclusion level. The impact of *Y. lipolytica* on the alpha and beta diversity of intestinal microbiota was minimal. However, significant synergistic correlation was observed for certain microbial taxa with *Y. lipolytica* supplemented diet and future studies could explore their potential functional implications on the gut dynamics. Hence, *Y. lipolytica* holds promise as a functional feed ingredient in aquaculture, influencing gut microbial diversity modestly while enhancing mucosal immunity in rainbow trout.

CRediT authorship contribution statement

Aprajita Singh: Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Aleksandar Vidakovic:** Writing – review & editing, Project administration, Methodology, Formal analysis, Conceptualization. **Abhijeet Singh:** Software, Formal analysis. **Johan Dicksved:** Writing – review & editing, Supervision, Methodology, Formal analysis, Data curation. **Anna Schnürer:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization. **Torbjörn Lundh:** Writing – review & editing, Validation, Supervision, Project administration, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

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

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.2024.741065.

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