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Local probiotic bacteria positively influence production parameters and resistance against *Providencia* infections in Nile tilapia (*Oreochromis niloticus*)

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ABSTRACT

This study evaluated the efficacy of dietary supplementation with two indigenous probiotic strains, *Lysinibacillus fusiformis* LFUG and *Priestia megaterium* PMUG01 in improving growth, health, and disease resistance in Nile tilapia juveniles. Each strain was administered at two concentrations (10^6 and 10^8 CFU.g^{-1} of feed) during a 60-day feeding trial. Regardless of concentration, probiotic-supplemented diets significantly improved growth performance indicators, including weight gain, biomass gain, specific growth rate (SGR), and feed conversion ratio (FCR). This was likely due to enhanced nutrient bioavailability and feed utilization. There were no notable changes in fish proximate nutrient composition or condition indices (condition factor, VSI and HSI), signifying that the probiotics did not adversely affect the nutritional quality and welfare of the fish. After the feeding trial, a 14-day challenge with virulent clinical *Providencia* sp. (strain PSNUG) revealed significant protection in probiotic-fed groups. This protection was attributed to the modulation of gut microbiota, suppression of pathogenic taxa and attenuation of pathogen virulence factors. Histological analysis showed reduced hepatic and splenic inflammation in treated fish, while molecular assays demonstrated modulation of immune markers (*IL1β*, *IL6*, and *CXCL8*), and the complement component *C3*, indicating enhanced mucosal immunity and reduced systemic inflammation. Overall, both strains showed promise as safe and multifunctional probiotics capable of boosting growth, immune competence, and disease resistance in Nile tilapia juveniles. These results support their potential for improving fish health and aquaculture productivity. Exploration of their applicability across other aquaculture species and environments is recommended to promote sustainable fish farming practices.

List of abbreviations: ABW, Average Body Weight; ANI, Average Nucleotide Identity; ARDC, Aquaculture Research and Development Centre; CARD, comprehensive antibiotic resistance database; COV, Coefficient of Variation; DDDH, digital DNA-DNA Hybridization; DNA, Deoxyribonucleic Acid; FAO, Food and Agriculture Organization of the United Nations; FCR, Feed Conversion Ratio; GALT, Gut-associated lymphoid tissues; GIT, Gastrointestinal Tract; HSI, Hepatosomatic Index; IgM, Immunoglobulin M; IP, Intraperitoneal; K, Condition Factor; NARO, National Agricultural Research Organization; NCBI, National Center for Biotechnology Information; OD, Optical Density; PCR, Polymerase Chain Reaction; QQ, Quorum Quenching; RNA, Ribonucleic Acid; RT-qPCR, Reverse Transcription Quantitative PCR; SCFA, Short-Chain Fatty Acid; VFDB, Virulence Factor Database.

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1. Introduction

The ongoing intensification of aquaculture aims to address the global deficit in aquatic animal protein for the growing human population, projected to reach 10 billion by 2050 (Gephart et al., 2020). Fish and seafood offer a healthier alternative to terrestrial meat, driving the expansion of aquaculture (Tacon, 2023; Tacon et al., 2020). However, in Sub-Saharan Africa (SSA), per capita aquatic food consumption remains lower than the global average due to rapid population growth outpacing fish supply (FAO, 2020, 2024; Muringai et al., 2022). Africa contributed 1.9 % to global aquaculture production, with aquatic animal output rising by 0.8 % in 2022, signifying increased intensification (FAO, 2024).

Yet, the shift toward intensive aquaculture systems has led to a rise in the incidence of viral, parasitic, and bacterial diseases, resulting in annual global economic losses estimated at over 10 billion USD (Mwainge et al., 2021; Subasinghe et al., 2023). Among these, bacterial infections are particularly problematic due to the vast diversity of opportunistic and obligate pathogens affecting various stages of aquatic organisms (Maldonado-Miranda et al., 2022; S. K. Nayak, 2020). These infections account for 10–15 % of production losses (Maldonado-Miranda et al., 2022). Consequently, antibiotic use has increased, especially in regions with weak regulatory frameworks (Bondad-Reantaso et al., 2023; Lulijwa et al., 2020; Pruden et al., 2013). This has exacerbated concerns regarding antibiotic resistance, disruption of microbial balance (dysbiosis), and residue accumulation in aquaculture products and ecosystems, raising food safety, public, and veterinary health safety issues (Aly and Albutti, 2014; Zhou and Chen, 2024).

This has spurred interest in non-antibiotic, biologically based alternatives, such as bacteriophages, plant extracts, prebiotics, and probiotics (Bondad-Reantaso et al., 2023; Kılıç and Gültekin, 2024; Munni et al., 2023; Yilmaz et al., 2022). Probiotics, the focus of this study, are live microbial supplements that offer health benefits to the host when correctly administered (Sokooti et al., 2022). These benefits include enhanced digestion and metabolism, immune stimulation, and improved water quality, all of which contribute to better growth and resilience in fish and shellfish (Etyemez Büyükdeveci et al., 2023; Hlordzi et al., 2020; Naiel et al., 2022; Xia et al., 2020).

While most commercial probiotics for aquaculture are allochthonous (originating from outside the host or its environment), indigenous, autochthonous strains, isolated from the host species or its habitat, are increasingly considered more effective (X. Huang et al., 2023; Yamashita et al., 2020). They are hypothesized to offer better adaptability, colonization efficiency, and tolerance to diverse environmental conditions characteristic of aquaculture systems, such as variations in salinity, temperature, pH and host immune responses (Melo-Bolívar et al., 2021; Soltani et al., 2019). The use of indigenous strains aligns with international biodiversity commitments such as the Nagoya Protocol (CBD, 2011) and the Kunming-Montreal Global Biodiversity Framework 2050 (KMGBF, 2022), promoting conservation, local innovation, and equitable benefit sharing.

Despite their potential, a key limitation in probiotic efficacy studies is the variability in environmental conditions, including water quality, microbial community dynamics, host stress levels, and strain-specificity, which can influence colonization success and immune outcomes (McFarland, 2021; McFarland et al., 2018; Rytter et al., 2023; Yarullina et al., 2015). These factors may affect the reproducibility and generalizability of probiotic effects across different aquaculture systems and geographic regions (Amin et al., 2023; Ariyanto and Anika, 2024; Sumon et al., 2022). Careful consideration of these limitations is essential when interpreting efficacy under controlled experimental versus field conditions (Rytter et al., 2023).

This study aimed to assess the efficacy of Ugandan probiotic strains in enhancing growth performance, microbiome and immune modulation, and disease resistance of juvenile Nile tilapia (*Oreochromis niloticus*) under experimental conditions. Additionally, the study evaluated their protective efficacy against a virulent local clinical strain of *Providencia* sp., an emerging bacterial pathogen in aquaculture (Fabian Chinedu et al., 2020; Khalim et al., 2021; Rajme-Manzur et al., 2023), while accounting for potential treatment-specific influences on outcomes.

2. Materials and methods

2.1. Probiotic bacterial selection and culture

Indigenous strains of probiotic bacteria, *Priestia megaterium* PMUG01 and *Lysinibacillus fusiformis* LFUG, were isolated from sediment samples collected from earthen Nile tilapia ponds at the Aquaculture Research and Development Center (ARDC), part of the National Agricultural Research Organization (NARO) in Uganda, following the protocol published by Rwezawula, Waiswa Mwanja, et al. (2025). Unlike the commonly used gut-based isolation methods, which are invasive, this sediment-based approach was designed to noninvasively select non-hemolytic, proteolytic-positive, and stress-tolerant (NaCl, heat, and pH) strains with strong quorum-quenching (QQ) and antivirulence capability. Pond sediments were targeted as they harbor diverse, ecologically adapted, and resilient bacterial communities capable of contributing to both fish health and pond ecosystem stability (Niu et al., 2025). Both strains were used at two different concentrations, T1 (10^6) and T2 (10^8) CFU.g^{-1} of feed. They were retrieved from a −80°C freezer, streaked on tryptic soya agar (TSA) (Hi-Media, Mumbai, India), and incubated for 24 h at 30°C. Single colonies were inoculated into 100 mL of tryptic soy broth (TSB) and incubated for 48 h at 30°C. Cultures were then centrifuged at 7000 x g at room temperature for 10 min. The supernatants were discarded, and the pellets were washed three times with sterile saline (0.85 g.L^{-1} - NaCl) before resuspending them in sterile saline to create stocks. The absorbance of the stocks was measured at 550 nm, and the cell density was estimated using McFarland's standard (BioMerieux, France) at a wavelength of 550 nm, assuming an OD_{550} corresponds to 1.2×10^9 CFU.mL^{-1}. This was used to prepare appropriate dilutions in normal saline, corresponding to the required concentrations for feed fortification with the bacteria.

2.2. Experimental diet preparation

The commercial feed (0.5–0.8 mm crumbles; Koudijs, Kampala, Uganda) contained 49 % crude protein, 4 % crude fiber, 6.5 % crude fat, 11 % moisture, 14 % ash, 1.3 % phosphorus, and 1.5 % calcium according to the manufacturer. For all diets, the feed was uniformly mixed with 200 mL.kg^{-1} fish oil (MPUTA, Kampala, Uganda) and sprayed with 200 mL.kg^{-1} of either bacteria suspensions (test treatments) or sterile saline (Control). Treatments were designated as: Control (0 CFU.g^{-1}), PMUGT1 (10^6 CFU.g^{-1}), PMUGT2 (10^8 CFU.g^{-1}), LFUGT1 (10^6 CFU.g^{-1}) and LFUGT2 (10^8 CFU.g^{-1}) (Fig. 1). Diets were air-dried at 30°C for 5 days with daily mixing to prevent caking and then stored in sterile airtight glass bottles at 4°C. Feeds were freshly prepared every three weeks over the 60-day trial. Fish were fed at 4 % of their average body weight (ABW) twice daily, with feeding response monitored.

2.3. Experimental fish

Nile tilapia juveniles (n = 1000) (1.7 ± 0.03 g) were acclimated for two weeks in 15 60-L glass tanks containing 50 L of water. After the acclimation period, fish were assessed for overall health based on physical and sensory indicators, and only those without visible signs of disease, deformity, or injuries were selected for the experiment. For each treatment, 150 healthy fish (50 per replicate, in triplicate) were selected (Fig. 1). Tanks were cleaned twice daily by siphoning debris before feeding, and 70 % of the water was replaced daily with aerated fresh water. Dissolved oxygen, pH, and temperature were monitored daily

throughout the experiment.

2.4. Experimental design of the feeding trial and sampling

Nile tilapia juveniles (n = 750) were randomly assigned to five treatment groups, each in triplicate (50 fish per replicate): Control (no probiotics), LFUGT1, LFUGT2, PMUGT1, and PMUGT2. Fish were stocked in 60-L glass aquaria containing 50-L of water at a rate of 1 fish. L^{-1}.

Fish were monitored daily for 60 days, with various samplings conducted at pre-defined intervals (Fig. 2). Every 10 days, fish (n = 90) were randomly selected from survivors for growth measurements (weight, total length, and standard length). At day 60, surviving fish from each treatment were euthanized (n = 9). Three were processed for proximate analysis, while hindgut tissues from the remaining six were collected for gut microbiome analysis. Ten days post-feeding, hindgut tissues from an additional six fish per group were sampled to assess the persistence of probiotics and microbiome changes after probiotic feeding cessation. During this period, fish were maintained on a basal probiotic-free diet. Peripheral blood (for leukocyte counts and serological assays), spleen, and midgut tissues (for immune gene expression) were collected from sedated fish at day 60. Full details of fish distribution and samplings are presented in Table 1.

2.5. Analysis of growth performance and proximate composition

Growth performance indicators, including weight gain (g), specific growth rate (SGR) (%.day^{-1}), biomass gain (BG-g), coefficient of variation (COV-%), skewness, feed conversion ratio (FCR), condition factor (K) and survival rate (%), were calculated and compared between treatments. At the end of the feeding trial, the viscerosomatic index (VSI) and hepatosomatic index (HSI) were determined and compared between treatments. The growth parameters were calculated according to the following formulae:

1. Weight gain (g) = Final weight (g) − Initial weight (g)
2. Percentage weight gain (%WG) $= \frac{\text{Mean final weight (g)} - \text{mean initial weight (g)}}{\text{Mean initial weight (g)}}$ x 100
3. SGR (%.Day^{-1}) $= \frac{(\text{lnFinal average body weight (g)} - \text{ln Initial average body weight (g)})}{(\text{Time of sampling (Days)})}$ x 100
4. BG = Final biomass(g) − Biomass at stocking(g)
5. COV(%) $= \left(\frac{\text{Standard deviation}}{\text{Average body weight}}\right)$x100
6. Skewness $= \frac{n}{(n-1)(n-2)} \sum \frac{(xi-\bar{x})^3}{s}$ where n is the number of fish, $\bar{x}$ is the sample mean, and s is the standard deviation.
7. Feed Conversion Ratio (FCR) $= \frac{\text{Quantity of feeds fed(g)}}{\text{Fish biomass gained(g)}}$
8. Survival rate (%) $= \frac{\text{Number of fish alive at the end of the experiment}}{\text{Number of fish stocked}}$x100
9. Condition factor (K)= $\left(\frac{\text{Average body weight(g)}}{(\text{Total length(cm)})^3}\right)$ x 100
10. Viscerosomatic Index (VSI) $= \frac{\text{Weight of the viscera(g)}}{\text{Body weight(g)}}$
11. Hepatosomatic Index (HSI) $= \frac{\text{Weight of the liver(g)}}{\text{Body weight(g)}}$

Proximate composition analyses [crude protein (%), crude lipid (%), dry matter (%) and ash (%)] of the feeds and fish (sacrificed) were conducted at the end of the feeding trial (day 60) to determine whether feed supplementation with probiotic bacteria influenced the proximate composition of the feed and fish, according to Horwitz (2006) and Wong et al. (2009). For each treatment, three freshly sacrificed fish were gutted and cleaned upon receipt. Each fish was weighed and dried at 60°C in an oven for 48 h, and the weight change was recorded. Fish were then crushed in a mortar to form a fine powder, from which analyses of crude protein, crude lipid, dry matter, and ash were done. Crude protein was determined using the Kjeldahl method. Crude lipid was determined using the Soxhlet method, while dry matter and ash were determined using a forced air oven method and a muffle furnace method.

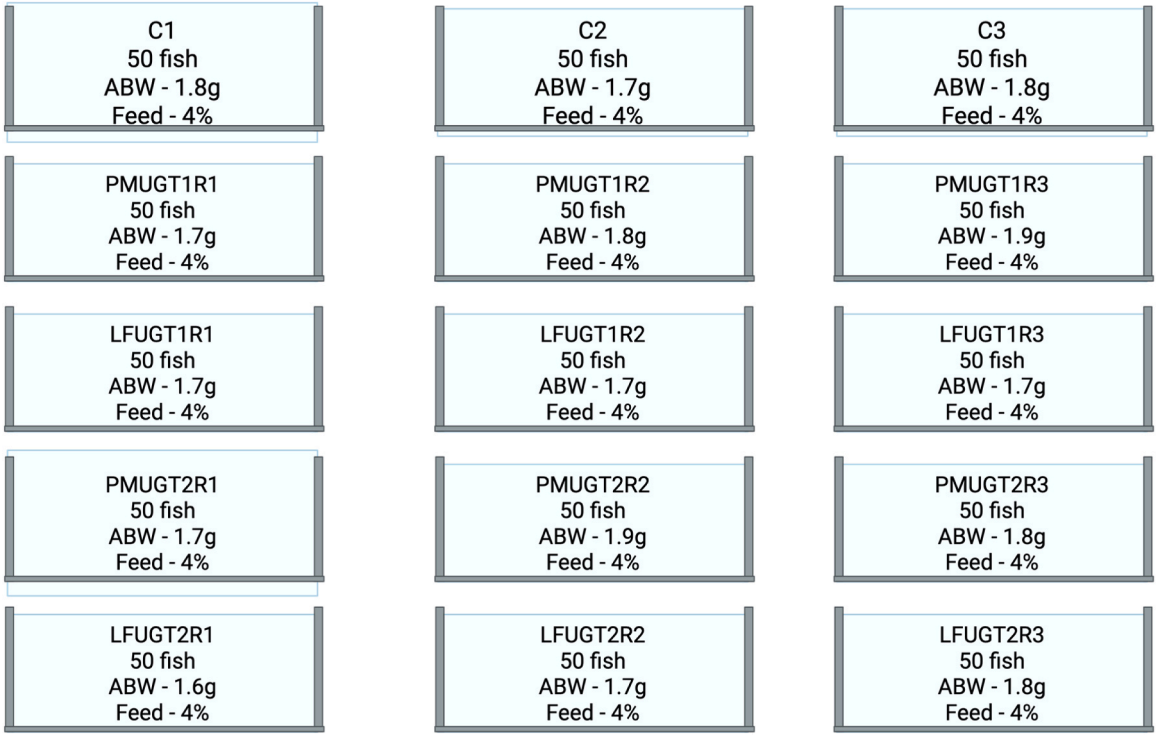

Fig. 1. The experimental design for the 60-day feeding trial with the two indigenous quorum quenching (QQ) probiotic bacterial strains LFUG and PMUG01. Each treatment had three replicates with 50 fish fed at a rate of 4 % of their average body weights (ABW) per day. C1, C2 and C3 represent the control treatments while the probiotic treatments are represented by their respective strain IDs for the two concentrations T1 and T2.

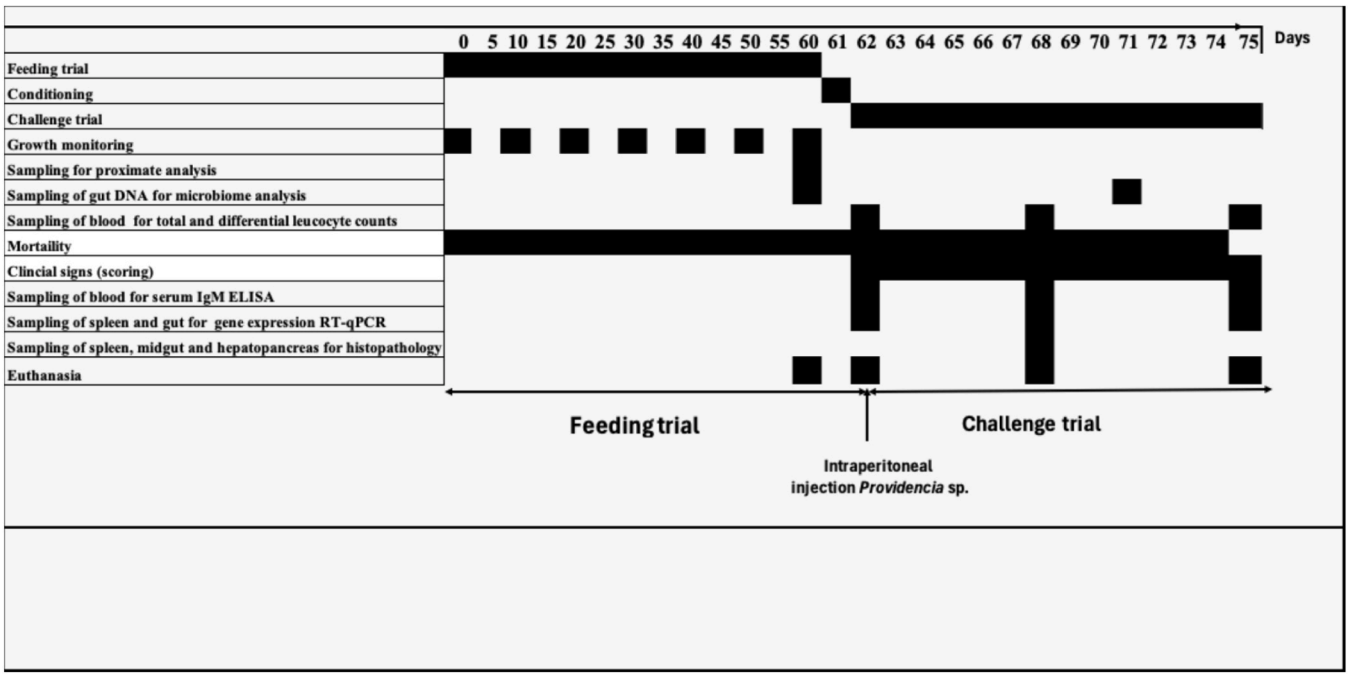

Fig. 2. Schematic presentation of the experimental design of the feeding trial. Five experimental groups including Control, PMUGT1, LFUGT1, PMUGT2 and LFUGT2, all in triplicate were sampled at several time points as indicated on the figure. After the feeding trial, fish were conditioned for a day before the challenge without feeding. 30 fish were randomly selected from each group and were challenged with *Providencia* sp. and monitored for 14 days while collecting different samples for various analyses at time points indicated in the figure. Euthanasia on the remaining fish from both the challenge and feeding trial occurred at the end of the challenge.

Table 1
An overview of the number of fish stocked and used during the different key samplings. Each treatment was set up in triplicate with 50 fish per replicate (150 fish per treatment). Sampling included growth monitoring every 10 days during the 60-day feeding trial and various biological samplings at the end of the trial (Day 60). Sample collection for microbiome analysis was conducted at day 60 (microbiome 1) and 10 days post-trial (microbiome 2) while feeding on a basal, probiotic-free diet.

Parameter	Control	LFUGT1	LFUGT2	PMUGT1	PMUGT2
Number stocked	150	150	150	150	150
Weight & Length	90	90	90	90	90
Proximate analysis	3	3	3	3	3
Microbiome 1	6	6	6	6	6
Microbiome 2	6	6	6	6	6
Leucocyte counts & serology	6	6	6	6	6
Immune gene expression	3	3	3	3	3

2.6. Probiotic effect on the gut microbiome

Genomic DNA was extracted from hindgut tissues using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Briefly, gut samples were collected on ice in sterile 2-mL microcentrifuge tubes, to which 1 mL of InhibitEX Buffer was added and vortexed thoroughly for complete homogenization. The homogenates were centrifuged at 17,000 x g for 1 min at room temperature, and 600 μL of the supernatant was carefully transferred to fresh sterile 2-mL tubes without debris. This supernatant was used for DNA extraction, and the resulting DNA was stored at −20°C until further analysis. DNA yield and quality were assessed prior to targeted full-length 16S rRNA gene sequencing on the PromethION platform (Oxford Nanopore Technologies, Oxford, United Kingdom) at PathoSense, Belgium, where the composition and diversity of the gut microbiome were analyzed and characterized.

Samples yielding over 100,000 reads were randomly subsampled to 100,000 reads. Reads were filtered based on quality and length, and only samples with sufficient reads post-filtering were retained. Rarefaction plots were generated through iterative subsampling to assess sequencing depth, and only samples reaching saturation were included in downstream analyses. Taxonomic classification of reads was conducted by mapping sequences against the SILVA reference database using the EMU algorithm. The relative abundance of all bacterial phyla, families, genera and species was determined. To assess microbial diversity and compositional shifts between treatments at the two sampling points, the Shannon alpha diversity and Bray-Curtis dissimilarity beta diversity metrics were computed. Furthermore, analyses of differentially abundant taxa were conducted to identify significant microbial shifts across treatments and time points.

2.7. Experimental challenge with Providencia sp

2.7.1. Pathogen identification and characterization

A virulent local strain of *Providencia* sp. was obtained from the Department of Biomolecular Resources and Biolab Sciences, College of Veterinary Medicine, Animal Resources and Biosecurity, Makerere University, Uganda. The strain was delivered to the PathoSense laboratory (Belgium) for genomic characterization.

High-molecular-weight DNA was extracted and subjected to long-read nanopore sequencing on a GridION device (Bokma et al., 2021). De novo genome assembly and downstream analyses were performed, assessing genome coverage, GC content, and completeness against a genus-specific database. Species-level identification was based on average nucleotide identity (ANI ≥95–96 %) and digital DNA-DNA hybridization (dDDH ≥70 %) thresholds (Yoon et al., 2017). The genome was screened for virulence and antimicrobial resistance (AMR) genes using the Virulence Factor Database (VFDB; B. Liu et al., 2019) and the comprehensive antibiotic resistance database (CARD; Alcock et al., 2023) in "conserved" mode (≥80 % identity and coverage). The final assembly was deposited in NCBI (Submission ID: SUB14747443;

BioProject: PRJNA1171306; BioSample: SAMN44238307; Accession: CP172972).

2.7.2. Culture conditions

For the challenge, the strain was cultured overnight at 30°C on brain heart infusion agar (BHIA) (Hi-Media, Mumbai, India). Single colonies were inoculated into brain heart infusion broth (BHIB) and incubated at 30°C for 24 h on a shaker (100 rpm). The cultures were centrifuged at 7000 x g for 10 min at room temperature, washed three times with sterile normal saline (0.85 $g.L^{-1}$ NaCl), and resuspended to prepare the infection stock. A relationship between optical density and cell density ($CFU.mL^{-1}$) was established by plating serial dilutions of known absorbance (OD_{550}) on BHIA (30°C, 24 h), enabling dose estimation for the challenge.

2.7.3. The challenge

A total of 180 Nile tilapia juveniles were used for the *Providencia* sp. challenge experiment, with 30 fish allocated to each of the six treatment groups: LFUGT1, LFUGT2, PMUGT1, PMUGT2, and two controls (NC - negative control and PC - positive control) (Fig. 3). The NC and PC groups were randomly selected from the control group used during the probiotic feeding trial. All groups were maintained in triplicate containers with 10 fish per replicate and were fed on a basal, probiotic-free diet throughout the challenge period.

Following a one-day acclimation period after the feeding trial, all groups except the NC were intraperitoneally (IP) injected on Day 62 with 0.2 mL of *Providencia* sp. PSNUG at a concentration of 1.72×10^8 $CFUmL^{-1}$. The NC group received 0.2 mL of sterile normal saline. The challenge dose was selected based on prior LD_{50} determination trials, which revealed a survival rate of (31 ± 11 %), closely approximating the LD_{50} and significantly lower than the unchallenged control ($P < 0.05$). Other tested doses showed the following survival rates: T1 (1×10^6 CFU/Fish) - 95 ± 7 %, T2 (1×10^7 CFU/Fish) - 70 ± 14 %, T4 (1×10^{10} CFU/Fish) - 0 ± 0 %. The uninfected control (0 CFU/Fish) showed a survival of 88 ± 12 %.

Before injection, fish were anaesthetized with clove oil (Naissance, Wales, UK) at 0.1 $mL.L^{-1}$ (v/v) for 5 min and subsequently transferred to well-aerated challenge tanks for recovery. The experiment lasted 14 days, during which morbidity and mortality were monitored and scored. Clinical signs, abnormal behavior, and daily mortalities were recorded. Moribund and freshly dead fish were necropsied to assess disease symptoms. *Providencia* sp. was re-isolated from the liver and spleen on violet-red bile agar (VRBA) to confirm infection. Sampling was conducted at specific time points post-infection to evaluate immune and pathological responses.

2.7.4. Total and differential blood leucocyte counts

Peripheral blood was collected from the caudal vein (*venipunctura caudalis*) of the fish (n = 6) for each treatment at two timepoints: day 7 (T7) and day 14 (T14) post-infection, in addition to the samples collected at the end of the feeding trial before the challenge (T0). To ensure consistency across datasets, leukocyte analyses were conducted using the same procedures applied at the end of the feeding trial. A drop of blood was used to prepare blood smears on clean glass slides, while the remaining portion was transferred into red-top non-heparinized tubes for serum collection. Blood smears were air-dried at room temperature, stored in glass slide holders, and stained with Hemacolor (Sigma-Aldrich, Darmstadt, Germany). Total and differential leucocyte count analyses were performed under a light microscope following established methodologies (Antache et al., 2014; Corrêa et al., 2017; De et al., 2003; Khunrang et al., 2023; Wang et al., 2021).

2.7.5. Serological analysis

Serum samples were collected from the portion of peripheral blood stored in the red-top non-heparinized tubes for the same time-points as leucocyte counts (T0, T7 and T14). They were used to quantify Immunoglobulin M (IgM) using a fish-specific IgM ELISA Kit (MyBiosource, San Diego, USA), following the manufacturer's guidelines. Samples were diluted (20x) and dispensed in a 96-well plate supplied with the kit. A standard curve from IgM standards (800–25 $\mu g.mL^{-1}$) was used to

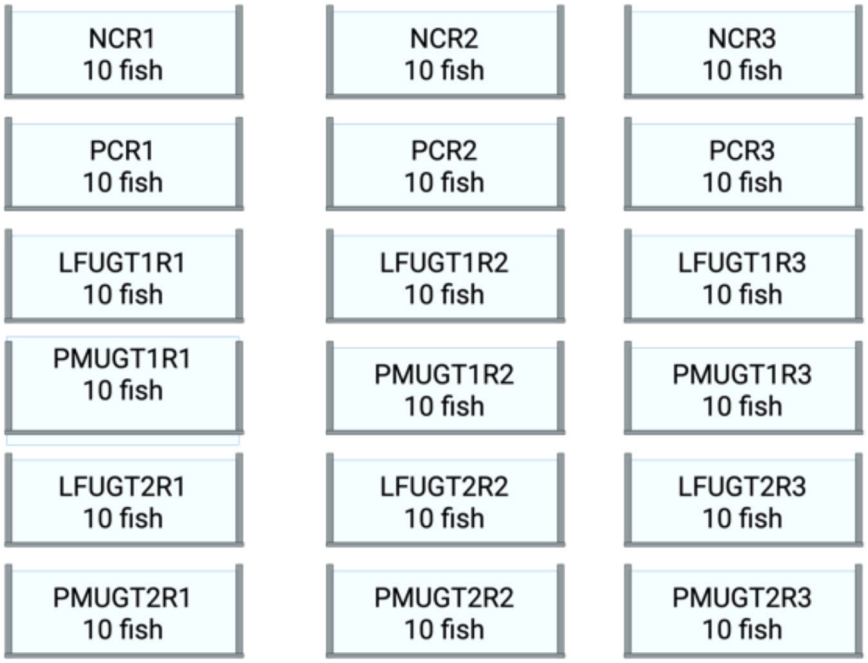

Fig. 3. The experimental design for the challenge assay with a virulent clinical *Providencia* species. Treatment groups included the negative (NC) and positive (PC) controls, LFUGT1, PMUGT1, LFUGT2 and PMUGT2. For each treatment, 3 replicates, each with 10 healthy fish with no signs of disease, deformity or injury randomly picked from the feeding trial treatments were used. The NC and PC contained fish from the feeding trial control treatment (no probiotics). All treatments were intraperitoneally infected with the pathogen except the NC, which was intraperitoneally injected with an equal volume of sterile normal saline.

determine IgM concentrations across treatment groups.

2.7.6. Immune gene expression analysis

2.7.6.1. Tissue collection and RNA preservation. Spleen and midgut tissues were collected from three fish per treatment group following the same schedule as peripheral blood collection (T0, T7 and T14). Immediately after dissection, tissues were preserved in 1.5-mL RNAlater tissue protect tubes (Qiagen, Benelux, Belgium), kept at 4°C for 24 h, then stored at −20°C until RNA extraction. Consideration of the spleen and midgut tissues for this study aligned with previous probiotic efficacy studies in Nile tilapia (Abarike et al., 2018; Cao et al., 2021; Dawood et al., 2024; Etyemez Büyükdeveci et al., 2023; Hashem et al., 2022; Ibrahim et al., 2021).

2.7.6.2. RNA extraction and purification. Total RNA was extracted using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's guidelines with minor modifications. Frozen tissues were thawed on ice, homogenized in RLT buffer containing β-Mercaptoethanol (10 μL.mL^{-1}; Sigma-Aldrich, Darmstadt, Germany) using a Kimble Pellet Pestle Motor for 2 min. They were centrifuged at 15,000 x g for 3 min (Awel-MF-20R, Blain, France). The supernatants were mixed with equal volumes of 70 % ethanol (Chem-lab nv, Belgium), transferred into RNeasy spin columns, and centrifuged at 15,000 x g for 30 s.

RNA samples were treated with RNase-Free DNase (Qiagen, Hilden, Germany) to eliminate residual genomic DNA. For purification, spin columns were washed twice with 500 μL of RPE buffer (prepared with 100 % ethanol), with centrifugation at 15,000 x g for 30 s (and 2 min for the second wash). Columns were then dried, and RNA was eluted with 30 μL of RNase-free water. RNA quality and concentration were measured using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, Delaware, USA). RNA samples with $A_{260/280}$ ratios between 1.9 and 2.2, and $_{A260/230}$ ratios between 1.7 and 2.2, were considered of acceptable purity and used for cDNA synthesis.

2.7.6.3. cDNA synthesis. First-strand cDNA was synthesized using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, Vilnius, Lithuania) in a 20 μL reaction containing: up to 1 μg total RNA, 4 μL 5X Reaction buffer, 1 μL Ribolock RNase Inhibitor (20 U·μL^{-1}), 2 μL dNTP mix (10 mM), 1 μL RevertAid H Minus M-MuLV Reverse Transcriptase (200 U·μL^{-1}), and nuclease-free water. Thermal cycling conditions were as follows: 25°C for 5 min (primer annealing), 42°C for 60 min (reverse transcription), and 70°C for 5 min (enzyme inactivation). The resulting cDNA was diluted with 180 μL of nuclease-free water to a final volume of 200 μL.

2.7.6.4. Quantitative real-time PCR (qPCR) and gene expression analysis. Relative expression of immune-related genes: complement component *C3*, interleukin-1 beta (*IL1β*), chemokine (C-X-C motif) ligand 8 (*CXCL8*), and interleukin-6 (*IL6*) was quantified in spleen and midgut samples using Maxima SYBR Green/ROX qPCR Master Mix (2X) (Thermo Scientific, Vilnius, Lithuania). Primer sequences and their sources are listed in Table 2. Reference genes elongation factor 1-alpha (*EF1α*), *Beta-actin* 1 & 2 were selected as the internal controls due to their previously validated expression stability in Nile tilapia under probiotic treatment and bacterial challenge conditions (Dawood et al., 2024; Standen et al., 2016; R. Xu et al., 2022; C. G. Yang et al., 2013). In our study, EF1α, Beta-actin 1 & 2 consistently exhibited minimal variation in CT values across all treatments and timepoints, supporting their suitability as stable reference genes for normalization.

Each 20 μL qPCR reaction contained: 12.5 μL SYBR Green/ROX Master Mix, 2 μL cDNA, 1 μL of each primer, and 3.5 μL nuclease-free water. The thermal cycling conditions included: 50°C for 2 min (pre-heating), 95°C for 10 min (initial denaturation), followed by 40 cycles of 95°C for 15 s (denaturation) and 60°C for 60 s (annealing/extension).

Table 2
Oligonucleotide primer sequences of the targeted genes and reference studies from which they were retrieved.

Target gene	Primer sequences (5' - 3')	Source
C3	F - GGTGTGGATGCACCTGAGAA R - GGGAAATCGGTACTTGGCCT	(Dawood et al., 2024)
IL-1β	F - TCAGTTCACCAGCAGGGATG R - GACAGATAGAGGTTTGTGCC	(Dawood et al., 2024)
CXCL8	F - CTGTGAAGGCATGGGTGTGGAG R - TCGCAGTGGGAGTTGGGAAGAA	(Dawood et al., 2024)
IL6	F - TAGAGAAGGAGTACCGCAGCA R - TGTGTGGTGTGTGGTTGTTTTG	(Cao et al., 2021)
EF1	F - TCAACGCTCAGGTCATCATC R - ACGGTCGATCTTCTCAACCA	(Dawood et al., 2024)
Beta-actin 1	F-TGACCTCAGACTACCTCATG R-TGATGTCACGCACGATTTCC	(Ren et al., 2020)
Beta-actin 2	F-ACAACCACACACCACACATTTC R-TGTCTCCTTCATCGTTCCAGTTT	(Abarike et al., 2018)

Primer amplification specificity and efficiency were assessed using melting curve analysis and standard curves generated from tenfold serial dilutions (neat to 10^{-4}) of pooled cDNA from all treatment groups.

Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method (R. Liu et al., 2024), comparing each sample to the T0 control group and normalizing against the reference genes.

2.7.7. Histopathology

Histopathological analysis was conducted on hepatopancreas, spleen, and midgut tissues collected from three fish per treatment group on day 7 post-challenge. Before fixation, gut tissues were flushed with normal saline. All samples were fixed in 10 % buffered formalin for 48 h in the dark, then rinsed and stored in 70 % ethanol. Tissues from each group were embedded in a single paraffin block, sectioned at 5 μm, and stained with Hematoxylin-Eosin (H&E) for microscopic evaluation. Histological assessment was performed collectively for the samples in each block, and results were reported as mean and median lesion scores per group.

Histological changes were graded based on severity according to Steckert et al. (2018) as follows: 0 = absent, 1 = minimal, 2 = slight, 3 = moderate, 4 = marked, and 5 = severe. In cases where grading was not applicable, lesions were recorded as present or absent.

2.8. Ethical approval

The study was conducted following an approved protocol (**CAES-REC-2024–79**) by the Research Ethics Committee of the College of Agricultural and Environmental Sciences, Makerere University, Uganda.

2.9. Statistical analyses

Prior to all statistical analyses, assumptions of normality and homogeneity of variances were tested to determine the appropriate statistical tests. Growth performance, serum IgM levels, and total and differential leukocyte counts, and immune gene expression data were analyzed using ANOVA (not assuming equal variances), followed by pairwise *t*-tests with pooled standard deviations where applicable. Weight distribution patterns over time were visualized using a heatmap. Survival data were evaluated using the Kaplan-Meier method with log-rank pairwise comparisons. The effect of probiotic supplementation on *Providencia*-induced mortality risk was further assessed using a Cox Proportional-Hazards Model, with the positive control serving as the reference group. Proximate nutrient composition and histopathological median scores per parameter were analyzed using the Kruskal-Wallis rank sum test. Where significant differences were detected, post-hoc pairwise comparisons were performed using Dunn's test with Holm correction. Overall patterns of tissue integrity were assessed using Bray-Curtis dissimilarity and visualized with Principal Coordinates Analysis

(PCoA) and a median-score heatmap. Microbiome alpha diversity (Shannon index) was compared using the Wilcoxon rank sum test. Beta diversity was assessed using the Bray-Curtis dissimilarity index and visualized via Principal Coordinates Analysis (PCoA). Differences in microbial community composition between time points were tested using PERMANOVA with 9999 permutations. Linear discriminant analysis effect size (LEfSe) was used to identify differentially abundant taxa across treatments and time points, with a significance threshold of LDA score > 2.0. All analyses were conducted at a 95 % confidence interval.

3. Results

3.1. Growth performance and proximate nutrient composition

Physiochemical water quality parameters remained within optimal ranges throughout the experiment (DO - 6.1 ± 0.3 mg.L^{-1}, Temperature - 24.7 ± 1.3 °C and pH - 8.2 ± 0.1). Growth performance parameters are summarized in Table 3.

Fish fed on LFUG and PMUG01-supplemented diets showed significantly improved weight gain than the control (307.1 ± 28.7 %) by day 60. Among probiotic treatments, PMUGT2 achieved the highest weight gain (451.9 ± 58.8 %), followed by LFUGT2 (426.5 ± 7.8 %), which significantly outperformed LFUGT1 (380.7 ± 39.9 %) but not PMUGT1 (386.3 ± 26.9 %). No significant difference was observed between PMUGT1 and LFUGT1. All probiotic treatments significantly increased biomass gain compared to the control ($P < 0.05$).

Table 3
Growth performance indicators of Nile tilapia juveniles after treatment with a commercial feed supplemented with the two putative probiotic strains LFUG and PMUG01 at two different concentrations T1 and T2 for 60 days. The concentrations T1 and T2 were 1×10^6 and 1×10^8 CFU.g^{-1} of feed. Data is presented as means±standard deviation, and values in the same row with different superscripts depict significant differences between the respective treatments. IABW – initial average body weight (g); FABW – final average body weight (g); AWG – average weight gain (g/%); BG – biomass gain (g); SGR – specific growth rate (%.day^{-1}); K – condition factor; COV – Coefficient of variation (%); FCR – feed conversion ratio; S – survival rate (%); HSI – hepatosomatic index; and VSI – viscerosomatic index.

Metric	Control	PMUGT1	PMUGT2	LFUGT1	LFUGT2
IABW	1.763 ± 0.032	1.786 ± 0.072	1.770 ± 0.096	1.688 ± 0.024	1.714 ± 0.058
FABW	7.179 ± 0.584	8.686 ± 0.626	9.797 ± 1.508	8.115 ± 0.720	9.025 ± 0.411
AWG	5.417 ± 1.988^{a}	6.901 ± 1.715^{bc}	8.034 ± 2.316^{e}	6.429 ± 1.590^{b}	7.305 ± 1.276 ^{cd}
%WG	307.1 ± 28.7^{a}	386.3 ± 26.9^{bc}	451.9 ± 58.8^{e}	380.7 ± 39.9^{b}	426.5 ± 7.8 ^{cd}
BG	243.5 ± 23.0^{a}	304.4 ± 38.7^{b}	342.3 ± 39.2^{b}	294.9 ± 25.6^{b}	336.4 ± 32.9^{b}
SGR	2.428 ± 0.285^{a}	2.728 ± 0.288^{b}	3.027 ± 0.490^{b}	2.616 ± 0.318^{ab}	2.867 ± 0.289^{a}
K	3.127 ± 0.654^{a}	3.000 ± 0.540^{a}	3.034 ± 0.660^{a}	3.034 ± 0.567^{a}	3.009 ± 0.581^{a}
COV	27.7 ± 3.2^{a}	22.6 ± 4.0^{b}	26.7 ± 5.5^{a}	22.8 ± 4.5^{b}	22.2 ± 5.9^{b}
FCR	1.282 ± 0.192^{a}	1.112 ± 0.151^{b}	1.050 ± 0.172^{b}	1.167 ± 0.158^{a}	1.056 ± 0.100^{b}
S	90 ± 2^{a}	88 ± 4^{a}	86 ± 8^{a}	92 ± 5^{a}	92 ± 7^{a}
HSI	9.063 ± 1.460^{a}	9.179 ± 1.726^{a}	8.589 ± 1.131^{a}	9.079 ± 1.246^{a}	8.199 ± 1.372^{a}
VSI	1.568 ± 0.507^{a}	1.448 ± 0.515^{a}	1.518 ± 0.254^{a}	1.446 ± 0.402^{a}	1.341 ± 0.497^{a}

Weight gain was assessed at 10-day intervals across treatments (Table 4). By day 10, only PMUGT2 showed a significant weight gain over the control ($P = 0.008$). By day 20, all probiotic treatments except LFUGT1 had significantly enhanced weight gain. This trend persisted through day 50, at which point LFUGT1 also showed significant improvement compared to the control ($P = 0.001$), up to the end of the trial.

Table 4
The mean weight gain changes in grams over the different sampling intervals for the different treatments. Data is represented as means±standard deviations of the weight gain. Values in the same row with different superscripts represent statistically significant differences compared to the control at a given time point.

Time (days)	Control	PMUGT1	PMUGT2	LFUGT1	LFUGT2
10	0.440 ± 0.573^{a}	0.495 ± 0.545^{a}	0.683 ± 0.76^{b}	0.466 ± 0.56^{a}	0.586 ± 0.685^{a}
20	0.958 ± 0.788^{a}	1.234 ± 0.676^{b}	1.328 ± 0.993^{b}	0.947 ± 0.616^{a}	1.206 ± 0.732^{b}
30	1.929 ± 1.102^{a}	2.214 ± 0.934^{b}	2.427 ± 1.304^{b}	2.505 ± 0.805^{a}	2.149 ± 0.811^{b}
40	2.890 ± 1.377^{a}	3.388 ± 1.319^{b}	3.947 ± 1.738^{b}	2.954 ± 1.136^{a}	3.362 ± 1.189^{b}
50	4.407 ± 1.817^{a}	5.441 ± 1.407^{b}	6.046 ± 2.127^{b}	5.286 ± 1.236^{b}	5.649 ± 1.253^{b}
60	5.417 ± 1.988^{a}	6.901 ± 1.715^{b}	8.034 ± 2.316^{b}	6.429 ± 1.590^{b}	7.305 ± 1.276^{b}

Cumulative survival trends across treatments were similar, with no significant differences observed (Table 3). Final survival rates ranged from (86 ± 8 %) in PMUGT2–92 % in both LFUG treatments, with intermediate values for PMUGT1 (88 ± 4 %) and the control (90 ± 2 %) (Supplementary Fig. S2 & Table 3).

Growth uniformity, assessed by the coefficient of variation (COV), differed significantly across treatments and time ($p < 0.05$) (Table 3 and Fig. 4A). The control group had the highest average COV (27.7 ± 3.2 %), while LFUGT2, LFUGT1, and PMUGT1 had significantly lower and comparable COVs (22.2–22.8 %). PMUGT2 showed a moderately high COV (26.7 ± 5.5 %), not significantly different from the control ($P = 0.961$). Except for PMUGT2, all probiotic treatments significantly improved growth uniformity ($P < 0.05$), as also reflected in the skewness coefficient heatmap (Fig. 4B).

No significant differences were observed among treatments in condition factor ($P > 0.05$), viscerosomatic index (VSI) ($P = 0.715$), hepatosomatic indices (HSI) ($P = 0.924$), or survival rate ($P > 0.05$). Specific growth rate (SGR) and feed conversion ratio (FCR) were significantly improved in all probiotic-fed groups compared to the control, except in the LFUGT1 treatment ($P = 0.388$; 0.454) (Table 3 and Supplementary Fig. S1).

There were no significant differences in the proximate nutrient composition of the experimental diets compared to the control (data not shown). Similarly, analysis of whole-body nutrient composition in fish showed no significant differences among treatments for all measured nutrients ($X^2 = 7.875$, df = 4, $P = 0.096$) (Supplementary Table S1).

3.2. Probiotic effect on the gut microbiome

The number of unique and shared species counts across the different treatments at the sampled time points are presented in Fig. 5. Alpha diversity, measured as the Shannon species diversity was assessed at the end of the 60-day feeding trial, and 10 days post-trial, after switching all treatments to a basal, non-supplemented diet. No significant differences were observed at day 60 ($P = 0.052$) (Fig. 6A). However, significant differences emerged 10 days post-feeding ($P = 0.002$), particularly between the control and PMUGT1 ($P = 0.043$) (Fig. 6B). Within-treatment changes between the two time-points were significant for only LFUGT2 ($P = 0.004$) and PMUGT1 ($P = 0.041$).

The Beta diversity (Bray-Curtis dissimilarity) also revealed no significant differences between the control and all probiotic groups at day 60 (Fig. 7A). At day 10 post-feeding trial, clear dissimilarities between the control and all probiotic treatments were observed except PMUGT1 (Fig. 7B). Furthermore, significant shifts in microbial communities over the two time-points were noticed for LFUGT1, LFUGT2, and PMUGT1, but not for the control or PMUGT2. This contrasts with alpha diversity findings, which only identified differences between the control and PMUGT1 at day 10 post feeding trial. After the feeding trial,

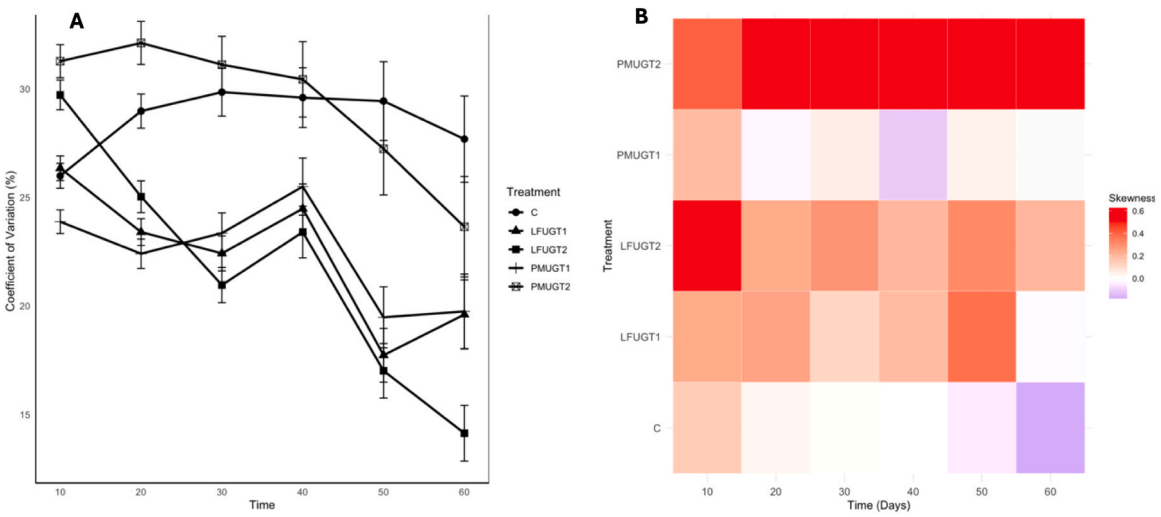

Fig. 4. Changes in weight distribution symmetry of fish over a 60-day feeding trial with probiotic-supplemented feeds. Panel A shows the mean coefficients of variation in body weight, while Panel B presents the skewness coefficients across different treatments at six sampling intervals.

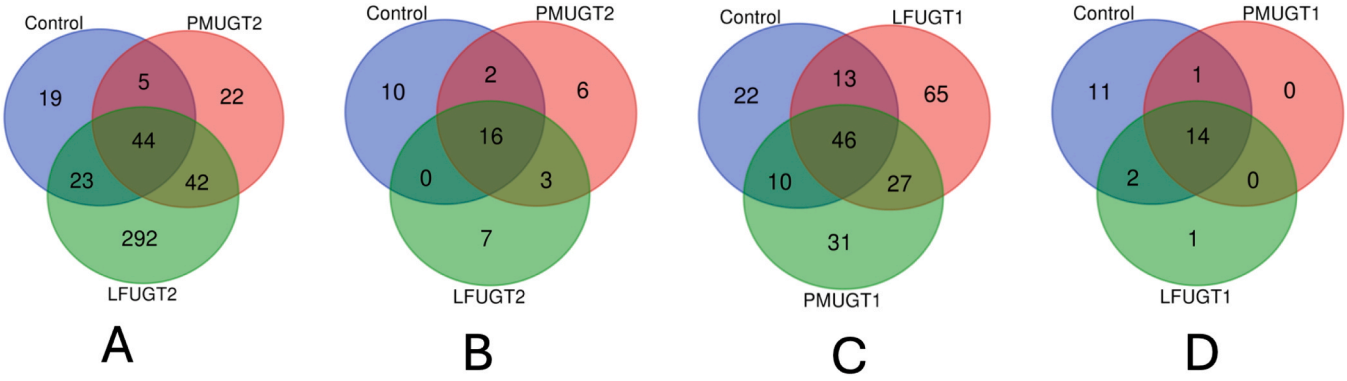

Fig. 5. Counts of the unique and shared species of the different treatments at the two time points. Panels A and C denote comparisons between the control and the probiotic treatments after the 60 days of the feeding trial. Panels B and D represent comparisons between the control and the probiotic treatments fed on a basal diet for 10 days after cessation of probiotic feeding.

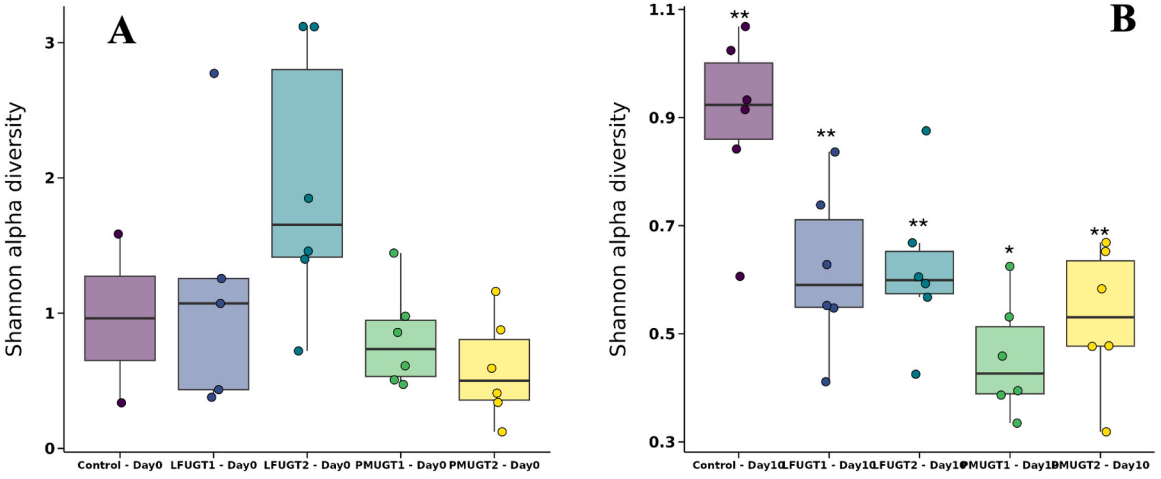

Fig. 6. Species-level α-Shannon diversity indices of the control and probiotic treatments at the end of the feeding trial (**Day 0**, Panel A) and ten days after cessation of probiotic feeding while fish were maintained on a basal diet without probiotics (**Day 10**, Panel B). Plots marked with different numbers of asterisks relative to the control indicate significant differences.

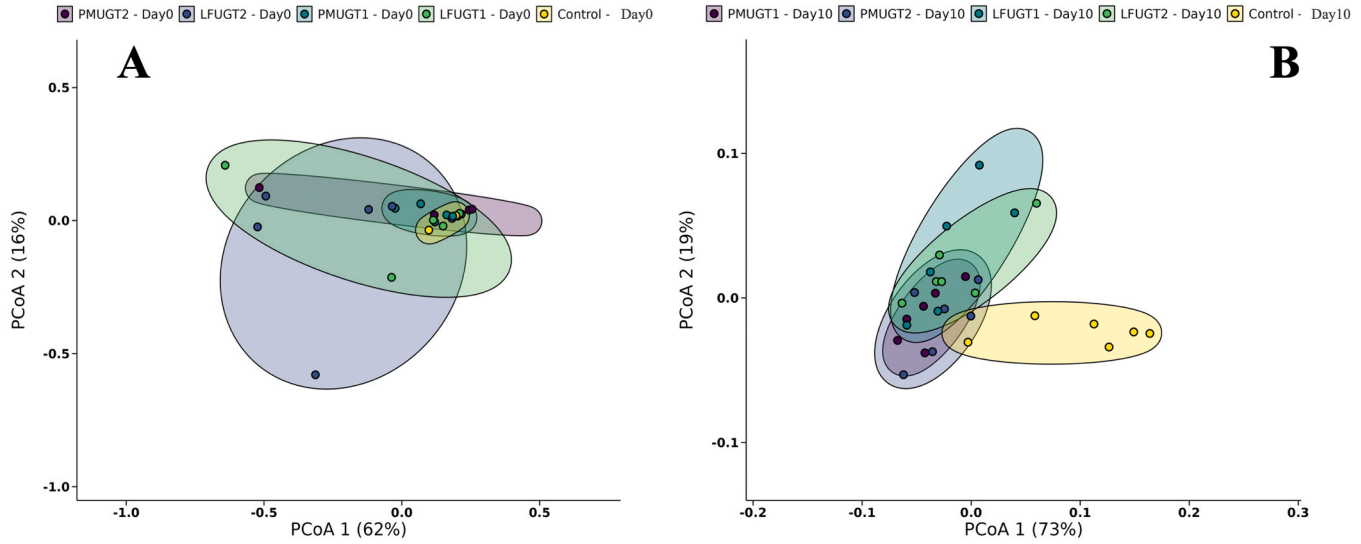

Fig. 7. Principal Coordinates Analysis (PCoA) plots based on Bray-Curtis dissimilarity indices showing differences in bacterial community composition (species level) between the control and probiotic treatments after the feeding trial (**Panel A - Day 0)** and ten days after cessation of probiotic feeding while fish were maintained on a basal diet without probiotics (**Panel B - Day 10**). Closer clustering indicates greater similarity between communities. No significant differences were observed at the end of the feeding trial (Day 0), whereas significant differences emerged between the control and all probiotic groups after ten days on a probiotic-free basal diet (Day 10).

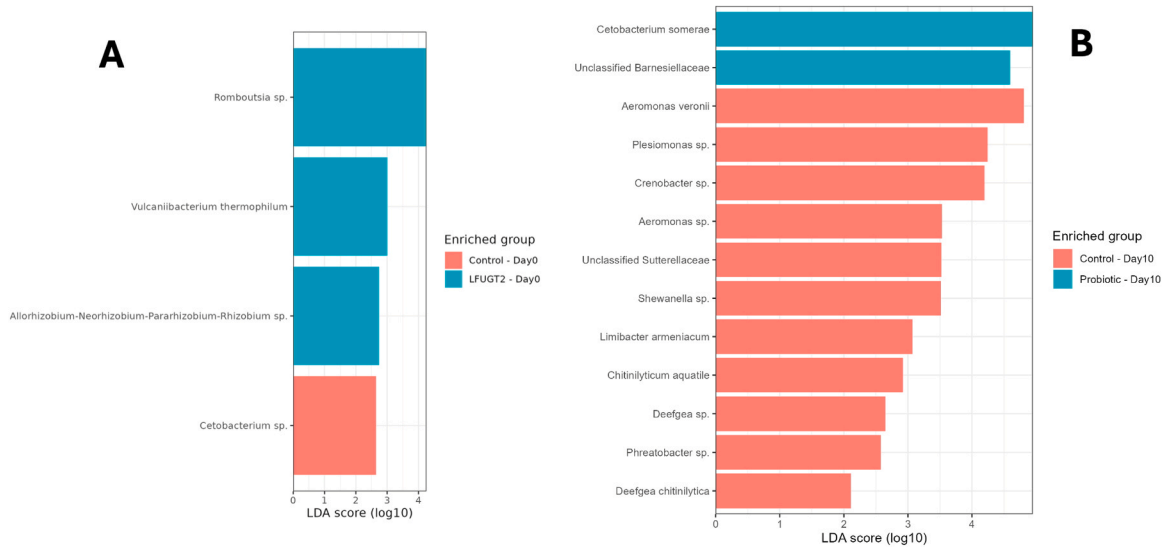

Fig. 8. Significantly differentially abundant bacterial species between the control and probiotic groups at the end of the feeding trial (**Panel A - Day 0**) and ten days after cessation of probiotic feeding while fish were maintained on a basal diet without probiotics (**Panel B - Day 10**). Only treatments showing species with significantly higher abundance than the control at either time point are presented.

differentially abundant species were only noticed between the control and LFUGT2 (Fig. 8A). LFUGT2 was enriched with *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* sp.., *Romboutsia* sp., and *Vulcaniibacterium thermophilum*. *Lysinibacillus* sp. was detected in the gut Nile tilapia juveniles fed in LFUGT2 group. In contrast, the control group was dominated by *Cetobacterium* sp. (Fig. 8A). Ten days post feeding trial, the probiotic-treated groups were significantly dominated by *Cetobacterium somerase* and an unclassified *Barnesiellaceae* sp. (Fig. 8B), whereas the control was dominated by *Aeromonas* sp., *Aeromonas veronii*, *Plesiomonas* sp., *Shewanella* sp., *Crenobacter* sp., and other taxa, as shown in Fig. 8B.

3.3. The challenge test

3.3.1. Molecular identification and characterization of the pathogen

Sequencing throughput from the GridION device revealed 528,522 reads, 11,740 N50 (bp)* value, and an output of 3219,017,042 bases. The final consensus genome was composed of a GC content (40.06 %), genome completeness (98.06 %), Genome size (4505,680 bp) and 3 contigs (1 chromosomal and 2 plasmid) with a coverage of 636x. The ANI (82.50 %) and dDDH (25.5 %) values indicated that the strain of this study belonged to the genus *Providencia* but could not be assigned a species-level identification when compared to the established threshold values of ANI (95–96 %) and dDDH (70 %). The closest type-strain genome belonged to *Providencia rettgeri* NCTC 11801. Therefore, it was tentatively named *Providencia* sp. strain PSNUG, awaiting further confirmation of its novelty and final declaration. The strain exhibited 4082, 22, 77 and 1 predicted coding DNA sequences (CDS), rRNA, tRNA and tmRNA respectively. Antimicrobial resistance gene screening revealed two known AMR genes (*CRP* - 81.83 % and *tet* (59) - 99.33. On the other hand, virulence gene screening in “conserved” mode

discovered one virulence gene (*sycB* – 80 %). The final genome assembly was submitted to NCBI under submission ID (SUB14747443), Bioproject (PRJNA1171306) and accession number (CP172972-CP172974).

3.3.2. Clinical signs and mortality

The daily and final survival rates, along with the observed clinical signs are presented in Fig. 9. Moribund fish were observed, especially in the positive control group, exhibiting clinical signs such as fin rot/erosion, fin base hemorrhages, skin ulcerations, scale loss, exophthalmia, belly distention, reddening and protrusion of the anus, and ascites. Necropsy also revealed oedema of the spleen and macroscopic lesions within the liver with hemorrhages. Acute infection, characterized by mortality within the first 24 h of challenge without showing distinct clinical signs except lethargy, was also observed. Comparing survival rates across treatments, there were significant differences between the negative control (NC) (97 ± 6 %) and positive control (PC) (35 ± 22 %) treatments ($P = 0.00046$), but not the other probiotic treatments (Fig. 9). Even though the NC treatment exhibited the highest survival rate, there was no significant difference compared to LFUGT2 and PMUGT2 (90 ± 10 %), PMUGT1 (87 ± 6 %), and LFUGT1 (83 ± 12 %). There were no significant differences in survival rates between the probiotic treatments (Fig. 9). Moreover, the Cox proportional hazards regression analysis revealed significant differences in hazard ratios among the treatment groups, with the PC as the reference group (Table 5). As expected, the NC (uninfected) exhibited the most substantial risk reduction (95.24 %), with the lowest significant hazard ratio (0.049), followed by LFUGT2 and PMUGT2 (0.148), PMUGT1 (0.197), and LFUGT1 (0.248). These results suggest a substantial reduction in *Providencia* sp.-induced mortality risk with PMUG01 and LFUG feed supplementation, irrespective of concentration (T1 or T2).

3.3.3. Immune parameter analysis

3.3.3.1. Total and differential blood leucocyte counts. Figs. 10 and 11, and Supplementary Table S2-S3 summarize total and differential leukocyte counts in Nile tilapia fed probiotic diets for 60 days and challenged with virulent *Providencia* sp. Blood samples were taken on day 0 (pre-challenge), and on days 7 and 14 post-challenge.

At day 0, all probiotic treatments significantly increased total leukocyte counts (TLCs), neutrophils, lymphocytes, monocytes, and basophils compared to both controls ($P < 0.05$).

By day 7, TLCs in probiotic groups dropped below the negative control, except in PMUGT2 ($P = 0.183$). Neutrophils and lymphocytes were also lower in all probiotic groups versus the negative control, with PMUGT2 maintaining higher levels. The positive control showed intermediate values. Monocyte and basophil counts were similarly reduced in all probiotic groups, except PMUGT2, which remained comparable to the negative control.

By day 14, TLCs rose across all groups, with probiotic treatments and the positive control exceeding the negative control. PMUGT2 had the highest TLCs, followed by PMUGT1, LFUGT2, and LFUGT1. Neutrophils and lymphocytes remained lower in all probiotic groups compared to the negative control, with PMUGT2 showing relatively higher values.

Table 5
The Cox regression analysis showing the % reduction in mortality by Juvenile Nile tilapia from a virulent clinical strain of *Providencia* sp. following a 60-day feeding trial with a commercial diet supplemented with our putative probiotic strains LFUG and PMUG01 at concentrations T1 and T2, using the positive control as the reference treatment for risk progression. The concordance index (0.779), likelihood ratio test ($\chi^2 = 30.61$, df = 5, $P < 0.00001$), Wald test ($\chi^2 = 28.93$, df = 5, $P < 0.00002$), and Score (logrank) test ($\chi^2 = 40.53$, df = 5, $P < 0.0000001$).

Treatments	Regression coefficients	Hazard ratios	% Risk reduction	z-score	P (>|z|)
LFUGT1	-1.393	0.248	75.179	-2.771	0.006
LFUGT2	-1.913	0.148	85.238	-3.079	0.002
NC	-3.021	0.049	95.124	-2.944	0.003
PMUGT1	-1.622	0.197	80.256	-2.948	0.003
PMUGT2	-1.910	0.148	85.185	-3.073	0.002

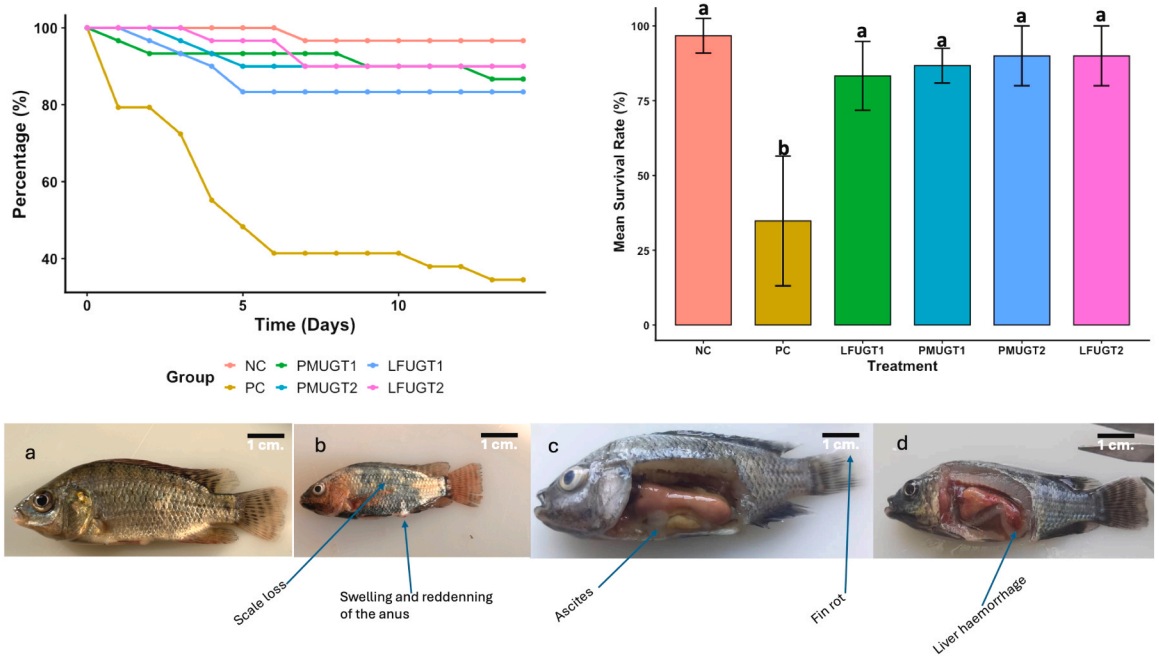

Fig. 9. The cumulative and final survival rates of juvenile Nile tilapia 14 days post infection with a clinical and virulent strain of *Providencia* sp. Data is presented as means±standard deviations and bars with different superscripts denote treatments with significant differences between each other. PC and NC represent the Positive and Negative control treatments. Image a represents normal fish from the NC while b, c and d represent fish from challenged treatments that exhibited clinical signs post *Providencia* sp. infection. Other signs such as oedema of the spleen, fin base hemorrhages, lethargy and abnormal swimming patterns were also observed in moribund fish.

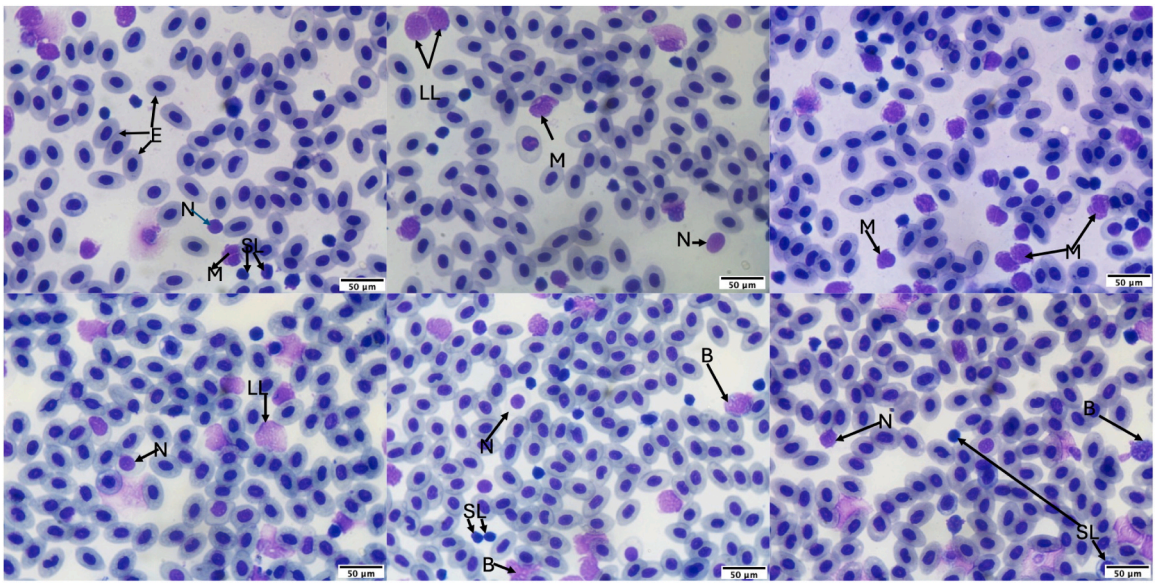

Fig. 10. Leucocytes of juvenile Nile tilapia (*Oreochromis niloticus*) viewed on smears stained with Hemacolor under a light microscope under 1000X magnification. E: erythrocytes; N: neutrophils; SL: small lymphocytes; M: monocytes; B: basophils; and LL: large lymphocytes.

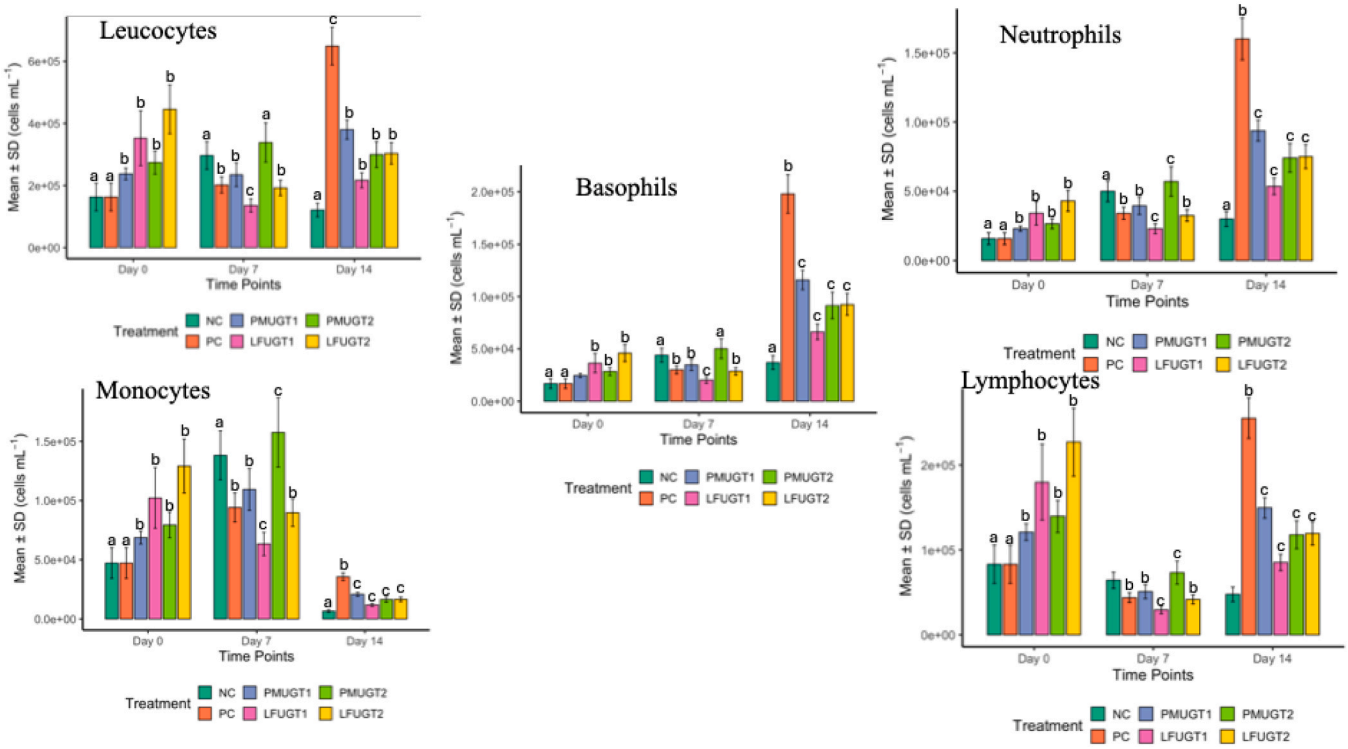

Fig. 11. Changes in Total Leucocyte Counts and Differential Leucocyte Counts in the peripheral blood of Nile tilapia juveniles fed on the different feed treatments and challenged with a clinical virulent strain of *Providencia* sp. Peripheral blood was sampled at the end of the feeding trial before the challenge (Day 0), 7 (Day 7) and 14 (Day 14) days after the challenge. Data is represented as the means±standard deviations (n = 6) and bars with different superscripts designate treatments that showed significant differences compared to the negative and positive controls.

Monocyte and basophil counts were elevated in all probiotic groups versus the negative control but remained below the positive control. LFUGT1 consistently had the lowest immune cell counts among probiotic treatments.

3.3.3.2. Serology. No significant differences in IgM levels were detected among treatments on days 0, 7 and 14 ($P < 0.05$). However, at day 0, all probiotic treatments exhibited elevated IgM levels compared to the controls (355.5 µg.mL^{-1}), except LFUGT2 (322.8 µg.mL^{-1}). By day 7, the IgM levels were comparable across most treatments (411.5–450.2 µg.mL^{-1}), with LFUGT1(325.5 µg.mL^{-1}) and LFUGT2 (328.8 µg.mL^{-1}) treatments showing slightly lower levels. Surprisingly, on day 14, LFUGT2 demonstrated an increase in IgM levels in LFUGT2 (596.8 µg.mL^{-1}), surpassing all other treatments, including the positive (531.5 µg.mL^{-1}) and negative control (468.8 µg.mL^{-1}). The remaining treatments showed minimal changes from day 7 to day 14.

3.3.3.3. Immune gene expression. Expression of *IL6*, *CXCL8*, *IL1β*, and *C3* was evaluated in the spleen and midgut of Nile tilapia juveniles fed probiotic-fortified diets after the feeding trial before the challenge (Day 0), and post-challenge (Days 7 and 14). Primer specificity and efficiency are presented in Supplementary Fig. S3. Results are summarized in Figs. 12 and 13 and Supplementary Table S4-S5.

3.3.3.3.1. Pre-challenge (Day 0). In the spleen, all immune genes were either downregulated or unchanged compared to the controls. In the midgut, *CXCL8* was markedly upregulated in PMUGT1 (~7-fold) and LFUGT2 (~1.4-fold). *IL1β* was significantly upregulated in all groups except PMUGT2, while *IL6* increased only in PMUGT1 (~3-fold) and LFUGT2 (~6-fold). *C3* was upregulated in PMUGT1 (~9-fold) and LFUGT1 (~1.3-fold) but downregulated in PMUGT2 and LFUGT2.

3.3.3.3.2. Early post-challenge (Day 7). In the spleen, the PC group showed strong upregulation of most markers (3–5-fold) compared to the NC group, except *C3*. This was matched or exceeded in probiotic-fed fish for *CXCL8*, except PMUGT1. *IL6* was highest in LFUGT2 (~7-fold), while in other groups it was significantly downregulated. *IL1β* was elevated in LFUGT1 and PMUGT2 but suppressed in PMUGT1 and LFUGT2. *C3* expression remained unchanged relative to NC but was lower in LFUGT1 and PMUGT2 compared to PC. In the midgut, *CXCL8* and *IL6* were significantly upregulated in all probiotic groups (vs PC), except LFUGT1. *IL1β* and *C3* were markedly induced in PMUGT1 and PMUGT2 (~5–80-fold), while suppressed in LFUG strains.

3.3.3.3.3. Late post-challenge (Day 14). In the spleen, all probiotic groups significantly upregulated *CXCL8* (~2–13-fold) compared to controls. *IL6* remained suppressed in most treatments, except LFUGT1 and PMUGT2. *IL1β* was strongly induced across all probiotic groups, except LFUGT2, while *C3* expression was elevated in all, except PMUGT1. In the midgut, all markers were suppressed in the PC. In probiotic treatments, they were significantly underregulated compared to the NC, except *IL1β* (PMUGT1), which still exhibited significant upregulation (~5-fold) compared to both controls.

3.3.3.4. Histopathological changes post-infection. Microscopic lesions were examined in the hepatopancreas, spleen, and midgut of juvenile Nile tilapia across the different treatment groups. In the hepatopancreas, macro-vesicular vacuolation indicative of steatosis was evident across all treatments (Fig. 14b&c). Mononuclear inflammatory cell infiltration was observed in the liver, particularly in the positive control (slight to moderate) and LFUGT1 (slight), but was absent in the negative control, LFUGT2, and PMUGT1. PMUGT2 showed minimal infiltration. Inflammation around the pancreatic acini was moderate in the positive control, present in 50 % of LFUGT1, and absent in LFUGT2 and PMUGT2 samples. Excess pancreatic zymogen granules were noted (Fig. 14b), and slight clusters of melano-macrophages were observed in 50 % of LFUGT1 samples. Cystic vacuolation of pancreatic acinar cells (Fig. 15a&b) was moderately present in the positive control and PMUGT1, minimal in LFUGT1, and absent in the negative control, PMUGT2 and LFUGT2.

In the spleen, slight congestion was recorded in all groups. Melano-macrophage clusters were markedly present in the positive control and both LFUG treatments, but only slightly evident in the negative control and PMUG treatments (Fig. 16).

In the midgut, the negative control and LFUGT2 exhibited slender villi with a high villus/crypt (V/C) ratio of 4:1 (Fig. 17b). Other treatments showed plump villi with lower V/C ratios (PC, PMUGT1 & PMUGT2–1:1 and LFUGT1–2:1) (Fig. 17a). Villi were extremely plump and nearly focally absent in PMUGT2. Minimal mononuclear inflammatory cell infiltration in the mucosal lamina propria was observed in all treatments, except for slight infiltration in PMUGT1 and the positive control. A mucous layer with debris was present on the mucosal surface in all groups.

Based on these observations, histopathological scores were computed and analyzed. Median-score analysis revealed significant differences among treatments ($X^2 = 19.3$, df = 5, $P = 0.002$). The positive control (PC) exhibited the most severe lesions across multiple parameters and tissues, with median scores significantly higher than those of the negative control (NC, $P = 0.01$), LFUGT2 ($P = 0.04$), and PMUGT2 ($P = 0.03$). No significant differences were observed between PC, LFUGT1, and PMUGT1 ($P > 0.05$). Multivariate analysis supported these findings, as Principal Coordinates Analysis (PCoA) of Bray-Curtis

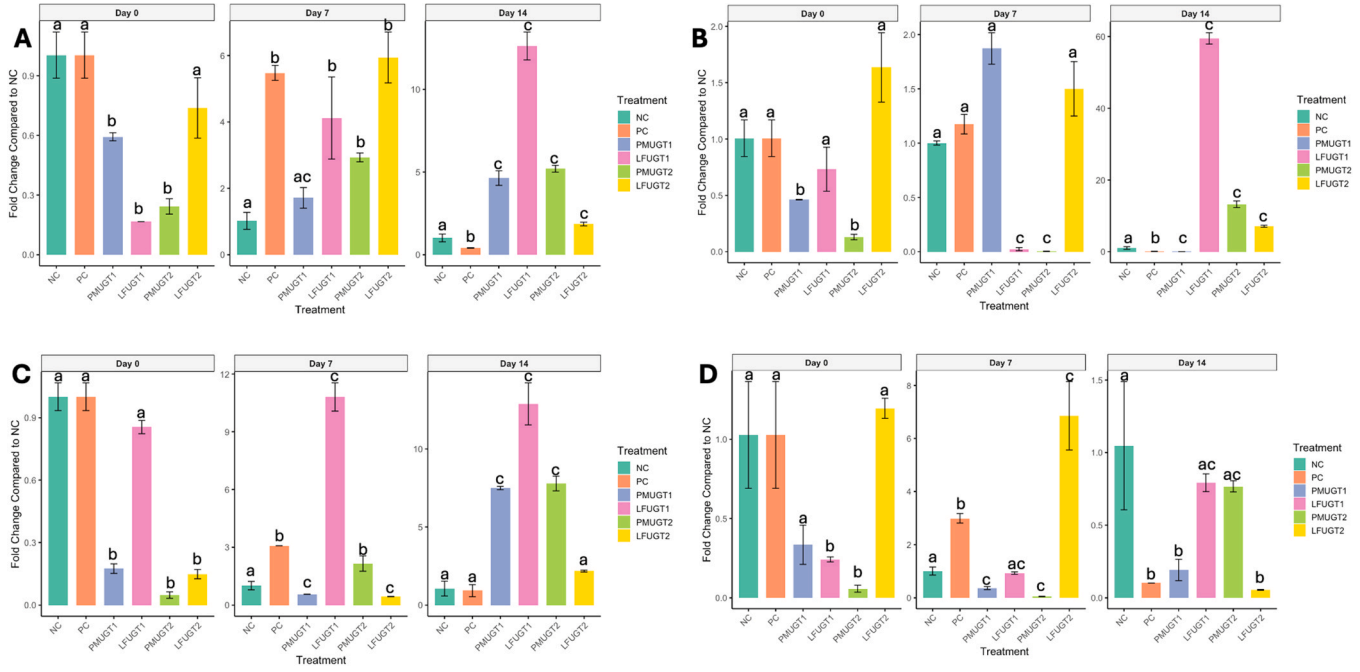

Fig. 12. Immune gene expression in the spleen of Nile tilapia juveniles after 60 days of feeding on a commercial diet supplemented with two local putative probiotic strains *Lysinibacillus fusiformis* LFUG and *Priestia megaterium* PMUG01 at two concentrations T1 – 1×10^6 CFU.g^{-1} and T2 – 1×10^8 CFU.g^{-1}, and a 14-day intraperitoneal challenge with a clinical strain of *Providencia* sp. PSNUG. The four genes included: **A** -Chemokine (C-X-C motif) ligand 8 (*CXCL8*); **B** - Complement component C3 (*C3*); **C** - Interleukin 1-beta (*IL1β*) and **D** - Interleukin 6 (*IL6*).

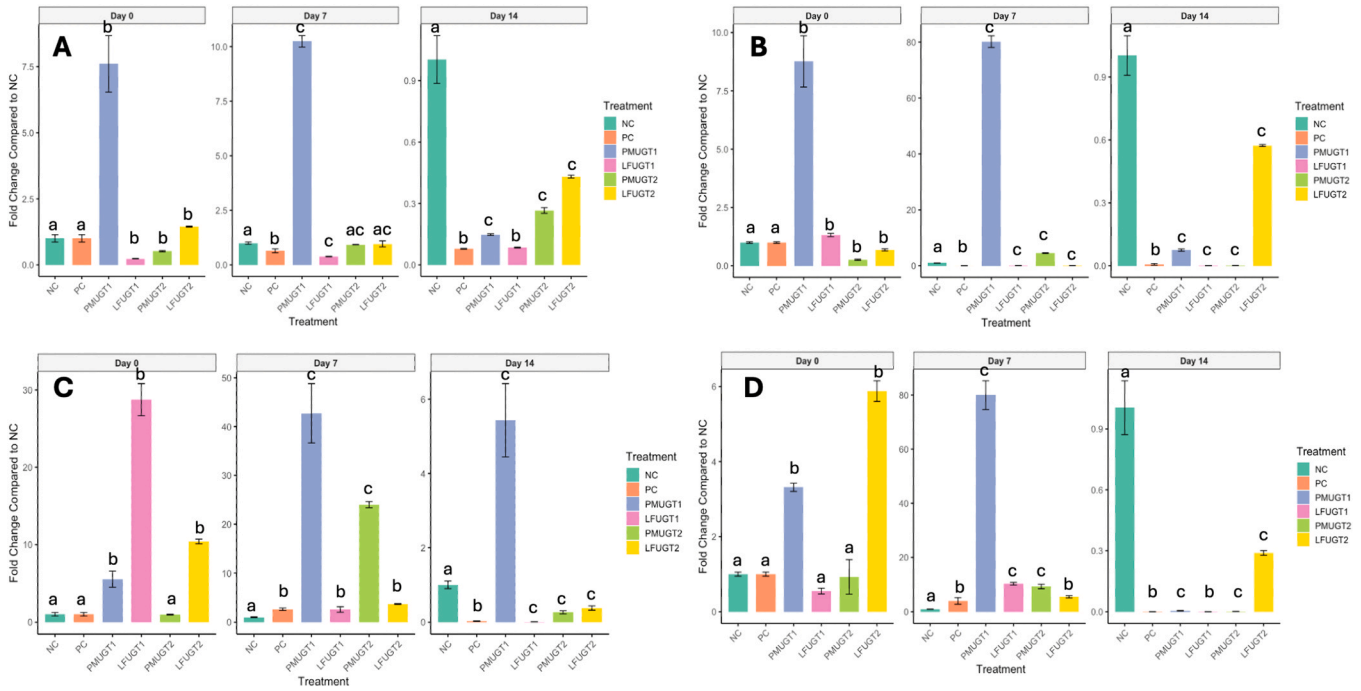

Fig. 13. Immune gene expression in the midgut of Nile tilapia juveniles after 60 days of feeding on a commercial diet supplemented with two local probiotic strains; *Lysinibacillus fusiformis* LFUG and *Priestia megaterium* PMUG01 at two concentrations T1 – 1×10^6 CFU.g^{-1} and T2 – 1×10^8 CFU.g^{-1}, followed by a 14-day intraperitoneal challenge with a clinical strain of *Providencia* sp. PSNUG. The four genes analyzed included: **A** -Chemokine (C-X-C motif) ligand 8 (*CXCL8*); **B** - Complement component C3 (*C3*); **C** - Interleukin 1-beta (*IL1β*) and **D** - Interleukin 6 (*IL6*). Bars representing the same gene at a given time point with different superscripts from the negative control (NC) and positive control (PC) indicate statistically significant differences ($P < 0.05$).

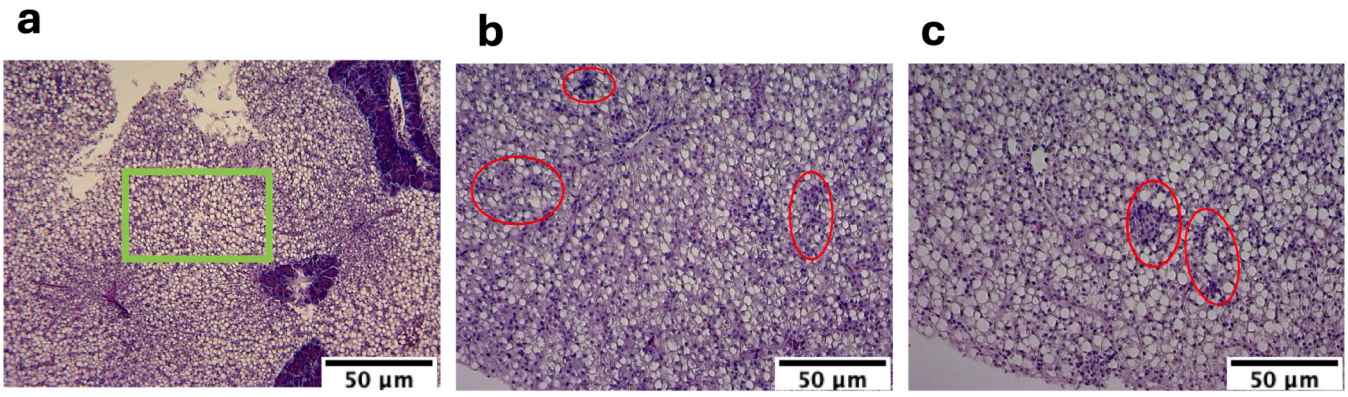

Fig. 14. Steatosis in the liver tissues of juvenile tilapia fed on different feed treatments and challenged with virulent *Providencia* sp. Fig. a represents the non-inflammation conditions (in green) while b and c represent a marked steatosis condition with mononuclear inflammatory cell infiltration (in red). A magnification of 400X was used to examine the tissues.

dissimilarities showed PC samples clustering separately from NC and probiotic-treated groups (Fig. 18A). LFUGT2 and PMUGT2 clustered closer to NC, indicating reduced tissue damage. PERMANOVA confirmed a significant overall treatment effect on histopathology profiles ($R^2 = 0.978$, F = 52.71, $P = 0.001$). Lesion severity across tissues and parameters was further illustrated by a heatmap of median scores (Fig. 18B), highlighting the protective effects of probiotic supplementation against *Providencia*-induced tissue damage. Supplementary Table S6 provides detailed scoring information, including all parameters and tissues assessed.

4. Discussion

To address the increasing demand for seafood through sustainable aquaculture, probiotics are gaining attention as non-antibiotic alternatives for promoting fish health and performance (Y. Deng et al., 2022; Tabassum et al., 2021). These beneficial microbes enhance feed utilization, stress tolerance, disease resistance, and overall host welfare (Y. Deng et al., 2022; El-Son et al., 2022).

In this study, supplementing Nile tilapia diets with *Lysinibacillus fusiformis* LFUG and *Priestia megaterium* PMUG01 significantly improved growth performance over 60 days, regardless of concentration. This aligns with previous findings that reported improved Nile tilapia growth with probiotic supplementation (Dawood et al., 2024; Ghalwash et al., 2022; Kuebutornye, Abarike, Sakyi, et al., 2020). Increased probiotic dosages yield diminishing economic returns in shrimp farming (Peñalosa-Martinell et al., 2021). Thus, lower concentrations (T1) could be more cost-effective than higher doses (T2).

The improved growth performance likely occurred due to enhanced appetite, feed intake and utilization, potentially driven by the

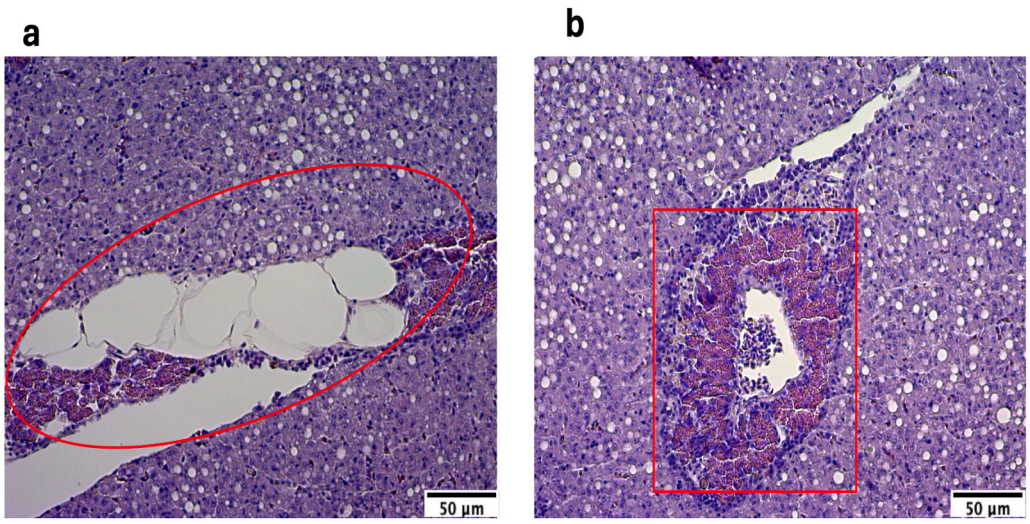

Fig. 15. Moderate cystic vacuolation of the pancreatic acinar cells (Fig. a) with excess pancreatic zymogen granules (Fig. b). A magnification of 400X was used to examine the tissues.

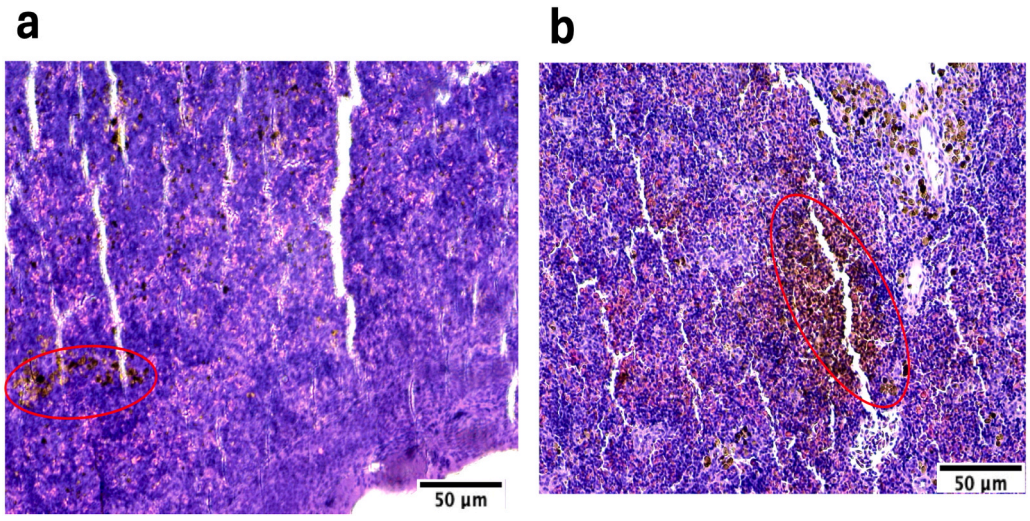

Fig. 16. Clusters of melano-macrophages (highlighted in red) were present in the spleen in all treatments. Fig. a represents a slight condition while Fig. b represents a marked condition. A magnification of 400X was used to examine the tissues.

probiotics' exo-enzymatic activities and biosynthesis of essential nutrients (Assan et al., 2022; Kuebutornye, Abarike, Sakyi, et al., 2020; Kuebutornye, Lu, Abarike, et al., 2020; Markowiak-Kopeć and Śliżewska, 2020; Rwezawula, Mwanja, et al., 2025; Rwezawula, Waiswa Mwanja, et al., 2025; Soltani et al., 2019). These effects were consistent with similar studies by Darafsh et al. (2020), Eissa et al. (2022) and Liao et al. (2022).

Notably, probiotic-fed groups, particularly LFUGT2, LFUGT1, and PMUGT1, exhibited lower coefficients of variation (COVs) and more symmetrical skewness in weight distributions. This implied enhanced size uniformity, which is critical for synchronized harvesting and marketability (da Costa Sousa et al., 2019; Lobo et al., 2014; Mehrim, 2009).

Although proximate composition remained unchanged across treatments, improved feed utilization may have resulted from probiotic-mediated degradation of anti-nutritional compounds (*e.g.*, phytase) and enhanced nutrient absorption (Amit et al., 2021). Previous studies similarly found no impact on proximate composition with probiotic use (Abd El-Naby et al., 2019; M. Liu et al., 2017). The observed proteolytic activity of both strains likely supported efficient protein digestion, addressing the costliest dietary component in aquaculture (Hossain et al., 2021; Rwezawula, Mwanja, et al., 2025; Rwezawula, Waiswa Mwanja, et al., 2025). Additionally, these probiotics may have contributed to improved water quality through microbial-mediated waste biodegradation and detoxification (Abdo et al., 2022; Hlordzi et al., 2020; Kuebutornye et al., 2022). Key physicochemical parameters, dissolved oxygen (6.1 ± 0.3 mg L^{-1}), pH (8.2 ± 0.1), and temperature (24.7 ± 1.3 °C), were maintained within optimal ranges throughout the experiment, consistent with the findings of Eissa et al. (2024) and Hendam et al. (2023), thereby reducing environmental stress on the fish. The survival and condition factors remained unaffected during the feeding trial, indicating that both strains were safe at the tested doses. This supports their suitability for application in Nile tilapia farming, aligning with prior biosafety assessments in *Artemia* nauplii and zebrafish embryos(Rwezawula, Mwanja, et al., 2025; Rwezawula, Waiswa Mwanja, et al., 2025).

Post-challenge with a virulent local *Providencia* sp. (PSNUG), survival was significantly better in probiotic-fed groups. *Providencia* spp. are emerging multidrug-resistant pathogens in aquaculture, causing severe mortalities and pose zoonotic risks (Baldissera et al., 2019; H. Cao

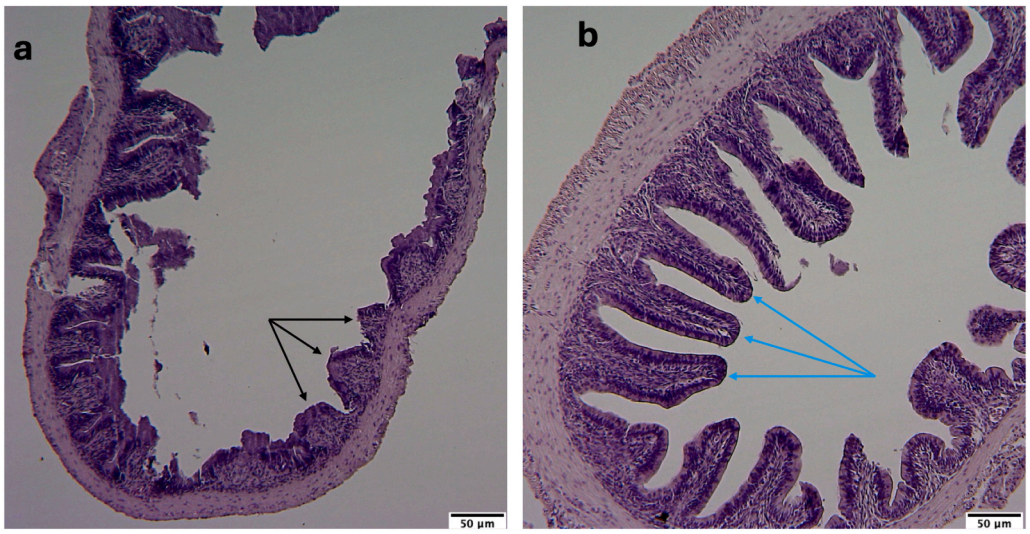

Fig. 17. The midgut tissues of Nile tilapia juveniles showing the plumpness and slenderness conditions of the villi. Fig. a - plump villi (black arrows) with lower villus/crypt (V/C) ratios (LFUGT1; 2:1 and 1:1; positive control, PMUGT1 and PMUGT2). Fig. b - slender villi (blue arrows) with high villus/crypt (V/C) ratios (negative control and LFUGT2). A magnification of 400X was used to examine the tissues.

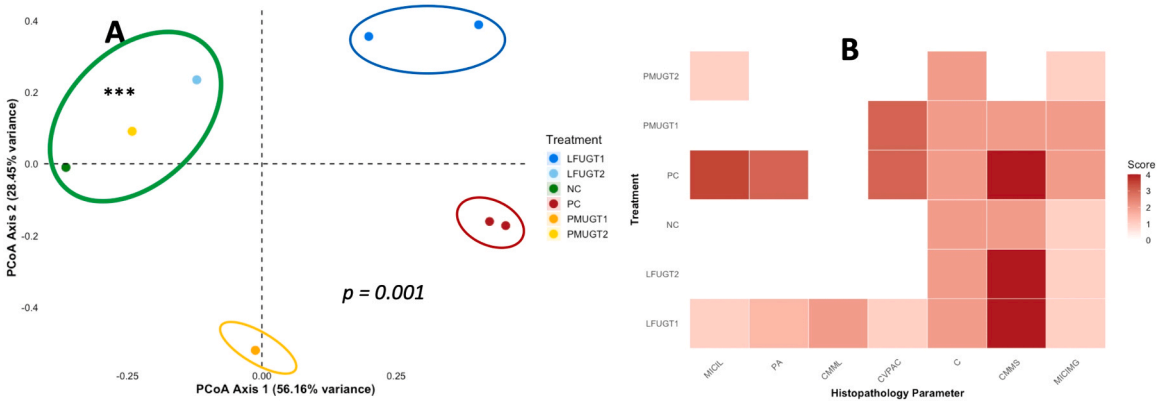

Fig. 18. Histopathology of Nile tilapia following probiotic supplementation and pathogen (*Providencia* sp.) challenge. Scores were based on findings in the spleen, liver, and midgut of juveniles (n = 3) after the feed trial on day 7 post-challenge. Scoring reflected the presence, absence, or severity of specific lesions (0 = Absent, 1 = minimal, 2 = slight, 3 = moderate, 4 = marked, 5 = severe). Panel A: PCoA of Bray–Curtis dissimilarities of histopathology scores, with colors and rings indicating treatment clustering. NC, PMUGT2, and LFUGT2 (green ring) showed significant protection (***) compared to PC (red ring), while PMUGT1 (orange ring) and LFUGT1 (blue ring) provided intermediate, non-significant protection. Panel B: Heatmap of median scores (0–5) per lesion type: MICIL = mononuclear inflammatory cell infiltration (liver), PA = pancreatic acini degeneration, CMML = melano-macrophage clusters (liver), CVPAC = cystic vacuolation of pancreatic acinar cells, C = congestion, CMMS = melano-macrophage clusters (spleen), MICIMG = mononuclear inflammatory cell infiltration (midgut).

et al., 2017; Mani et al., 2021; Murata et al., 2001; Rajme-Manzur et al., 2023). The PSNUG genome encoded virulence (*sycB*) and resistance genes (*CRP*, *Tet(59)*), highlighting the need for probiotic alternatives to mitigate such virulent and MDR pathogens (Bugrysheva et al., 2017; Walker and Miller, 2004). Probiotics may suppress pathogen virulence through enzymatic detoxification of protein-based toxins (Chizhayeva et al., 2022; Kumar Bajaj et al., 2015; Rwezawula, Mwanja, et al., 2025; Rwezawula, Waiswa Mwanja, et al., 2025). Hence, our proteolytic probiotic strains may have neutralized pathogen-derived proteins like T3SS toxins, thus reducing PSNUG virulence (Chizhayeva et al., 2022; Kumar Bajaj et al., 2015). Moreover, competitive exclusion, immune modulation, and quorum quenching may have contributed to the observed protection (Melo-Bolívar et al., 2022; Rwezawula, Mwanja, et al., 2025; Rwezawula, Waiswa Mwanja, et al., 2025; Xue et al., 2020).

Full-length 16S rRNA sequencing on the PromethION platform can detect bacteria at ~90 CFU.mL^{-1} or ≥ 5 % relative abundance, depending on sequencing depth, primers, and host DNA interference (Lin et al., 2023; Zhang et al., 2023). In our study, only *Lysinibacillus* sp. was detected in fish from LFUGT2, suggesting LFUG's better persistence in the gut and a likely contribution to improved performance, while PMUG01 remained undetected, possibly due to lower colonization efficiency or niche-specific localization (Alcolea-Medina et al., 2023). Although alpha and beta diversity indices showed no significant changes, probiotic-fed groups exhibited higher relative richness and abundance of possibly beneficial symbionts over opportunistic taxa. These microbial shifts likely supported improved digestion, immune modulation, and disease resistance (Adeoye et al., 2016; Etyemez Büyükdeveci et al., 2023; Shija et al., 2023). Beneficial microbes produce digestive enzymes, short-chain fatty acids (SCFAs), and antimicrobial compounds, maintaining gut health and promoting growth (B. Wang et al., 2023; Xue et al., 2020). Additionally, gut microbial communities modulate gut-associated lymphoid tissues (GALT), cytokine signalling, and epithelial cell development (Bates et al., 2006; S. K. Nayak, 2010b; Rawls et al., 2004), with disruptions linked to inflammation and immune dysfunction (Bates et al., 2007; Nie et al., 2017). Maintaining microbial balance is thus critical for intestinal homeostasis

and disease resilience.

Immune analysis showed probiotic-induced leukocytosis, indicating improved immune readiness (Opiyo et al., 2019; Srisapoome and Areechon, 2017; Y. Zhao et al., 2022). Elevated leukocyte counts and immune gene expression patterns (*CXCL8, IL6, IL1β, C3*) were consistent with former studies on probiotic immunostimulation (Opiyo et al., 2019; Ren et al., 2020; Y. Zhao et al., 2022). Following the feeding trial and prior to challenge, gene expression patterns indicated localized immune priming in the midgut, the primary site of host-microbe interaction, particularly in PMUGT1 and LFUGT2 groups, while systemic responses in the spleen were suppressed, suggesting a compartmentalized immunomodulatory effect (Elbahnaswy and Elshopakey, 2020; K. Q. Nguyen et al., 2022). Overall, upregulation of immune genes (*e.g., IL1β, IL6, CXCL8,* and *C3*) in gut and spleen tissues post-feeding and or post-challenge, suggested strain-specific and dose-dependent LFUG and PMUG01-induced immune priming, localized and systemic immune response enhancement (Elbahnaswy and Elshopakey, 2020; K. Q. Nguyen et al., 2022). For instance, Chemokine (C-X-C motif) ligand 8 (*CXCL8*) upregulation, observed across treatments, likely regulated neutrophil recruitment and inflammation, contributing to localized immune responses (Elbahnaswy and Elshopakey, 2020; K. Q. Nguyen et al., 2022). Also, interleukin (*IL6*)'s promotion of B-cell maturation and antibody production further emphasizes its role in enhancing immunity (Elbahnaswy and Elshopakey, 2020; Li et al., 2023; Nayak, 2010). Similarly, *IL1β*'s upregulation, suggests its involvement in countering PSNUG virulence through macrophage activation and lymphocyte mobilization, which are critical in defusing microbial invasion and colonization (Etyemez Büyükdeveci et al., 2023). Furthermore, upregulation of the complement component (*C3*) was probably pivotal in pathogen opsonization and the complement cascade, emphasizing its role in enhancing mucosal and systemic defense mechanisms (Giang et al., 2018). Serum IgM levels remained largely unchanged across treatments, consistent with the observed localized mucosal immune priming rather than systemic immune activation. This pattern aligns with findings in *Oreochromis niloticus* and *Oncorhynchus tshawytscha*, where probiotics primarily enhance cellular innate and mucosal responses rather than systemic humoral immunity (Naftal Gabriel et al., 2022; Soto-Dávila et al., 2024). Systemic IgM production is a delayed adaptive process that requires B-cell activation, antigen-specific stimulation, or vaccination (Guimarães et al., 2022). Moreover, immunoglobulin T (IgT), which is strongly associated with mucosal immunity, would likely have provided a more sensitive and relevant measure of the immune response (B. Wang et al., 2023).

Histopathological analyses of vital organs, gut, hepatopancreas and spleen, further validated the protective effects of probiotics and provided insights into host-pathogen-probiotic interactions. Infections with *Providencia* sp. commonly trigger inflammation and tissue disruptions (Moustafa et al., 2021), as seen in the positive control group, where moderate inflammatory cell infiltration, hepatocellular steatosis, and elevated melano-macrophage aggregates were observed, typical signs of pathogen-induced stress (Dawood et al., 2024; Ruiz et al., 2020). Still, probiotic-treated groups, particularly LFUGT2, PMUGT2, and PMUGT1, exhibited reduced inflammatory infiltration and lower melano-macrophage aggregation in the hepatopancreas and spleen, suggesting modulated immune activation and reduced tissue stress. Despite steatosis being present across all groups, a common effect of commercial feeds (Bilen, B. 2013; Coz-Rakovac et al., 2005), probiotic groups showed fewer degenerative changes, indicating potential hepatoprotective effects. Mild-moderate cystic vacuolation of pancreatic acinar cells was noted in some treatments (PC, LFUGT1 and PMUGT1), signifying slight feed-related stress (Gewaily et al., 2021). Interestingly, excess zymogen granules are normally present in stressed fish, and while observed across treatments, their lower density in probiotic groups again supported a mitigated stress response (Nikiforov-Nikishin et al., 2023). Furthermore, gut histology revealed further probiotic benefits. Fish fed on probiotic-supplemented diets, particularly LFUGT2, showed slender villi with higher V/C ratios comparable to the negative control, which are associated with reduced mucosal inflammation and efficient nutrient absorption (Barba-Vidal et al., 2017; Gewaily et al., 2021). In contrast, the positive control exhibited plumper villi and lower villus-to-crypt (V/C) ratios, indicating stress and or inflammation. Mucosal debris layers were observed across groups, likely due to environmental influences, but slight lamina propria inflammation was seen outside the positive control and PMUGT1.

Generally, *L. fusiformis* LFUG and *P. megaterium* PMUG01 demonstrated potential as safe, indigenous probiotic candidates that promoted growth, feed utilization, size uniformity, disease resistance, tissue integrity, gut microbiome and immune modulation in Nile tilapia juveniles.

5. Conclusions

After a 60-day feeding trial with a commercial diet supplemented with our indigenous autochthonous strains, *Lysinibacillus fusiformis* LFUG and *Priestia megaterium* PMUG01 at concentrations (T1 – 10^6 CFU.g^{-1} and T2 – 10^8 CFU.g^{-1}), Nile tilapia juveniles demonstrated better growth performance and overall health compared to the control group. Both strains, irrespective of the concentration, significantly improved growth rate, feed utilization, and immunity. Both strains modulated the gut microbiome and immune gene expression without inducing mortality, confirming their biosafety. Remarkably, fish that received probiotic-supplemented diets showed reduced mortality and tissue damage following a challenge with virulent clinical *Providencia* sp. PSNUG.

These findings emphasize the potential of using indigenous probiotics to reduce antibiotic dependence, enhance fish resilience to pathogens, and sustainably boost productivity in aquaculture. Given the critical role of Nile tilapia in food security, livelihoods, and economic development in regions like Sub-Saharan Africa, these results contribute to the advancement of environmentally friendlyand locally adapted aquaculture practices. Further validation in other species and real-world settings is essential to inform broader adoption and policy support.

CRediT authorship contribution statement

Everlyne Ajore: Methodology, Investigation, Data curation. **Brian Lota:** Methodology, Investigation, Data curation. **Parisa Norouzitallab:** Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Kartik Baruah:** Validation, Supervision, Methodology, Investigation, Conceptualization. **Peter Bossier:** Writing – review & editing, Validation, Supervision, Methodology, Investigation, Funding acquisition, Conceptualization. **Daisy Vanrompay:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Philip Rwezawula:** Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Jesca Nakavuma:** Validation, Supervision, Methodology, Conceptualization. **Catherine Agoe:** Methodology, Investigation, Data curation. **Mwanja Wilson:** Validation, Supervision, Resources, Funding acquisition, Conceptualization. **Victoria Namulawa:** Validation, Supervision, Resources, Conceptualization.

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Declaration of Competing Interest

The authors declare the following financial interests/personal

relationships which may be considered as potential competing interests: Philip Rwezawula has patent #EP 24212619.1 pending to European Patents Office (EPO). Daisy Vanrompay has patent #EP 24212619.1 pending to European Patents Office (EPO). Peter Bossier has patent #EP 24212619.1 pending to European Patents Office (EPO). Peter Bossier is a section editor of the journal. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.aqrep.2025.103259.

Data availability

The datasets generated and/or analyzed during the current study are available in the NCBI database under Bio projects - PRJNA1094437 and PRJNA1171306

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