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Bacillus indicus and *Bacillus subtilis* as alternative health and colouration promoters to synthetic astaxanthin in cyprinid aquaculture species.

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Abstract

One of the largest challenges for the sustainable development of global aquaculture is the threat of infectious diseases. Preventative strategies that reduce antibiotic use are required to ensure fish health, minimise infectious diseases and subsequent pharmaceutical interventions. Recent strategies involve health-promoting feed supplements, such as astaxanthin and probiotic bacteria. Astaxanthin, a widely used carotenoid, offers colouration and antioxidant properties that can improve fish growth and fish survival when challenged with a pathogen. Probiotics can provide fish with a range of health benefits ranging from enhanced feed digestion, synthesis of vitamins, boost of innate immune response and active defence against potential pathogens.

In this study, we tested if novel probiotic blends (*Bacillus subtilis* and/or *Bacillus indicus*) can be used as alternative health and/or colouration supplements to astaxanthin in two cyprinid species, mirror carp (*Cyprinus carpio*) and Red Comet goldfish (*Carassius auratus auratus*). Using experimental feed trials and 16S rRNA microbial profiling, the impact of the probiotic on fish growth and microbial community within the distal gastrointestinal tract was assessed. In addition, in mirror carp, blood

samples were tested for immunology and haematological parameters, while in goldfish colouration of the skin was analysed.

Mirror carp fed astaxanthin showed significantly increased growth whereas *B. substilis /B.indicus* supplementation had non-significant effects on growth performance. Our results provide the first insights into how the supplementation of astaxanthin changes the microbial composition in cyprinid species. In mirror carp, astaxanthin and the probiotic blend induce a significant shift in gut microbial communities. Mirror carp fed *B. substilis/ B.indicus* showed several indices of potential microbial and health benefits such as increased diversity, an abundance of potentially beneficial bacteria and enhancement of the phagocytic activity and creatinine blood levels. However, no effect on colouration, growth or the microbial community was found in goldfish, highlighting substantial species-specific differences in response to probiotics, in two closely related cyprinid species. Further research into the efficacy and site of colonization of supplemented bacteria in fish gastrointestinal tracts, and the mechanisms underlying the observed shifts in the host microbiota, is required to fully understand species-specific responses to probiotic supplementation.

Keywords: Microbiota, Probiotics, Astaxanthin, Mirror carp, Goldfish, Fish health,

Introduction

Infectious diseases are one of the biggest burdens to the sustainable growth of the aquaculture industry, resulting in high treatment costs and losses in production (Bank, 2014; Pettersen *et al.*, 2015). Globally, antibiotics are widely used to treat and prevent bacterial diseases. However, due to the increasing prevalence of antibiotic resistance, their use can potentially severely harm the environment, humans, and reduce

treatment efficiency (Dawood, Koshio and Esteban, 2018; Pérez-Sánchez, Mora-Sánchez and Balcázar, 2018; Lulijwa, Rupia and Alfaro, 2020; Schar *et al.*, 2020). Hence, the application of antibiotics in aquaculture is becoming increasingly restricted in Europe and the development of alternative approaches is a research priority. Vaccinations are a powerful and efficient method to mitigate a variety of diseases, but vaccinations are not yet available for all diseases and fish species. Cost implications also limit the application of vaccines in many countries (Miccoli *et al.*, 2021). Therefore, alternative methods are urgently needed to boost fish health and reduce the risk of disease outbreaks. Recent health-promoting strategies involve supplements added to feeds to improve fish health and improve disease resistance (Dawood, Koshio and Esteban, 2018).

The carotenoid astaxanthin is a widely used feed supplement with well-known health benefits for the host and is also used as a colourant to enhance consumer perception. In Atlantic salmon (*Salmo salar*), astaxanthin is a commonly used feed additive to induce the colouration of the flesh, while in some cyprinid species (e.g., goldfish) astaxanthin can improve skin colouration (Lim *et al.*, 2018). In addition, as a health promoter, astaxanthin has strong antioxidant capacities, increases stress resistance, and enhances immune responses, generally strengthening disease resistance (Sadraddin *et al.*, 2019; Chang and Xiong, 2020; Lim *et al.*, 2021). Astaxanthin has been shown to increase survival during exposure to bacterial (e.g. *Aeromonas hydrophila* in common carp *Cyprinus carpio*) and viral pathogens (e.g. *Vibrio alginolyticus* in Asian sea bass *Lates calcarifer*) and increase growth performance significantly (Sadraddin *et al.*, 2019; Lim *et al.*, 2021). However, fish cannot synthesise astaxanthin *de novo* and therefore it needs to be provided in aquaculture via feeds

(Guerin, Huntley and Olaizola, 2003). In nature, astaxanthin is exclusively synthesised by a variety of microorganisms such as algae (e.g., *Haematococcus pluvialis*) and yeasts (e.g., *Phaffia rhodozyma*). Currently in aquaculture, astaxanthin is almost exclusively produced synthetically (Lim *et al.*, 2018) and due to high production costs, its application is restricted to high-value fish species (Stachowiak and Szulc, 2021).

Probiotics are increasingly used in aquaculture for a variety of health-promoting properties. Probiotic treatments typically consist of spores of single or multiple bacteria species, delivered via feeds or added directly into the rearing water (Merrifield, Dimitroglou, *et al.*, 2010). Spores are intended to germinate and colonise the host gastrointestinal tract or other mucosal surfaces (Li *et al.*, 2019). Once ingested, probiotic bacteria may modify the host mucosal microbiota, such as increasing bacterial community diversity, a widely described indicator for healthy fish (Legrand *et al.*, 2020). In addition, supplemented bacteria can synthesise enzymes (e.g. amylase, lipase, and protease) that can enhance host feed digestion, improving nutrient availability and growth performance (Assan *et al.*, 2022). Moreover, some probiotic bacteria produce antimicrobial compounds and thus directly inhibit the growth of pathogens. Probiotic applications can strongly influence fish immunocompetence including increased levels of phagocytic activity, respiratory burst, lysozyme and immune gene expression (Newaj-Fyzul *et al.*, 2007; Kuebutornye *et al.*, 2020; Shi *et al.*, 2020). Despite the range of potential health benefits for the host, the main bottleneck of probiotic application is inconsistent outcomes between experimental studies. There remains a lack of knowledge on the colonization of probiotic species in the gastrointestinal tract of fish. Whilst probiotic species are intended to settle long-term or temporarily in the intestine of the fish, the majority of microbial studies cannot

provide evidence for their permanent establishment in the host gastrointestinal tract (Li *et al.*, 2019; H. Zhang *et al.*, 2021).

The predominant probiotic taxa currently used in aquaculture belong to the genus *Bacillus*, particularly *B. subtilis,* the application of which has demonstrated strong disease resistance properties including increased survival against pathogenic *Aeromonas spp.* in rainbow trout (*Oncorhynchus mykiss*), Dabry's sturgeon (*Acipenser dabryanus*) and crucian carp (*Carassius carassius*) (Newaj-Fyzul *et al.*, 2007; Di *et al.*, 2019; Liu *et al.*, 2022). A potential novel probiotic *Bacillus indicus*, isolated first from an aquifer in India and recently from human faeces, offers promising beneficial properties including the synthesis of carotenoids (Suresh *et al.*, 2004; Duc *et al.*, 2006; Sy *et al.*, 2013, 2015b). These unique properties raise the prospect for aquaculture production to use *B. indicus* as an alternative colourant and health promotor to expensive synthetic astaxanthins. In this study, we test astaxanthin against novel probiotic products in two cyprinid species: mirror carp (*Cyprinus carpio*) and red comet goldfish (*Carassius auratus auratus*). In mirror carp, *Bacillus indicus* and *Bacillus subtilis* were tested as a probiotic blend. In goldfish, we tested *Bacillus indicus* alone and in combination with astaxanthin. Using experimental trials and 16S rRNA microbiota profiling, we compare the effects of *Bacillus spp.* supplements and traditional astaxanthin on fish growth performance and the microbial communities of the gastrointestinal tract in both cyprinid species. In addition, in carp, we assess health parameters via haematological and immunology analyses. In goldfish, we assess if partial or complete substitution of astaxanthin by *B. indicus* can enhance skin colouration.

Methods

Feeding trials and sampling procedures

Feeding trials were carried out at the Pontus research facility (Aberdare, Wales), using two cyprinid species; Mirror carp (*Cyprinus carpio*) and Red Comet goldfish (*Carassius auratus auratus)*, both supplied by Rodbaston Aquaculture.

Carp and goldfish were acclimatised for two weeks in two separate recirculation aquaculture systems (RAS, carp: 12 x 200L tanks, goldfish: 20 x 70 L glass aquariums) before the start of the feeding experiment. Both RAS systems are equipped with an ultraviolet disinfection unit to ensure no probiotic contamination between tanks. Animal handling procedures were approved by the Pontus research animal ethics committee.

At the start of the carp trial, 240 fish (40.38 g ± 0.39) were randomly distributed into 12 tanks (200 L), with quadruplicate tanks per treatment group (20 fish per tank). Fish were raised for 7 weeks on one of three experimental diets ([1] negative control: standard feed, [2] probiotic diet: standard feed + 0.36 g/kg *Bacillus indicus* + 1 g/kg *Bacillus subtilis*, [3] positive control: standard feed + 40 mg/kg astaxanthin). The three feeds were formulated and produced in cooperation with SPAROS (Olhão, Portugal) and Microbiome LABS UK Ltd (West Yorkshire, United Kingdom), and composed of a standard diet with a supplemented probiotic blend (*B. indicus* and *B. subtilis*) or astaxanthin as additives. All diets were formulated to meet the principal nutritional requirements of mirror carp (Table S 1). A proximate analysis was carried out for all experimental diets. Fish were fed to satiation by hand, five times a day and feed intake was recorded daily. Throughout the trial, tanks were exposed to a 12:12 h light: dark regime. Water quality parameters in the RAS system were maintained at 21 °C (±1

°C), >80 % oxygen saturation, pH 7.25 (± 0.3), < 0.02 mg/l ammonia, < 0.6 mg/l nitrite and < 75 mg/l nitrate, following optimal welfare conditions for carp.

For the goldfish trial, 100 fish (12.18g ± 0.17) were randomly distributed into 20 tanks (5 fish per tank, 70 L), with quadruplicate tanks per treatment group. For the experimental part of the trial, fish were raised for 8 weeks on one of five experimental diets ([1] negative control: standard feed, [2] standard feed + 3.3 g/kg *Bacillus indicus*, [3] standard feed + 1.65 g/kg *Bacillus indicus* + 20mg/kg astaxanthin, [4] standard feed + 0.99 g/kg *Bacillus indicus* + 28mg/kg astaxanthin, [5] standard feed + 40 mg/kg astaxanthin) (Table S 2). The experimental feed was formulated and produced in cooperation with SPAROS and Microbiome LABS UK Ltd and composed of standard diet with supplemented astaxanthin or a partial/complete replacement of astaxanthin by a probiotic additive (*B. indicus*). All feeds were formulated following the nutritional requirements of goldfish. The goldfish were fed to satiation by hand, twice a day and feed intake was recorded daily. Throughout the trial, tanks were exposed to a 12:12 h light: dark regime. Water quality parameters in the RAS system were maintained at 29 °C (±1 °C), >80 % oxygen saturation, pH 7.8 (± 0.15), < 0.1 mg/l ammonia, < 1 mg/l nitrite and < 150 mg/l nitrate, following optimal welfare conditions for goldfish.

Growth performance

For the assessment of growth performance, batch weights (total biomass per tank) were taken at weeks 0, 4 and 7/ (8 for goldfish) of the trials. Fish were starved for 24 h prior to weighing. Growth performance was measured using specific growth rate (SGR); percentage body weight gain per day.

$$\mathrm{SGR} = \frac{\left(\mathrm{Ln}(\text{End Batch Weight}) - \mathrm{Ln}(\text{Start Batch Weight})\right) \times 100}{\text{Number of Days}}$$

In addition, feed intake (FI) was calculated as percentage of body weight per day.

$$\mathrm{FI} = \left(\frac{\text{Feed Consumed}}{\text{Bodyweight}}\right) \times 100$$

The feed conversion ratio (FCR) was calculated as the ratio of feed intake to weight gain.

$$\mathrm{FCR} = \frac{\text{Feed Consumed}}{\text{Weight increase}}$$

Mortalities were recorded and summarized as overall survival (%).

Carp health assessment

In addition to growth performance, an overall health assessment for the carp was carried out by Moredun Scientific (MS). For the health assessment, samples from the fish head, kidney, whole blood and plasma were collected from 5 fish per tank (20 per treatment). The health analysis was processed at MS and included a haematological and immunological analysis (Metochis *et al.*, 2016). For the immunological analyses, a range of parameters were measured: total protein, total plasma IgM, plasma peroxidase activity, plasma anti-protease activity, plasma lysozyme activity, plasma complement activity, respiratory burst activity, macrophage activity and B and T lymphocytes in the blood (Table S 6). The haematological analysis measured several blood characteristics (Table S 7).

Goldfish pigmentation evaluation

Throughout the goldfish trial, the pigmentation of the fish skin was assessed. Pigmentation samples were taken at weeks 0, 3, 6 and 8 of the experiment. For the

pigmentation analysis, all fish in each tank were individually photographed in a photographic chamber and the pictures were further processed using ImageJ (ImageJ v1.8.0_172, (Siegenthaler, Mondal and Benvenuto, 2017)). The colour parameters used were L* (Lightness) which ranges from 0 for black and 100; a* for red/green chromaticity and b* for yellow and blue chromaticity, following the recommendations of the International Commission on Illumination (CIE, 1976, (Robertson, 1977)). From these values, the hue (Hab) and Chroma (Cab) values were calculated. Hue, namely the observable colour (e.g., red, blue, yellow), is an angular measurement where 0° indicates a red hue, 90° denotes a yellow hue, 180° green and 270° blue and is calculated by the equation: Hab = arctan ($b*/a*$). Chroma is an expression of saturation or intensity of the colour (Figure 3, Table S 5) attained and is expressed by the equation: Cab = ($a*2$+ $b*2$)0.5.

Statistical evaluation

Growth performance indicators, the health assessment results, and the pigmentation evaluation were tested in R for normality (Shapiro-Wilk test) and homogeneity of variance (Levene's test). If normality and homogeneity were confirmed, significant ($p < 0.05$) differences between treatment groups were determined using a Tukey pairwise *post hoc* analysis of the ANOVA results.

Microbiota profiling

For microbiota analyses, 3 random carp and 4 random goldfish from every tank were sampled (12 per treatment group carp/ 20 per treatment group goldfish) at the end of the experiment. Fish were knocked on the head and killed by the destruction of the brain. Microbiota swab samples were taken from the distal intestine. Based on

previous probiotic and microbial studies the distal intestine shows the highest microbial diversity and likelihood of probiotic colonization (Newaj-Fyzul *et al.*, 2007; Merrifield, Harper, *et al.*, 2010). For taking the swab samples, the whole intestine was removed using a sterile dissection kit. A 1 cm long piece of the distal intestine was cut, opened, and faecal residues removed using sterile distilled water, followed by rubbing the mucosal surface with a swab (sterile rayon bud swab, MWE). All swab samples were immediately frozen and stored at - 80°C until DNA extraction.

Total DNA was extracted from each intestinal microbiota swab sample using the Qiamp DNA mini kit, following manufacturer instructions. Extracted DNA was stored immediately at -20 °C. A subset of the samples was quantified using the Qubit BR DNA assays to verify successful DNA extraction. PCR amplification and library preparation were performed by 2-step PCR targeting of the V1-V2 region of the 16S rRNA gene. First round of PCR amplification used 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 338R (5'- TCTGCTGCCTCCCGTAGGAGT -3') primers with the addition of universal tails (Bohmann *et al.*, 2021) and were performed in triplicate for each extraction sample. The PCR reaction volume was 25 ul, including 12.5 ul PCR mix (NEB Q5 Hotstart High fidelity PCR master mix), 0.5 ul of each primer (10 µM), 10.5 ul H_2O and 1 ul of DNA. The cycling protocol was as follows: 98 °C for 30 s., 35 cycles of 98 °C for 10 s., 55 °C for 30 s., 72 °C for 30 s. and final elongation at 72 °C for 10 min. PCR products were visualised by agarose gel electrophoresis to ensure successful amplification. Negative controls for DNA extractions and PCRs, and a mock community (ZymoBIOMICS Microbial Community Standard) as a positive control, were included for sequencing. PCR round 1 triplicate were pooled and cleaned using Agencourt AMPure XP beads according to

manufacturer instructions (bead: sample ratio 0.9:1). The second round of PCR introduced Illumina adapter sequences and unique, dual indexes for sample identification (Bohmann *et al.*, 2021). PCR round 2 conditions were as above using 15 cycles. Final PCR products were quantified using Qubit BR DNA assays and pooled equimolarly (absolute amount of 80 ng). Pooled samples were bead cleaned together in a single tube (bead: sample ratio 0.9:1). The cleaned libraries were sequenced using an Illumina MiSeq v2 2 x 250 bp run at Bangor University Centre for Environmental Biotechnology. Raw sequence data are available at the NCBI Short Read Archive (SRA) under accession (PRJNA800661).

Paired-end demultiplexed sequencing reads were imported into Quantitative Insights Into Microbial Ecology 2 (QIIME2,(Hall and Beiko, 2018)). Sequences were then quality filtered, trimmed, dereplicated, chimeras rejected, and pair-end reads merged in QIIME2 using DADA2 with standard settings (--p-trunc-len-f 225, --p-trunc-len-r 196, --p-max-ee-f/r 2, --p-trunc-q 2, minimum overlap = 12 bp, no mismatch). Reads were clustered by 99% identity using the de-novo function. Classification of Amplicon Sequence Variants (ASVs) was performed using a scikit-learn naive Bayes machine-learning classifier trained using sequences representing the bacterial V1 – V2 rRNA region available from the SILVA database (https://www.arb-silva.de/download/archive/qiime;Silva_138, downloaded 14.12.2021), and taxonomic classifications were based on the q2-feature classifier in QIIME2. The classifier then assigned taxonomic information to representative sequences of each ASV. The QIIME2 output was further processed in RStudio (Version 4.0.3) with the package “phyloseq” (McMurdie and Holmes, 2013). Rarefaction analysis was used to determine sufficient read depth and samples with less than 10,000 sequences were excluded.

Subsequent filtering excluded taxa with less than 100 reads, taxa found in only one sample and taxa annotated as *Mitochondria* and *Chloroplast*. After raw read processing, no negative control samples retained sufficient quality or quantity of reads to be considered further. R-software was used to analyse significant differences in alpha (pairwise Wilcoxon signed-rank test) and beta (pairwise Adonis) diversity measures. Significant differential abundance of ASVs between fish fed the prebiotic blend and the control (no prebiotic) was determined using DESeq2 (FDR-corrected $p < 0.05$). The PICRUSt2 package (Douglas *et al.*, 2020) was used for functional prediction of the microbial communities (p-max-NSTI = 2). Significant differences in metabolic pathway abundances between treatment groups were determined using DESeq2.

Results

Over the 7-week carp trial, no mortalities were observed across all tanks (survival 100%). In carp, specific growth rate (SGR) ($p = 0.03$) and end weight ($p = 0.03$) were significantly increased in fish fed astaxanthin compared to the control group (Figure 1, Table S 3). Carp fed the probiotic showed greater SGR compared to the control, however, the difference was not significant to the other treatment groups ($p = 0.13$) (Figure 1). Similar results revealed the end weight of the carp (control: 125.50 ± 6.52a, probiotic: 136.48 ± 8.41ab, astaxanthin: 141.13 ± 1.66b, Table S 3). Feed intake (FI) and feed conversion ratio (FCR) were not significantly different among the treatment groups (Table S 3). Across the tested immunological parameters of the blood analysis, the phagocytic activity was significantly greater in fish fed the probiotic compared to the control ($p = 0.005$) and fish fed with astaxanthin ($p = 0.044$) (Figure 2 A). Lysozyme levels were increased in fish fed both supplements, although not significant (Figure 2

B, Table S 6). For the haematological analysis, significant differences between the experimental groups were found for creatinine, lipase, low-density lipoprotein and magnesium ($p < 0.05$) (Figure 2 C-F, Table S 6).

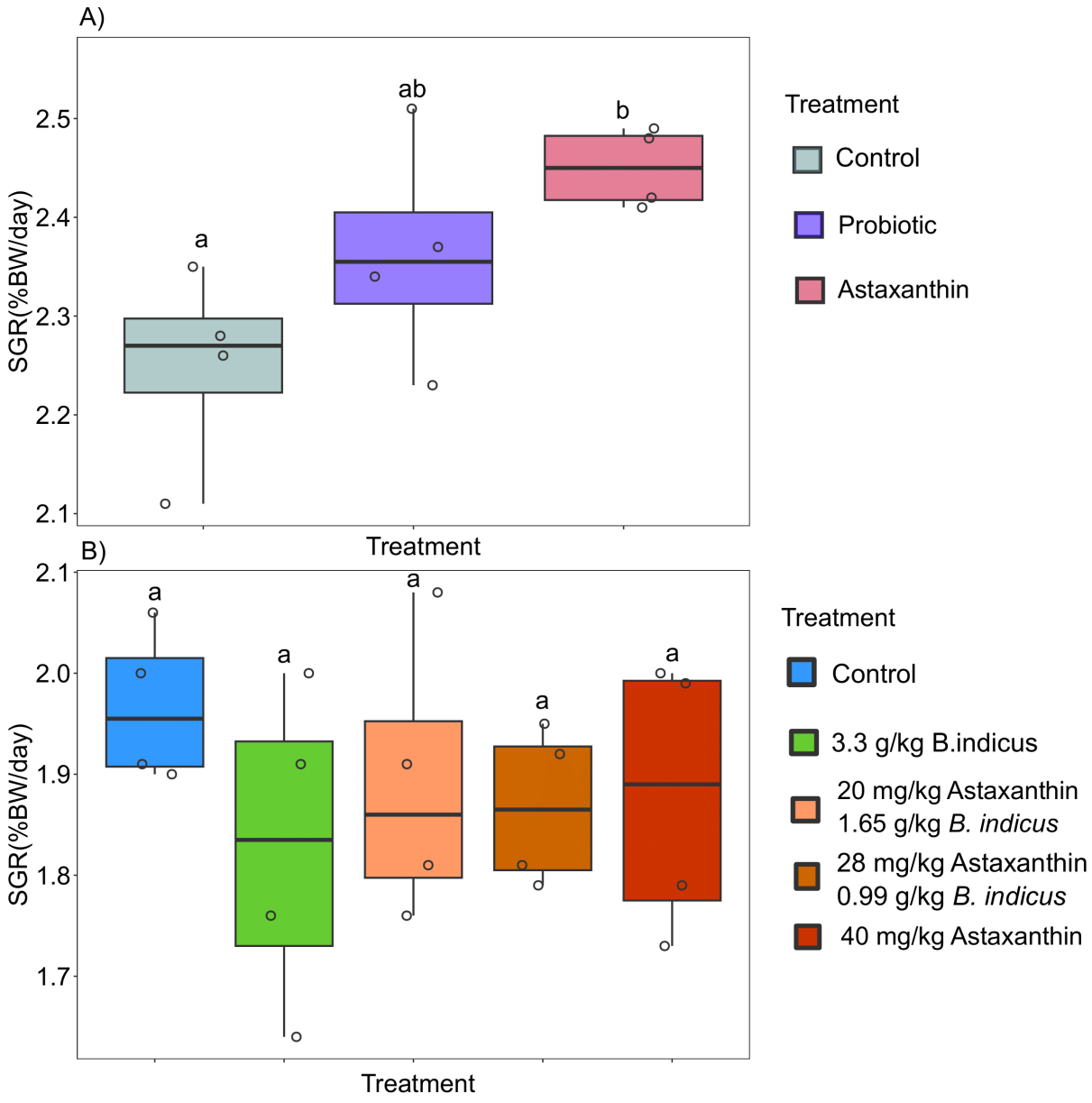


Figure 1: A) Specific Growth Rate (SGR) in carp, Control: standard feed, Probiotic: standard feed + 0.36 g/kg *Bacillus indicus* + 1 g/kg *Bacillus subtilis*, Astaxanthin: standard feed + 40 mg/kg astaxanthin, letters indicate significant ($p<0.05$) differences between the treatment groups). B) SGR in goldfish

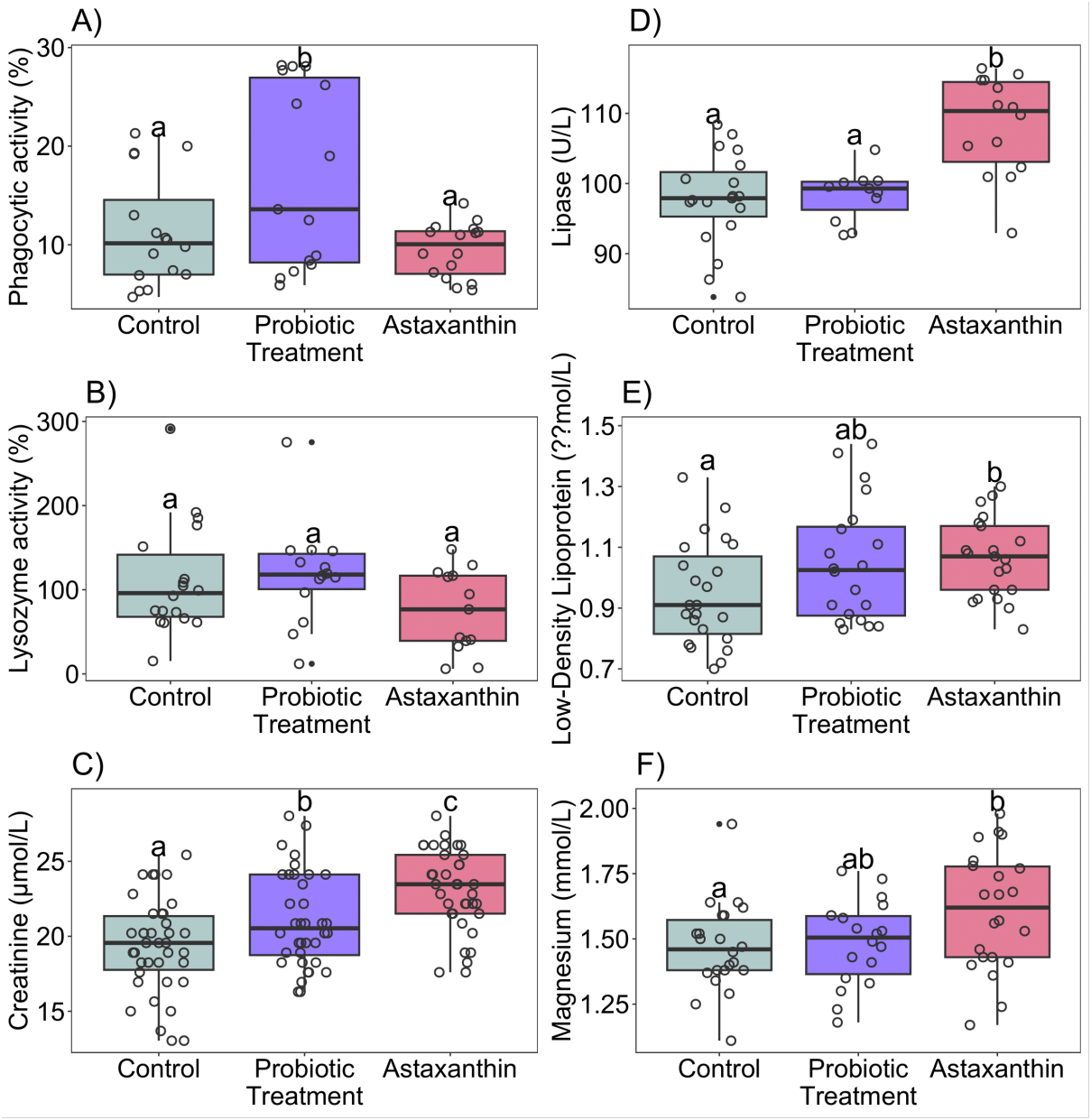


Figure 2: Carp immunological results: A) Phagocytic activity, B) Lysozyme activity, haematological analysis: C) Creatine, D) Lipase, E) Low-Density Lipoprotein, F) Magnesium. Letters indicate significant ($p<0.05$) differences between the treatment groups. Control: standard feed, Probiotic: standard feed + 0.36 g/kg Bacillus indicus + 1 g/kg Bacillus subtilis, Astaxanthin: standard feed + 40 mg/kg astaxanthin).

For the goldfish trial, no significant results were determined for any growth performance indicators (SGR, FI, FCR) between the experimental groups (Table S 4). Over the 8-week experimental period, no mortalities occurred across all tanks (survival 100%). However, the pigmentation analysis revealed a significant colouration effect of

the skin for fish fed astaxanthin, indicated by significantly increased chroma values compared to the control ($p < 0.0001$) Figure 3, Table S 5). In contrast, the probiotic experimental diet did not affect chroma levels.

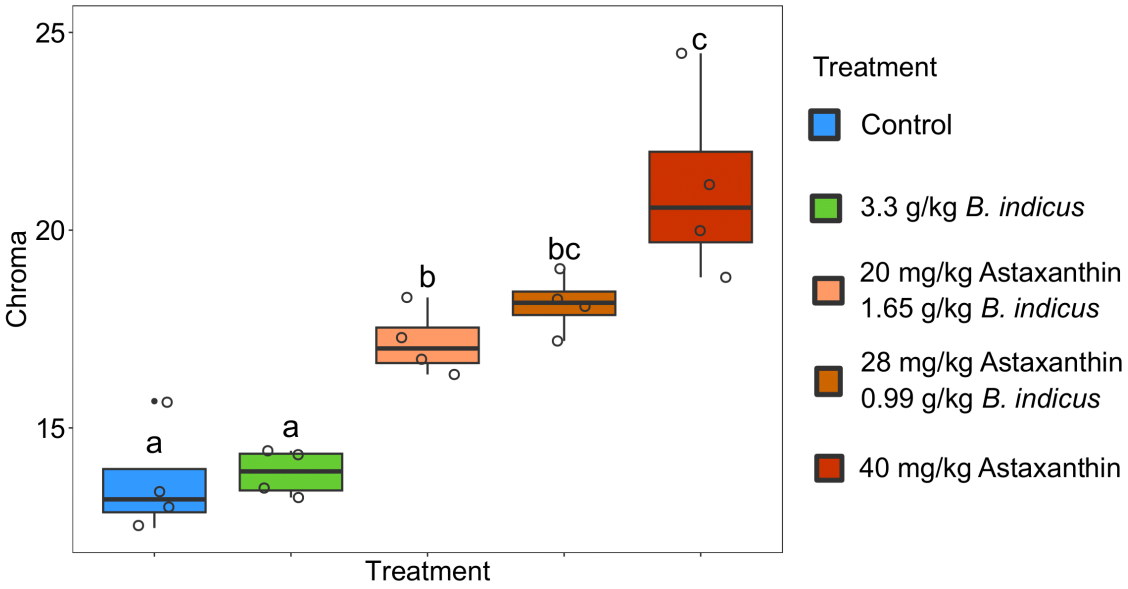


Figure 3: Goldfish chroma levels of the skin, letters indicate significant ($p<0.05$) differences between the treatment groups.

Microbiota profiling

Overall, a total of 6 million raw read pairs were produced from the 110 sequenced samples. After filtering and data pre-processing, a total of 5.2 million reads (average reads per sample 42,276 range = 24,114– 332,946) were retained. Rarefaction curves confirmed that a minimum read depth of 10,000 reads was sufficient to reach saturation of diversity in the intestine of carp and goldfish. For diversity tests, gastrointestinal samples were rarefied to the smallest number of reads. Carp samples were rarefied to 24,114 reads per sample and goldfish intestine samples were rarefied to 25,545 reads per sample. In total, 535 ASVs for the carp and 409 ASVs for the goldfish were retained for further analysis.

Shannon alpha diversity on ASV level was significantly increased in fish fed the probiotic ($p = 0.04$) and astaxanthin ($p = 0.003$) compared to the control group. Similar to Shannon diversity, Chao1 was higher for carp fed the probiotic and astaxanthin, although only astaxanthin induced significant changes ($p = 0.008$, Figure 4 A). Moreover, beta diversity analyses revealed a significant shift in the microbial community in fish-fed astaxanthin compared to the control group ($p = 0.006$). The second-biggest driver of group differences was the probiotic supplement, although not significant (Figure 4 A & C).

In carp, *Proteobacteria* and *Fusobacteria* represent the dominant phyla of the microbial community in the distal gastrointestinal tract (Figure 5 A.). At the genus level, the bacterial community was dominated by *Cetobacterium* and *Aeromonas*. In addition, deseq2 analysis revealed a vast amount of significant differential ASVs between the carp fed a supplement and the control group. Among the differentially abundant ASVs, the majority were significantly more abundant in carp fed a supplement (including various ASVs of the genus *Bacillus*), with few ASVs significantly more abundant in the control group (e.g., *ZOR0006*, *Roseomonas* and *Comamonas*) (Figure 6). For fish fed astaxanthin, all significantly different ASVs were more abundant in fish fed the supplement compared to the control group including *Chryseobacterium*, *Runnella* and *Streptococcus* species Figure 6. Overall, out of all (37) significant differentially abundant genera, 51.4 % (19) were shared between both supplements, while 45.7 % (17) of the genera are only differentially abundant in the probiotic treatment group. One single genus was exclusively found in the astaxanthin treatment group (Table S 8).

The metabolic prediction analysis with PICRUSt and subsequent statistical assessment with Deseq2 revealed significantly different metabolic pathways between the treatment groups in carp (Figure 7, Figure S 1). In carp fed astaxanthin and the probiotic, the majority were classed as degradation (e.g., carbohydrates and aromatic compounds) and generation of precursor metabolites and energy (e.g. TCA cycle and glycolysis), which were increased compared to the control group (Figure 7 B & C, Figure S 1 B & C). In contrast, metabolic pathways involved in biosynthesis (e.g., amino acids and metabolic regulators) were increased in the control group (Figure 7 A, Figure S1 A). Comparing the two supplements, more metabolic pathways were increased in carp fed the probiotic compared to astaxanthin (36 pathways vs 31) (Figure 7, Figure S1).

The microbial community in the goldfish indicated no significant differences in alpha or beta diversity measures (Figure 4 A & C), with very similar taxa dominating the microbial communities in all treatment groups (Figure 5 B). Again, *Proteobacteria* and *Fusobacteria* were the main phyla and similar to the carp, *Cetobacterium* was by far the most dominant genus, followed by *Aeromonas* and *Bacteroides* (Figure 5 B). In goldfish, only two differential abundant bacteria were found at ASV level. *Methylotenera* was consistently reduced between all treatment groups versus the control. In fish fed 40mg/kg of astaxanthin, *Gordonia* was more abundant compared to the control.

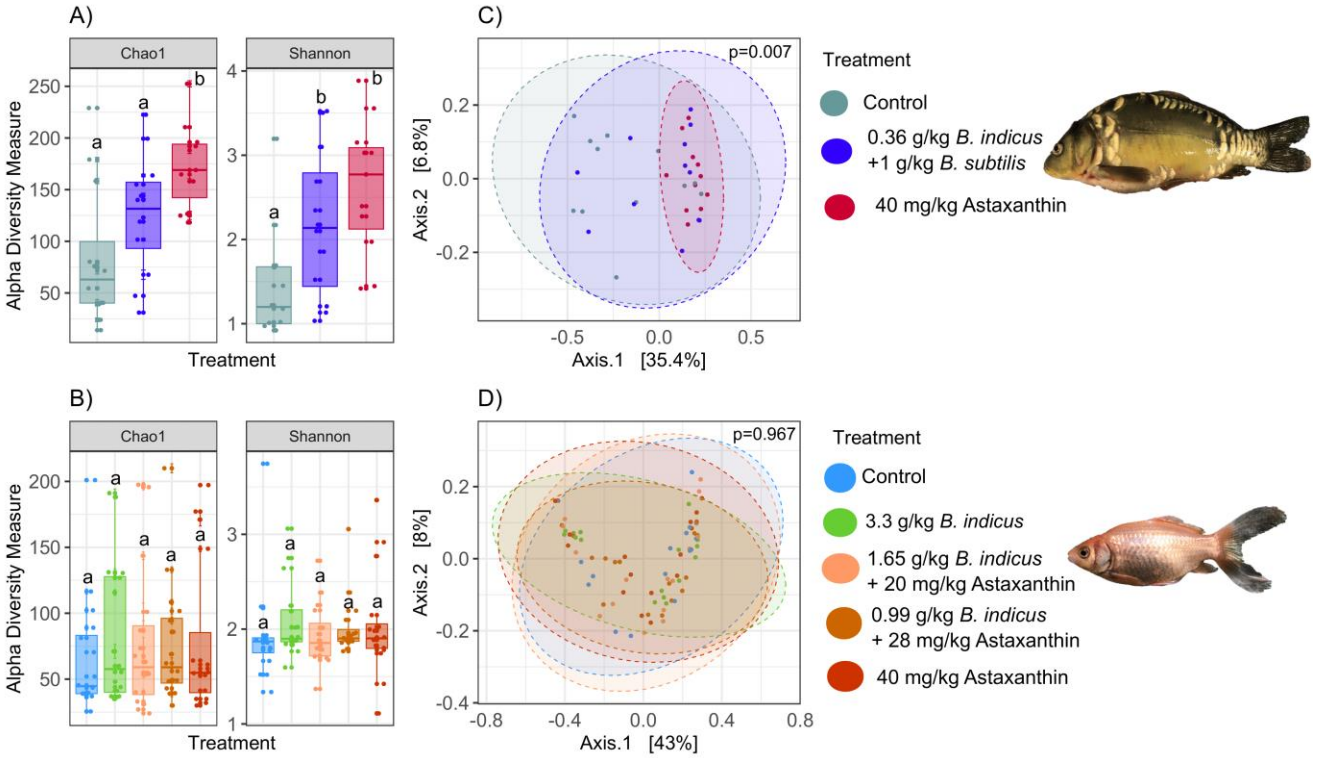
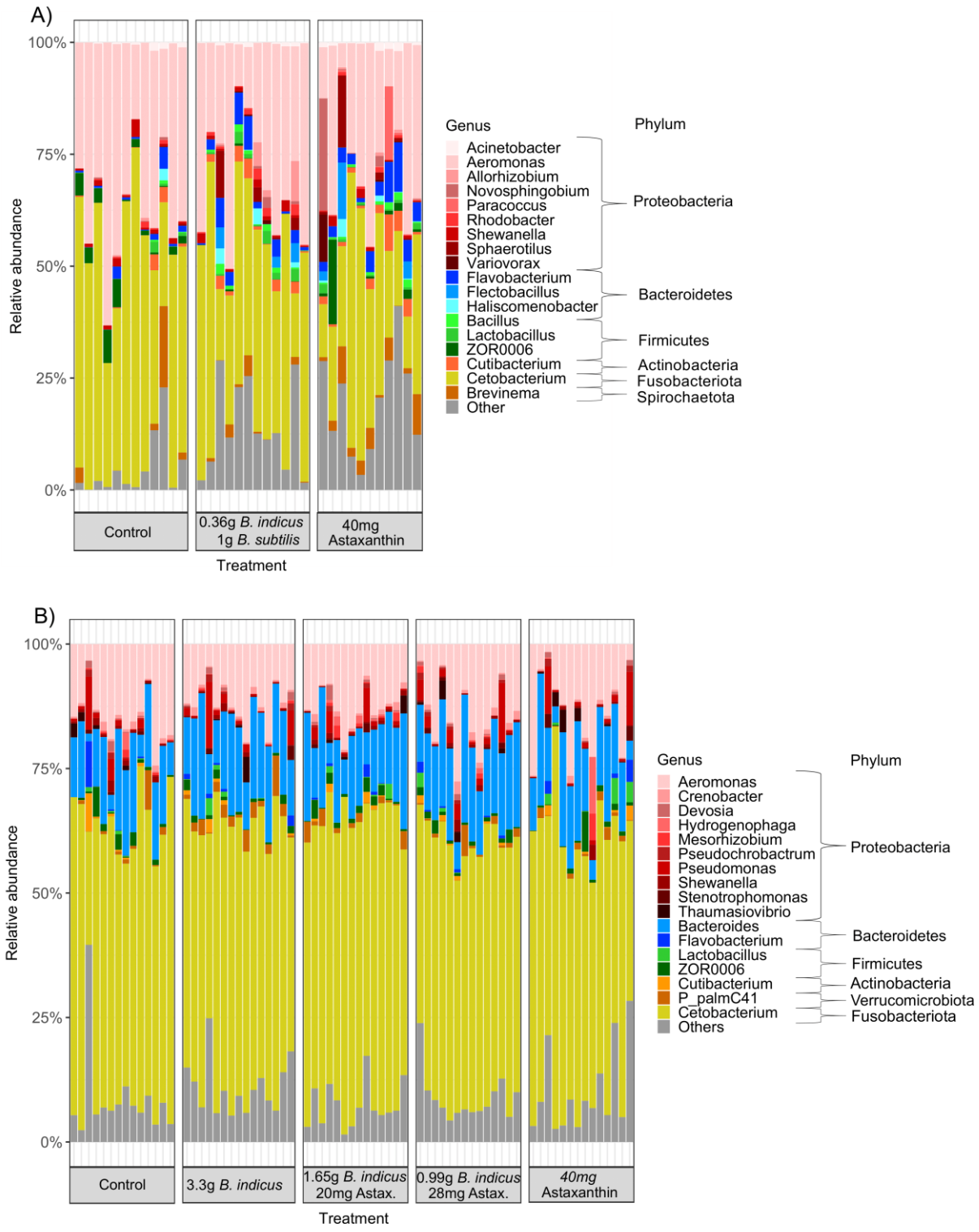


Figure 4: Diversity measures of the microbial community in carp (A & C) and goldfish (B & D) under probiotic inclusion levels and the supplementation of astaxanthin. Alpha diversity was measured by Chao1 and Shannon indices in the A) goldfish and B) carp. PCoA of beta diversity values of C) goldfish and D) carp communities (unweighted Unifrac distances). Ellipses indicate 95% confidence.



372

373 Figure 5: Relative abundance of the top 20 genera of the microbial community of carp
374 (A) and goldfish (B), colour shades separate taxa at the Phylum level.

375

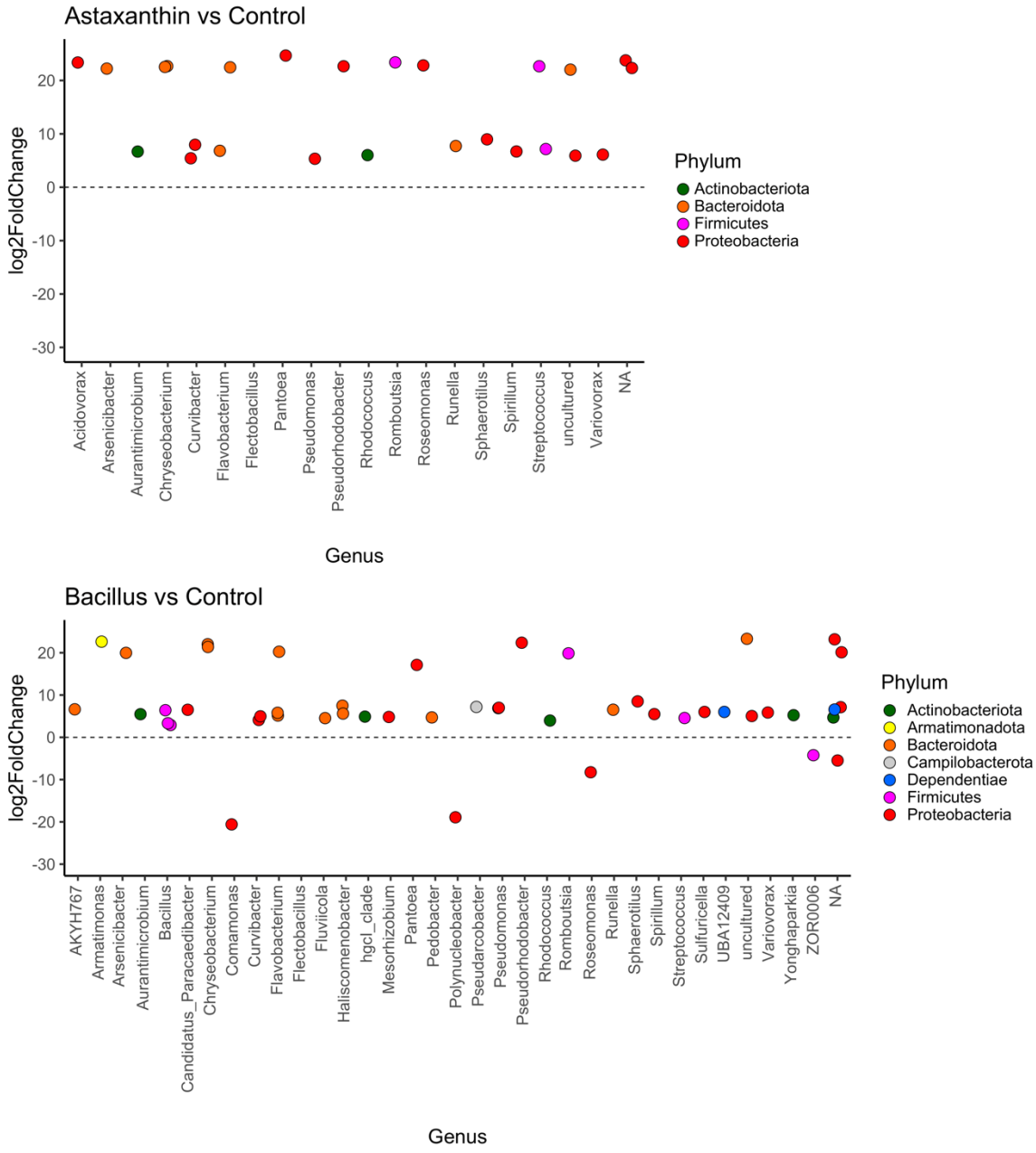
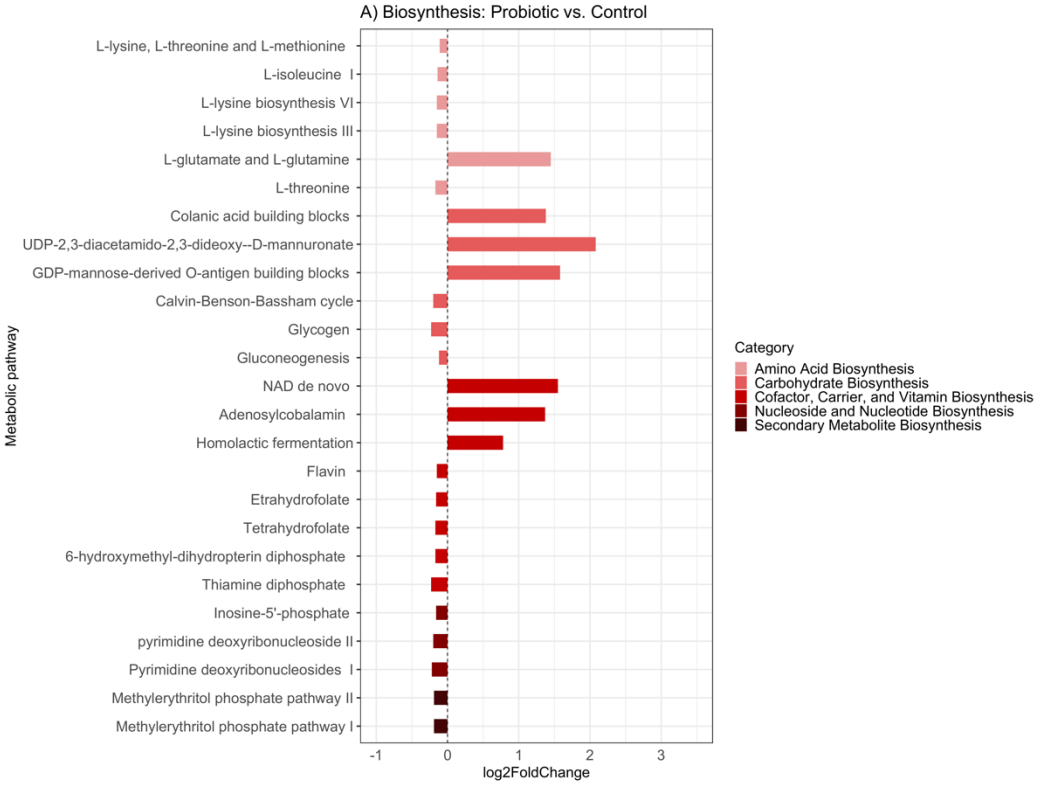
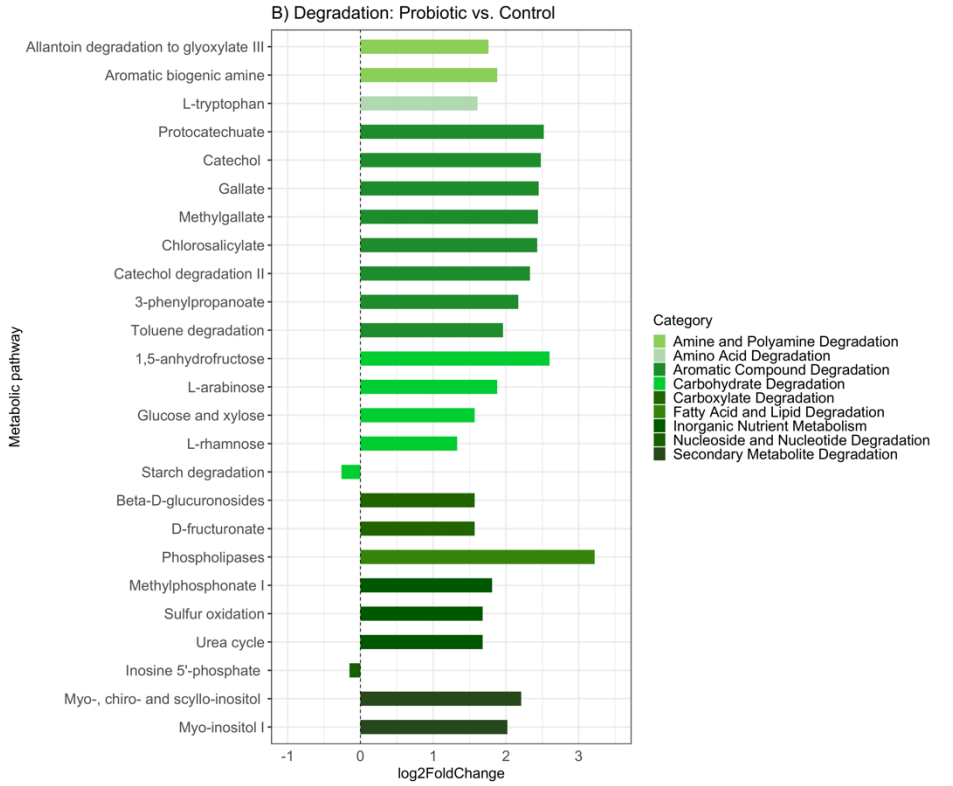


Figure 6: Microbial ASVs with significantly different abundances (FDR-corrected p value < 0.05) in carp between astaxanthin and *Bacillus* vs control, determined via DESeq 2 analyses. Taxa above the dotted line are more abundant in the supplement groups, below the line taxa are more abundant in the control. ASVs summarized at the x-axis to genus level, colours distinguish between Phylum levels.


A) Biosynthesis: Probiotic vs. Control
L-lysine, L-threonine and L-methionine
L-isoleucine I
L-lysine biosynthesis VI
L-lysine biosynthesis III
L-glutamate and L-glutamine
L-threonine
Colanic acid building blocks
UDP-2,3-diacetamido-2,3-dideoxy--D-mannuronate
GDP-mannose-derived O-antigen building blocks
Calvin-Benson-Bassham cycle
Glycogen
Gluconeogenesis
NAD de novo
Adenosylcobalamin
Homolactic fermentation
Flavin
Etrahydrofolate
Tetrahydrofolate
6-hydroxymethyl-dihydropterin diphosphate
Thiamine diphosphate
Inosine-5'-phosphate
pyrimidine deoxyribonucleoside II
Pyrimidine deoxyribonucleosides I
Methylerythritol phosphate pathway II
Methylerythritol phosphate pathway I
Metabolic pathway
-1
0
1
2
3
log2FoldChange
Category
Amino Acid Biosynthesis
Carbohydrate Biosynthesis
Cofactor, Carrier, and Vitamin Biosynthesis
Nucleoside and Nucleotide Biosynthesis
Secondary Metabolite Biosynthesis


B) Degradation: Probiotic vs. Control
Allantoin degradation to glyoxylate III
Aromatic biogenic amine
L-tryptophan
Protocatechuate
Catechol
Gallate
Methylgallate
Chlorosalicylate
Catechol degradation II
3-phenylpropanoate
Toluene degradation
1,5-anhydrofructose
L-arabinose
Glucose and xylose
L-rhamnose
Starch degradation
Beta-D-glucuronosides
D-fructuronate
Phospholipases
Methylphosphonate I
Sulfur oxidation
Urea cycle
Inosine 5'-phosphate
Myo-, chiro- and scyllo-inositol
Myo-inositol I
Metabolic pathway
-1
0
1
2
3
log2FoldChange
Category
Amine and Polyamine Degradation
Amino Acid Degradation
Aromatic Compound Degradation
Carbohydrate Degradation
Carboxylate Degradation
Fatty Acid and Lipid Degradation
Inorganic Nutrient Metabolism
Nucleoside and Nucleotide Degradation
Secondary Metabolite Degradation

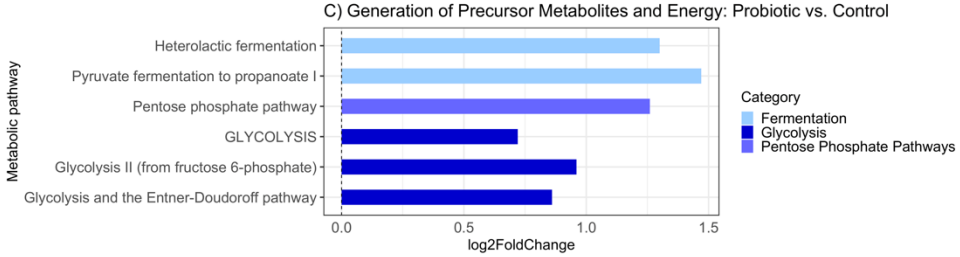

C) Generation of Precursor Metabolites and Energy: Probiotic vs. Control
Heterolactic fermentation
Pyruvate fermentation to propanoate I
Pentose phosphate pathway
GLYCOLYSIS
Glycolysis II (from fructose 6-phosphate)
Glycolysis and the Entner-Doudoroff pathway
Metabolic pathway
0.0
0.5
1.0
1.5
log2FoldChange
Category
Fermentation
Glycolysis
Pentose Phosphate Pathways

Figure 7: Results of metabolic pathway predictions using PICRUSt and differential abundance analysis with Deseq2 between the control and the probiotic supplement. A maximum of 25 significant ($p < 0.05$) pathways are summarized in each plot.

Discussion

The present study assessed the suitability of novel probiotic blends (*B. indicus & B. subtilis*) to replace astaxanthin as health and/or colouration promoters in two cyprinid species (mirror carp and goldfish), using combinations of growth performance indicators, gastrointestinal microbiota profiling, haematology/immunology and skin colour metrics. We demonstrate that supplementation of both the probiotic and astaxanthin in carp substantially shifted their gastrointestinal microbial communities and improved several immune/health indices. However, only astaxanthin supplementation significantly increased growth rates. In contrast, no supplement tested significantly changed the growth or the gastrointestinal microbiota in goldfish. In addition, *B. indicus* did not affect the colouration of the skin. As part of our microbial metabarcoding analyses, we did not detect the supplemented bacteria species in the distal intestine of either fish species. Our results demonstrate variable effects of probiotics even in closely related species, highlighting the need for further in-depth experiments to establish the efficacy and site of colonization of the supplemented bacteria in the fish gastrointestinal tract, and the mechanisms underlying the observed shifts in the host microbiota.

In our goldfish experimental study, we assessed *B. indicus* as a potential replacement for astaxanthin as a colourant of the skin. The colouration analysis determined a significant positive correlation between increasing astaxanthin levels and the Chroma

("orangeness") of the skin, as expected from the results of comparable studies (Paripatananont *et al.*, 1999a). However, the supplementation of *B. indicus* resulted in no impact on skin colouration (Figure 3, Table S 4). *B. indicus* was selected for this experiment based on its ability to synthesise carotenoids (Khaneja et al., 2010; Sy et al., 2013). *B. indicus* was originally sourced from human faeces and a substantial change in host environmental conditions could prevent the probiotic colonization and/or synthesis of carotenoids in the fish gastrointestinal tract (Duc *et al.*, 2006). Alternatively, the carotenoids produced by *B. indicus* may be unable to be utilised by fish. Further work to improve understanding of carotenoid uptake and metabolism in fish will be critical in finding alternatives to synthetic astaxanthin (Sy *et al.*, 2015b; Li *et al.*, 2019).

In carp, only astaxanthin-supplemented growth performance was significantly improved (Figure 1, Table S 3). Although not statistically significant, probiotic supplementation also showed a trend towards higher growth rates (Figure 1, Table S3). However, longer experimental trials and/or adjustments of the probiotic inclusion levels are required to conclusively determine its efficacy for aquaculture productivity. *B. subtilis* is a widely used probiotic with variable effects on growth performance in fish. Studies in grass carp, tilapia, and trout, demonstrate probiotic supplementation with *B. subtilis* increases growth performance significantly (Bagheri *et al.*, 2008; Abarike *et al.*, 2018; Guo *et al.*, 2022; Liu *et al.*, 2022), while (Merrifield, Harper, *et al.*, 2010; Di *et al.*, 2019) reported no impact on growth in trout and sturgeon respectively. In contrast, astaxanthin is a more established growth supplement with predominantly consistent improvements in performance in a variety of fish species (Lim *et al.*, 2018; Sadraddin *et al.*, 2019; Abdulrahman, 2020; Wu and Xu, 2021). To our knowledge, *B.*

indicus has not been tested so far as a probiotic feed additive in any fish species but is considered a promising candidate species due to its ability to produce carotenoids (Khaneja *et al.*, 2010; Sy *et al.*, 2015a), and thus provide similar health and/or colouration benefits as astaxanthin. In our study of goldfish, no effect on growth performance was detected when astaxanthin or *B. indicus* was added to the feed. No literature is available for *B. indicus* in fish, nevertheless, our results for goldfish fed astaxanthin are similar to previous studies, suggesting no effect of astaxanthin on growth performance in this species (Xu *et al.*, 2006). Although no effect on growth performance, the supplementation of astaxanthin can significantly increase survival in the juvenile stage of goldfish (Paripatananont *et al.*, 1999b; Xu *et al.*, 2006; Yeşilayer *et al.*, 2011).

Significantly improved growth in carp fed astaxanthin was supported by our haematological analysis of blood samples. Results of the haematological analysis revealed significantly increased levels of creatinine, lipase, lipoprotein and magnesium in fish fed astaxanthin (Table S 7, Figure 2). As demonstrated in other fish studies, increased lipase, lipoprotein and creatinine levels indicate enhanced lipid and protein metabolism, thus explaining the greater growth of the carp fed astaxanthin in this study (Jyothi and Narayan, 2000; Kulkarni and Pruthviraj, 2016; Wu and Xu, 2021).

Metabolic rate and nutrient digestion, and hence, the growth rate of fish is strongly linked to their gastrointestinal microbial community. Gut microbiota plays a key role to support nutrient acquisition e.g., by the production of enzymes and/or synthesis of vitamins (Llewellyn *et al.*, 2014). In addition, gut microbiota contributes to the health of the fish by enhancing immune defence mechanisms and pathogen resistance (Llewellyn et al., 2014; Merrifield and Rodiles, 2015; Perry et al., 2020). Although a

widely used health and growth promoter in aquaculture, our study gives the first insights into how the supplementation of astaxanthin changes the microbial composition in cyprinid species. Overall, we find the distal gastrointestinal microbial community of goldfish and carp is composed predominantly of *Fusobacteriota, Proteobacteria*, *Bacteroidetes*, *Firmicutes* and *Spirochaetota* phyla and is dominated by the genera *Cetobacterium* and *Aeromonas* (Figure 5), resembling microbial community profiles in similar studies of cyprinid species (Li *et al.*, 2015; J. Zhang *et al.*, 2021).

No significant differences between the treatment groups were determined for the dominant phyla and genera. However, at the ASV level in carp, both the probiotic blend and astaxanthin treatment resulted in a significant shift of abundance in many bacterial ASVs compared to the control group. Interestingly, whilst many ASVs (51.4 %) were similarly altered by both supplements, the probiotic blend altered a greater number of ASVs compared to astaxanthin (Table S 8). In addition, our results indicate a substantial alteration in the functioning of the microbial community in carp fed astaxanthin or probiotics (Figure 7, Figure S 1), with the probiotic inducing a wider range of impacts on metabolic pathways including degradation and the generation of precursor metabolites and energy (Figure 7). Increased microbial degradation of, for example, carbohydrates or amino acids may result in improved nutrient digestion and hence the improved growth observed. The probiotic supplement of *B. indicus/B. subtilis* increased the abundance of three *Bacillus* ASVs significantly. Importantly, various *Bacillus* species are considered beneficial bacteria, offering a wide spectrum of nutritional and immune-boosting properties for the host (Kuebutornye, Abarike and Lu, 2019; Kuebutornye et al., 2020).

We demonstrate significantly increased microbial alpha diversity and distinct beta diversity in carp fed the probiotic blend and astaxanthin (Figure 4). However, no differences in diversity measures were found between the goldfish treatment groups. Greater microbial diversity has been strongly linked with improved growth, health and survival in fish (Li et al., 2017; de Bruijn et al., 2018). In contrast, dysbiosis, a loss of microbial diversity and/or expansion of potentially harmful bacteria, is common in sick and slow-growing fish (Infante-Villamil, Huerlimann and Jerry, 2021). Our results are similar to previous studies of *B. subtilis* supplementation in various fish species, which show greater microbial diversity, enhanced immune response with increased disease resistance, and higher stress tolerance (Kuebutornye, Abarike and Lu, 2019; Kuebutornye *et al.*, 2020; Du *et al.*, 2021). In contrast, there are no previously published microbiota studies of *B. indicus* supplementation in fish.

In addition to the abundance of promising beneficial bacterial taxa and increased microbial diversity, the immunology analysis of the head kidney in carp revealed promising results induced by the probiotic. Phagocytic activity was significantly increased in fish fed the probiotic (Figure 2). Increased phagocytic activity suggests a stimulation of the fish's nonspecific immune response through the probiotic supplement, that can enhance overall host disease resistance (Rahimi *et al.*, 2022). Our findings resemble study outcomes in various fish species and crustaceans demonstrating increased phagocytic activity when being fed *B. subtilis*. Moreover, authors report that *B. subtilis* increased survival when being challenged with a pathogen (*Vibrio alginolyticus* or Singapore grouper iridovirus (SGIV)) (Newaj-Fyzul *et al.*, 2007; Tseng *et al.*, 2009; Zhou *et al.*, 2019). Taken together, the significant impact

of the probiotic supplement on the microbial community and the immunological stimulation suggests a positive effect on the carp's health.

Despite the probiotic treatments substantially altering gut microbiota, the supplemented species could not be detected using 16S rRNA profiling of the distal intestine in carp and goldfish. One possible explanation for this result is that *B. indicus/B. subtilis* colonize a different, non-examined part of the gastrointestinal tract and/or colonize temporarily the digesta (Gajardo et al., 2016; Zhang et al., 2019). Moreover, we confirmed *B. indicus* in the feed of carp and goldfish, while *B. subtillis* could not be detected in the carp feed (Figure S 2). This suggests that we should detect any present *B. indicus* in the sampled section of the gut. *B. subtillis* could not be determined on the species level with the primers used for the molecular work, however, it may be still present and only assigned to Genus level. Among microbiota profiling studies of probiotics, only a few detect the supplemented bacteria long-term in the gastrointestinal tract (Wanka *et al.*, 2018; Di *et al.*, 2019; Li *et al.*, 2019; Shi *et al.*, 2020; Guo *et al.*, 2022). Ideally, probiotic supplemented bacteria establish long-term on the mucosal surface of the gut or provide beneficial functions while passing through the digestive tract of the host. Understanding if and how probiotic bacteria colonise host gastrointestinal tracts is crucial for the successful application of probiotics in aquaculture (Merrifield, Dimitroglou, *et al.*, 2010; Merrifield, Harper, *et al.*, 2010). The majority of currently used probiotic bacteria are selected based on *in vitro* experiments of their potential beneficial properties such as antagonistic activity, enzyme production and colonization ability (Banerjee and Ray, 2017; Li *et al.*, 2019). The often-seen poor or short-term colonization of the fish gastrointestinal tract could be due to the origin of the probiotics. Similar to *B. indicus,* many other probiotic

bacteria are sourced from exogenous, non-fish related, terrestrial environments (Li *et al.*, 2019; Wuertz, Schroeder and Wanka, 2021) and the substantial change in the host environment (e.g., pH, temperature) may prevent their growth in the fish gastrointestinal tract. Whilst some exogenously sourced probiotics have been used successfully in a variety of fish species, a greater focus on developing probiotics from naturally fish-associated microbes may prove beneficial (Wanka *et al.*, 2018; Di *et al.*, 2019).

Despite the positive effect of astaxanthin on the skin colouration in goldfish, no significant differences in growth and the microbial community occurred between the treatment groups for this fish. Physiological and/or immunological differences between carp and goldfish may impact the processing of astaxanthin and probiotics in the gastrointestinal tract, leading to different effects on the microbial community in the distal intestine (López-Olmeda, 2017). Moreover, the higher temperature used for raising the goldfish could result in a more robust microbial intestinal community that remains relatively unperturbed by the addition of dietary astaxanthin and/or probiotics (Merrifield and Rodiles, 2015; Vera *et al.*, 2023). Although goldfish and carp are closely related, our results suggest strong species-specific modes of action of the probiotic and astaxanthin (Wuertz, Schroeder and Wanka, 2021). This highlights the pressing need for future research to uncover the underlying species-specific mechanisms of probiotic impacts on fish microbiota and health to increase the broad applicability of such products in aquaculture.

Conclusion

Overall, the supplementation of a probiotic blend (*B. subtilis* and *B. indicus)* has the potential for promoting gut microbial health and improving immune parameters in

mirror carp. However, it is not as effective as a growth promoter as astaxanthin. Carp fed the probiotic showed a significant alteration in the microbial community, similar to astaxanthin, including several indices of potential health benefits such as significantly increased microbial diversity, the abundance of potentially beneficial bacteria and enhanced immunity (increased phagocytic activity). In contrast, no effect on growth or the microbial community was found in goldfish. These substantial differences between closely related species in supplementation outcomes highlight the need for further research into the species specificity of probiotic applications. In addition, our microbial metabarcoding analyses did not detect the supplemented bacteria species in the distal intestine of either fish species. Therefore, to improve the board-scale applicability of probiotics in aquaculture, further research to gain insights into the efficacy and site of colonization of supplemented bacteria in fish gastrointestinal tracts, and the mechanisms underlying observed shifts in host microbiota and links with growth and immunity are urgently needed.

Acknowledgements

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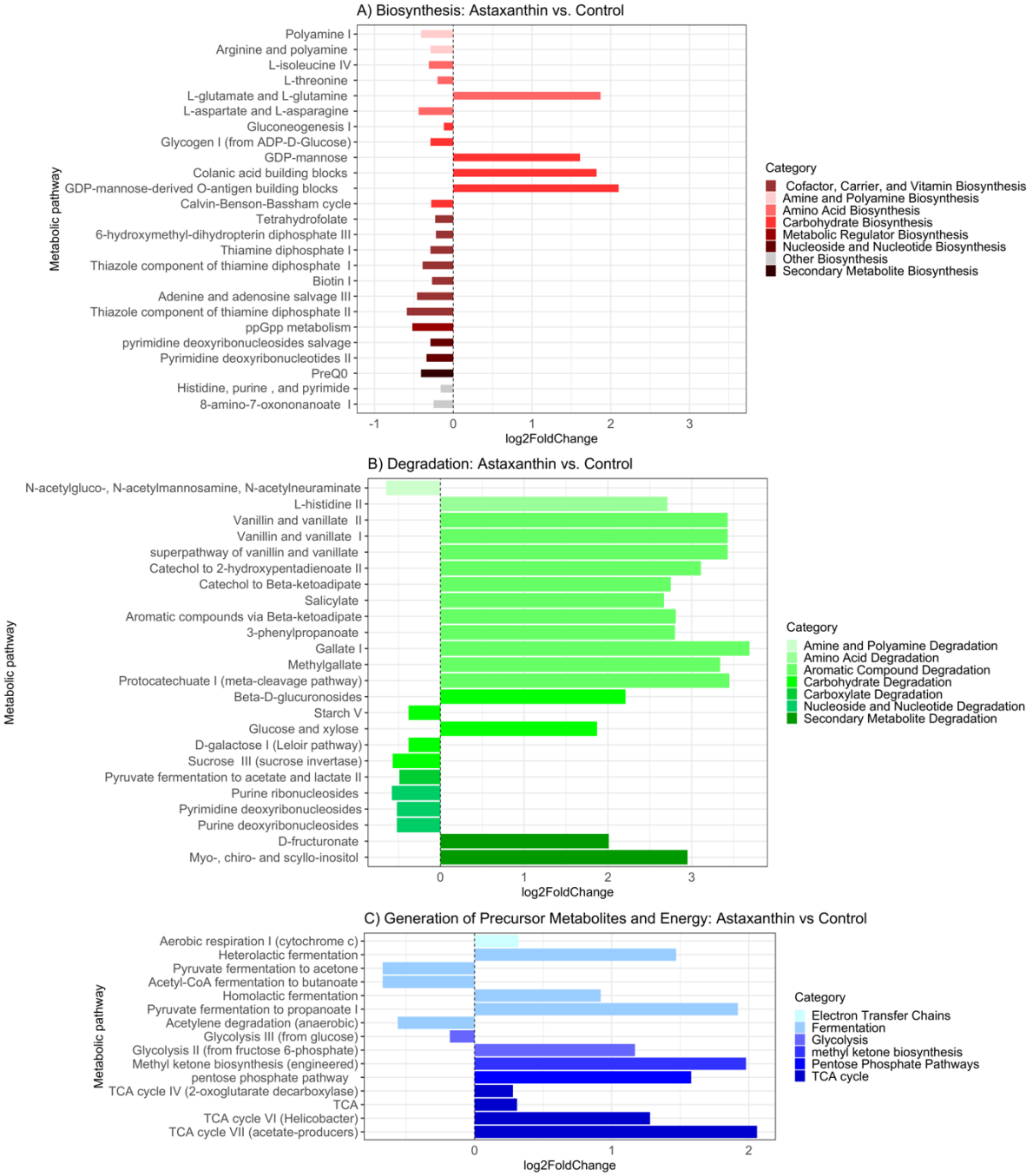
Appendix



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590 Figure S 1: Results of metabolic pathway predictions using PICRUSt and differential
591 abundance analysis with Deseq2 between the control and Astaxanthin as a
592 supplement. A maximum of 25 significant ($p < 0.05$) pathways are summarized in each
593 plot.

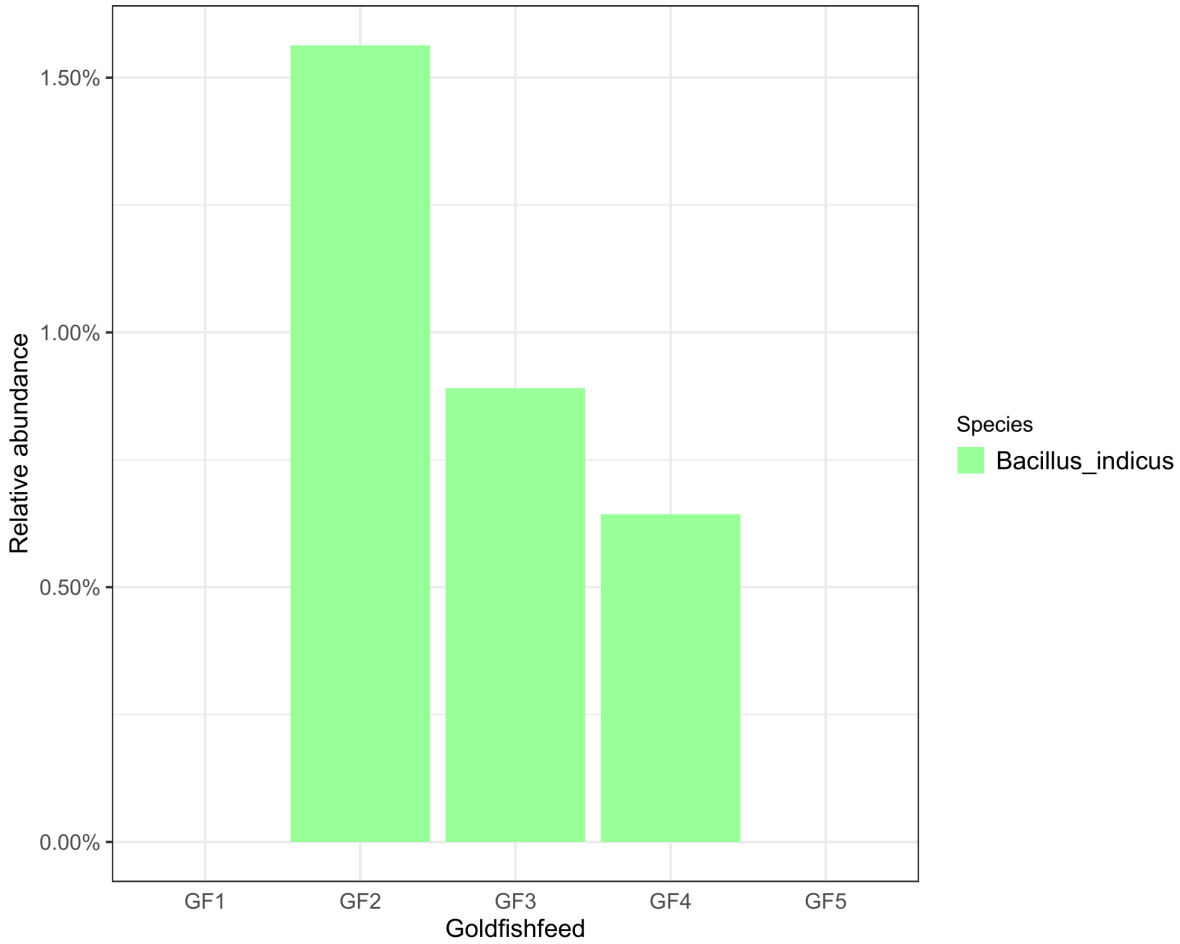

Figure S 2: Goldfish feed, Bacillus indicus

*T*able S 1: Feed formulation based on the nutritional requirement of Mirror carp

Diet	1[Control]	2 [0.36 g/kg B. indicus + 1 g/kg B. subtilis]	3 [40mg/kg astaxanthin]
Wheat meal [g/kg]	423.8	421.6	423.2
Poultry meal [g/kg]	120	120	120
Fishmeal [g/kg]	105	105	105
Soybean meal [g/kg]	90	90	90
Corn gluten meal [g/kg]	65	65	65
Rapeseed meal [g/kg]	65	65	65
Sunflower meal [g/kg]	47.5	47.7	47.5
Fish oil [g/kg]	19	19	19
Rapeseed oil [g/kg]	15	15	15
Vitamin premix [g/kg]	10	10	10
Mineral premix [g/kg]	10	10	10
L-Phenylalanine [g/kg]	8.1	8.1	8.1

L-Lysine [g/kg]	7.9	7.9	7.9
DL-Methionine [g/kg]	6.3	6.3	6.3
Monocalcium phosphate [g/kg]	6	6	6
Antioxidant powder [g/kg]	2	2	2
CarophyllPin10%Astaxanthin [g/kg]	0	0	0.4
B. indicus spores [g/kg]	0	0.36	0
B. subtilis HU 58 spores [g/kg]	0	1	0
Total [g]	1000	1000	1000

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603 Table S 2: Feed formulation based on the nutritional requirement of Red Comet
604 goldfish

Diet	1 [Control]	2 [3.3 g/kg B. indicus]	3 [1.65 g/kg B. indicus + 20 mg/kg astaxanthin]	4 [0.99 g/kg B. indicus + 28 mg/kg astaxanthin]	5 [40 mg/kg astaxanthin]
Wheat meal [g/kg]	274.5	270.7	272.4	273	274
Fishmeal [g/kg]	200	200	200	200	200
Potato starch [g/kg]	75	75	75	75	75
Wheat gluten [g/kg]	71.2	71.7	71.5	71.4	71.3
Soybean meal [g/kg]	71.5	71.5	71.5	71.5	71.5
Poultry meal [g/kg]	71.5	71.5	71.5	71.5	71.5
Brewer's yeast [g/kg]	71.5	71.5	71.5	71.5	71.5
Haemoglobin powder [g/kg]	71.5	71.5	71.5	71.5	71.5
Soybean oil [g/kg]	5	5	5	5	5
Fish oil [g/kg]	51	51	51	51	51
Soy lecithin [g/kg]	5	5	5	5	5
B. indicus spores [g/kg]	0	3.3	1.7	1	0
Vitamin premix [g/kg]	10	10	10	10	10

Mineral premix [g/kg]	10	10	10	10	10
DL-Methionine [g/kg]	6	6	6	6	6
L-Phenylalanine [g/kg]	4	4	4	4	4
Antioxidant powder Verdilox [g/kg]	2	2	2	2	2
B. indicus spores [g/kg]	0	3.3	1.7	1	0
CarophyllPin 10% astaxanthin [g/kg]	0	0	0.2	0.3	0.4
Vitamin C35 [g/kg]	0.3	0.3	0.3	0.3	0.3
Total [g]	1000	1000	1000	1000	1000

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606 Table S 3: Summary of average growth performance indicators of mirror carp on
607 experimental diets. SGR = Specific Growth Rate, FI=Feed Intake, FCR=Feed
608 Conversion Ratio, Standard deviation added.

Diet	1 [Control]	2 [standard diet + 0.36g/kg *B.indicus* & 1g/kg *B.subtilis]*	3 [standard feed + 40mg/kg astaxanthin]
Start weight (g)	39.87 ± 0.71	40.82 ± 0.55	40.45 ± 0.61
SGR (% bw d-1)	2.25 ± 0.09a	2.36 ± 0.10ab	2.45 ± 0.03b
FI (% bw d-1)	2.43 ± 0.12	2.56 ± 0.10	2.59 ± 0.10
FCR (kg feed/kg gain)	1.11 ± 0.05	1.14 ± 0.05	1.12 ± 0.03
End weight (g)	125.50 ± 6.52a	136.48 ± 8.41ab	141.13 ± 1.66b
Survival (%)	100.00	100.00	100.00

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615 Table S 4: Summary of average growth performance indicators of goldfish on
616 experimental diets including (±) standard deviation SGR = Specific Growth Rate,
617 FI=Feed Intake, FCR=Feed Conversion Ratio,

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Diet	1 [Control]	2 [standard diet + 3.3g/kg *B. indicus*	3 [standard feed + 20mg/kg astaxanthin + 1.65 g/kg *B. indicus*]	4 [standard feed + 28mg/kg astaxanthin + 0.99 g/kg *B. indicus*]	5 [standard feed + 40mg/kg astaxanthin]
Start weight (g)	12.15 ± 0.62	12.50 ± 0.50	12.20 ± 0.61	12.05 ± 0.73	12.00 ± 0.63
SGR (% bw d-1)	1.96 ± 0.07	1.83 ± 0.14	1.89 ± 0.12	1.87 ± 0.07	1.99 ± 0.12
FI (% bw d-1)	3.70 ± 0.24	3.76 ± 0.20	3.71 ± 0.26	3.66 ± 0.16	3.86 ± 0.12
FCR (kg feed/kg gain)	1.94 ± 0.17	2.14 ± 0.25	2.01 ± 0.23	2.01 ± 0.06	2.10 ± 0.14
End weight (g)	36.60 ± 2.56	34.85 ± 2.75	35.25 ± 2.84	34.25 ± 0.94	34.30 ± 1.10
Survival (%)	100.00	100.00	100.00	100.00	100.00

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630 Table S 5: Summary of the pigmentation results of goldfish on experimental diets,
631 including (±) standard deviation. Letters indicate significant ($p<0.05$) differences
632 between the experimental groups.

Diet	1 [Control, standard diet]	2 [standard diet + 3.3g/kg *B.indicus*	3 [standard feed + 20mg/kg astaxanthin + 1.65 g/kg *B.indicus*]	4 [standard feed + 28mg/kg astaxanthin + 0.99 g/kg *B.indicus*]	5 [standard feed + 40mg/kg astaxanthin]
Lightness	79.80 ±0.56	79.25 ± 0.59	78.71 ± 0.10	78.28 ± 0.98	79.65 ± 1.11
Hue	1.17 ± 0.08	1.17 ± 0.04	1.13 ± 0.04	1.14 ± 0.04	1.15 ± 0.03
Chroma	**13.63 ± 1.22a**	**13.86 ± 0.51a**	**17.17 ± 0.73b**	**18.14 ± 0.65bc**	**21.11 ± 2.11c**

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635 Table S 6: Summary of the carp immunological analysis including head kidney, whole
636 blood and plasma samples, standard deviation added. Letters indicate significant
637 ($p<0.05$) differences between the treatment groups.

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Analysis	1 [Control, standard diet]	2 [standard diet + 0.36g/kg *B. indicus* & 1g/kg *B. subtilis*	3 [standard feed + 40mg/kg astaxanthin]
Total protein	30.7 ± 3.5	29.7 ± 4.4	29.7 ± 5.0
Total plasma IgM	3.21 ± 0.44	3.24 ± 0.62	3.23 ± 0.55
Plasma peroxidase activity	0.208 ± 0.158	0.272 ± 0.246	0.194 ± 0.157
Plasma anti-protease activity	80.2 ± 1.6	79.4 ± 1.9	78.9 ± 2.6
Plasma lysozyme activity	74.7 ± 48.91	118.2 ± 60.41	111.3 ± 65.82
Plasma complement activity	103.8 ± 26.8	134.3 ± 112.5	123.3 ± 36.7
Respiratory burst activity (NBT +PMA values)	0.426 ± 0.057	0.463 ± 0.07	0.434 ± 0.049
Respiratory burst activity (NBT +PMA values)	0.430 ± 0.067	0.423 ± 0.05	0.429 ± 0.049
Phagocytic activity	**11.2 ± 5.7a**	**16.9 ± 9.0b**	**9.5 ± 2.8a**

B and T lymphocytes in blood (%)	3.0 ± 1.3	3.4 ± 1.4	3.6 ± 1.5

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640 Table S 7: Summary of the carp haematological analysis of blood samples, standard
641 deviation added. Letters indicate significant ($p<0.05$) differences between the
642 treatment groups.

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Analysis	1 [Control, standard diet]	2 [standard diet + 0.36g/kg *B. indicus* & 1g/kg *B. subtilis*	3 [standard feed + 40mg/kg astaxanthin]
Alanine Aminotransferase (U/L)	19.23 ± 2.97	18.49 ± 2.70	19.34 ± 3.11
Albumin (g/L)	13.26 ± 0.71	13.00 ± 0.84	13.31 ± 0.79
Aldosterone (U/L)	203.63 ± 91.99	174.81 ± 84.97	177.36 ± 79.40
Alkaline Phosphatase (U/L)	51.19 ± 19.7	51.76 ± 20.31	48.49 ± 20.83
Ammonia (µmol/L)	482.40 ± 147.14	476.60 ± 137.99	507.09 ± 124.87
Amylase (U/L)	119.63 ± 16.66	121.81 ± 14.80	125.67 ± 13.68
Calcium (mmol/L)	2.09 ± 0.33	2.10 ± 0.29	2.09 ± 0.31
Carbon dioxide (mmol/L)	15.36 ± 1.82	15.09 ± 1.72	15.26 ± 1.56
Chloride (mmol/L)	114.20 ± 3.62	112.74 ± 4.12	112.51 ± 3.44
Copper (µmol/L)	9.11 ± 0.82	8.67 ± 1.51	8.99 ± 1.45
Creatine Kinase (U/L)	10249.34 ± 3699.69	9243.67 ± 3279.61	9664.28 ± 3803.71
Creatine Kinase-MB (U/L)	16988.48 ± 7210.19	14942.18 ± 5214.25	15627.40 ± 6024.35
Creatinine (µmol/L) ***	**19.34 ± 3.11[a]**	**21.06 ± 3.14[b]**	**23.10 ± 2.66[c]**
Globulin (g/L)	11.43 ± 0.89	11.11 ± 1.08	11.00 ± 0.86
High-Density Lipoprotein (mmol/L)	2.97 ± 0.32	2.85 ± 0.39	3.06 ± 0.27
Iron (µmol/L)	1.39 ± 1.06	1.25 ± 0.95	1.41 ± 0.72
Lactate (mmol/L)	3.27 ± 0.86	3.29 ± 0.70	3.49 ± 1.00
Lactate Dehydrogenase (U/L)	794.15 ± 600.45	686.97 ± 546.76	725.53 ± 625.97
Lipase (U/L) ***	**97.75 ± 6.67[a]**	**98.31 ± 3.63[a]**	**108.24 ± 7.02[b]**

Low-Density Lipoprotein (µmol/L) ***	**0.95 ± 0.17[a]**	**1.05 ± 0.20[ab]**	**1.06 ± 0.13[b]**
Magnesium (mmol/L) ***	**1.47 ± 0.17[a]**	**1.49 ± 0.16[ab]**	**1.61 ± 0.23[b]**
Phosphorus (mmol/L)	2.86 ± 0.56	2.92 ± 0.60	2.90 ± 0.60
Potassium (mmol/L)	2.64 ± 0.34	2.61 ± 0.33	2.59 ± 0.30
Total Bilirubin (µmol/L)	4.72 ± 1.77	5.36 ± 2.13	4.62 ± 1.06
Total Cholesterol (mmol/L)	4.36 ± 0.54	4.31 ± 0.55	4.39 ± 0.54
Total Iron-Binding Capacity (µmol/L)	33.96 ± 4.47	34.39 ± 5.03	35.26 ± 4.08
Total Protein (g/L)	24.72 ± 1.48	24.08 ± 1.94	24.45 ± 1.63
Triglycerides (mmol/L)	3.17 ± 0.61	3.10 ± 0.58	3.00 ± 0.56
Zinc (µmol/L)	67.25 ± 7.88	62.47 ± 8.29	67.40 ± 8.32

Table S 8**:** Comparison of differential abundant (ASVs) genera in carp between probiotic vs. control and astaxanthin vs. control

Probiotic (45.7 %)	Common (51.4 %)	Astaxanthin (2.9%)
AKYH767 *Armatimonas* *Bacillus* *Candidatus_Paracaedibacter* *Comamonas* *Fluviicola* *Haliscomenobacter* *hgcI_clade* *Mesorhizobium* *Pedobacter* *Polynucleobacter* *Pseudarcobacter* *Sulfuricella* *UBA12409* *Yonghaparkia* *ZOR0006*	*Arsenicibacter* *Aurantimicrobium* *Chryseobacterium* *Curvibacter* *Flavobacterium* *Flectobacillus* *Pantoea* *Pseudomonas* *Pseudorhodobacter* *Rhodococcus* *Romboutsia* *Roseomonas* *Runella* *Sphaerotilus* *Spirillum* *Streptococcus* *uncultured* *Variovorax*	*Acidovorax*

Metabolic Pathways comparison:

Biosynthesis decreased:

Bacillus 38.9%	Common 11.1%	Astaxanthin 50%
Thiamine diphosphate Pyrimidine deoxyribonucleosides I pyrimidine deoxyribonucleoside II Methylerythritol phosphate pathway I Methylerythritol phosphate pathway II 6-hydroxymethyl-dihydropterin diphosphate Inosine-5'-phosphate Etrahydrofolate Flavin L-lysine biosynthesis III L-lysine biosynthesis VI L-isoleucine I Gluconeogenesis L-lysine, L-threonine and L-methionine	Glycogen Calvin-Benson-Bassham cycle Tetrahydrofolate L-threonine	Thiazole component of thiamine diphosphate II ppGpp metabolism Adenine and adenosine salvage III "L-aspartate and Lasparagine" PreQ0 Polyamine I Thiazole component of thiamine diphosphate I Pyrimidine deoxyribonucleotides II L-isoleucine IV pyrimidine deoxyribonucleosides salvage Thiamine diphosphate I Arginine and polyamine Biotin I 8-amino-7-oxononanoate I 6-hydroxymethyl-dihydropterin diphosphate III Histidine, purine, and pyrimidine Gluconeogenesis I

Biosynthesis increased:

Bacillus 50%	Common 37.5%	Astaxanthin 12.5%
Homolactic fermentation Adenosylcobalamin NAD de novo UDP-2,3-diacetamido-2,3-dideoxy--D-mannuronate	Colanic acid building blocks L-glutamate and L-glutamine GDP-mannose-derived O-antigen building blocks	GDP-mannose

Degradation decreased:

Bacillus 11%	Common 11%	Astaxanthin 77%
Inosine 5'-phosphate	Starch	N-acetylgluco-, N-acetylmannosamine, N-acetylneuraminate Purine ribonucleosides Sucrose III (sucrose invertase)

		Purine deoxyribonucleosides Pyrimidine deoxyribonucleosides Pyruvate fermentation to acetate and lactate II D-galactose I (Leloir pathway)

Degradation increased:

Bacillus 48.5%	Common 21.2%	Astaxanthin 33%
L-rhamnose L-tryptophan Urea cycle Sulfur oxidation Allantoin degradation to glyoxylate III Methylphosphonate I L-arabinose Aromatic biogenic amine Toluene degradation Myo-inositol I Catechol degradation II Chlorosalicylate Catechol Protocatechuate 1,5-anhydrofructose Phospholipases	D-fructuronate Beta-D-glucuronosides Glucose and xylose 3-phenylpropanoate Myo-, chiro- and scyllo-inositol Methylgallate Gallate	Salicylate L-histidine II "Catechol to Beta-ketoadipate " Aromatic compounds via Beta-ketoadipate Catechol to 2-hydroxypentadienoate II superpathway of vanillin and vanillate Vanillin and vanillate I Vanillin and vanillate II Protocatechuate I (meta-cleavage pathway)

Generation of precursor metabolites decreased:

Bacillus	Common	Astaxanthin 100%
		Acetyl-CoA fermentation to butanoate Pyruvate fermentation to acetone Acetylene degradation (anaerobic) Glycolysis III (from glucose)

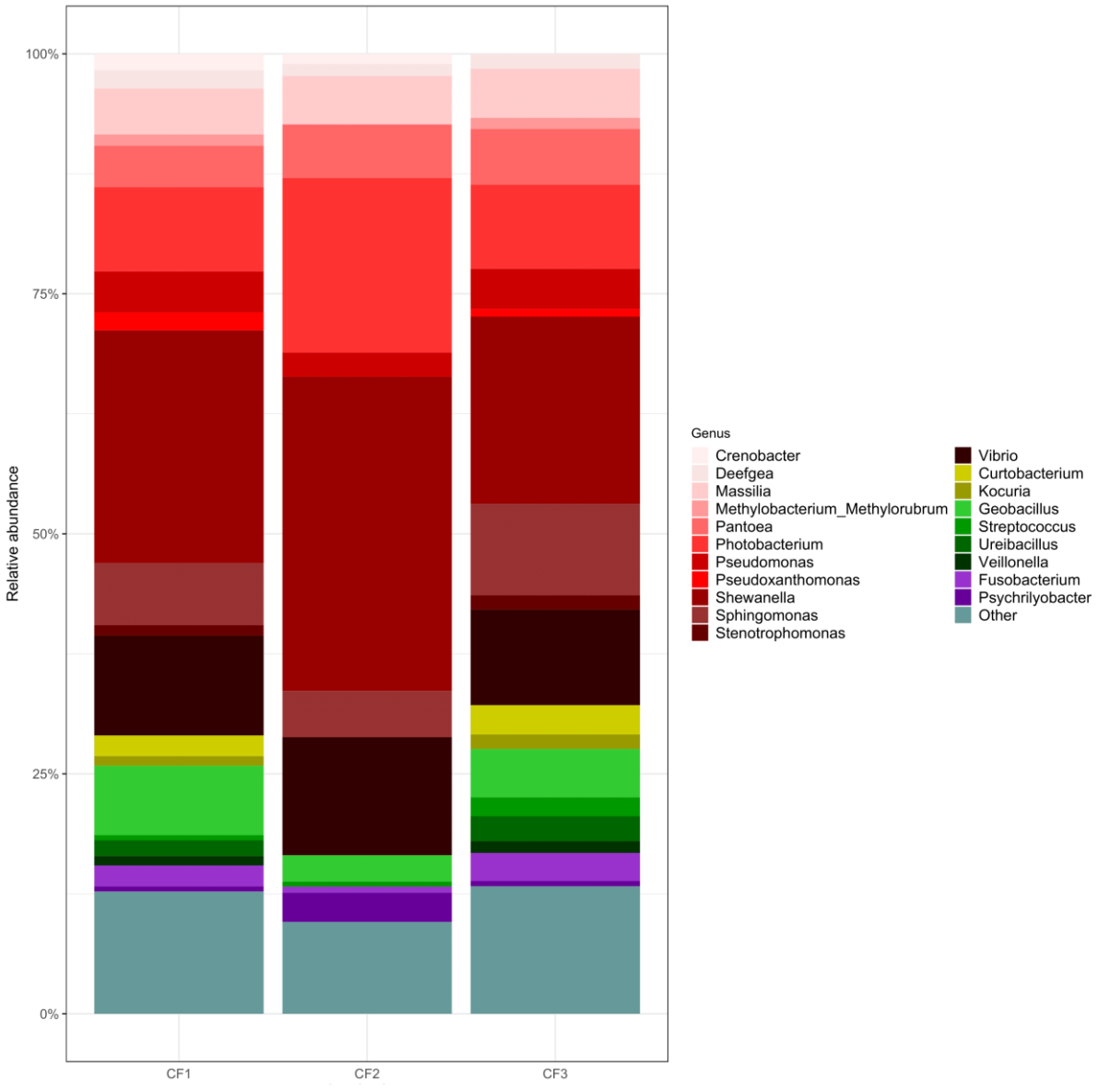
Generation of precursor metabolites increased

Bacillus 21.4%	Common 21.4%	Astaxanthin 57.1%

Glycolysis and the Entner-Doudoroff pathway GLYCOLYSIS Pentose phosphate pathway	Glycolysis II (from fructose 6-phosphate) Pyruvate fermentation to propanoate I Heterolactic fermentationbuilding blocks	TCA cycle IV (2-oxoglutarate decarboxylase) TCA Aerobic respiration I (cytochrome c) Homolactic fermentation TCA cycle VI (Helicobacter) "pentose phosphate pathway " Methyl ketone biosynthesis (engineered) TCA cycle VII (acetate-producers)

666

667



668

Figure 6: Carp feed

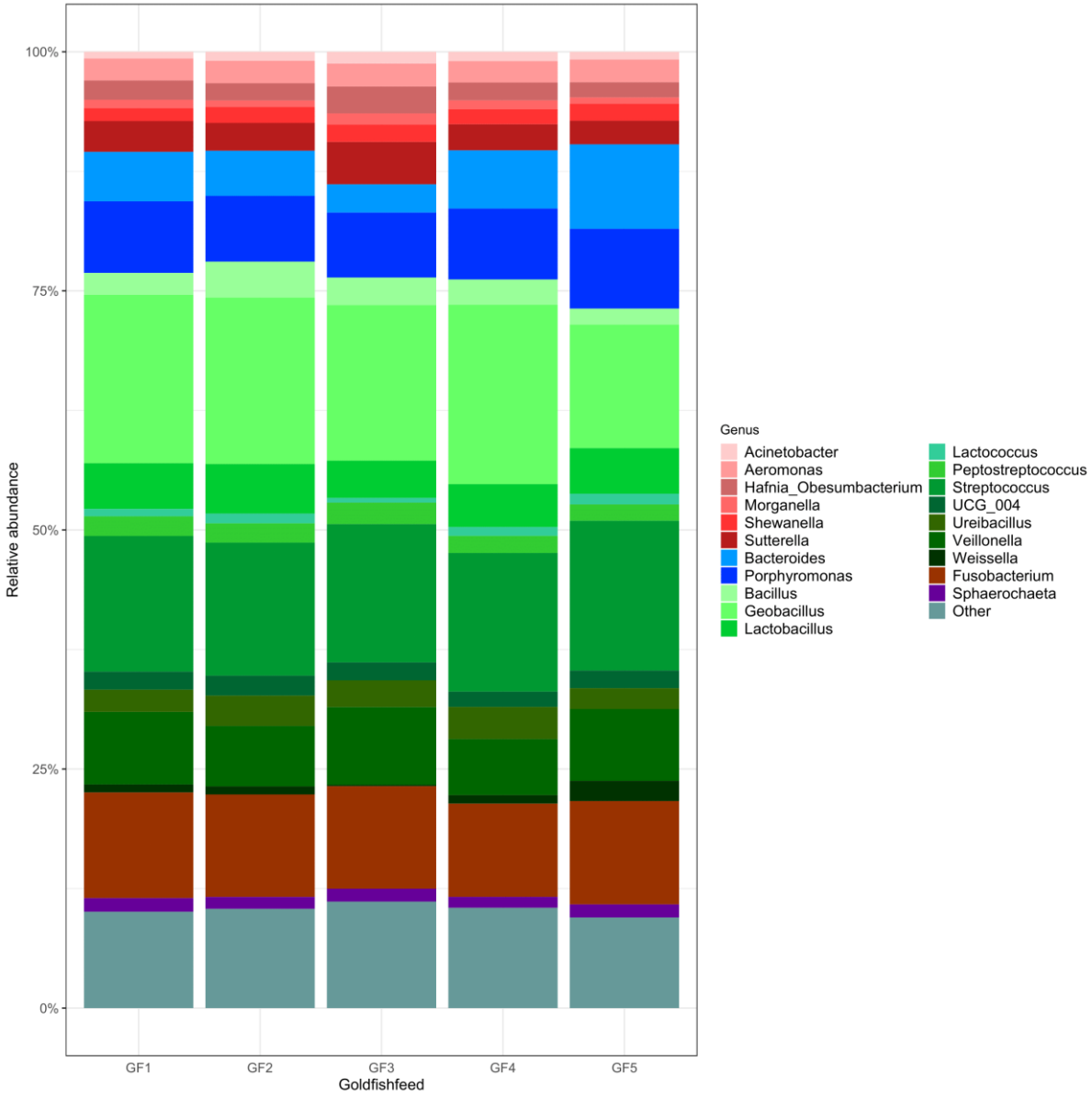


Figure 7: Goldfish feed

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