# Supplementation of Rose Petal (*Rosa rubiginosa* L.) as Functional Feed Additive Enhances Growth, Pigmentation, Immune Response, and Gut Health in Goldfish (*Carassius auratus*)

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# Abstract

Natural additives are increasingly valued in ornamental fish aquaculture. Rose petal contains bioactive compounds, yet their effects on goldfish (*Carassius auratus*) remain underexplored. This study assessed the impacts of graded dietary rose petal supplementation (0, 5, 10, 20, and 40 g/kg; RP-0 to RP-40) over an 8-week feeding trial on growth performance, skin pigmentation, serum antioxidant status, intestinal gene expression, and gut microbiota composition. Fish fed RP-supplemented diets, particularly at 40 g/kg, exhibited significantly higher final weight and weight gain than the RP-0 group ($p < 0.05$), without adverse effects on survival or feed conversion ratio. Skin redness (a*) and yellowness (b*) increased significantly in a dose-dependent manner ($p < 0.05$ at RP-20/40 for a*, RP-40 for b*). Serum antioxidant capacity improved with increasing RP levels, as indicated by higher ABTS and SOD activities and lower MDA levels ($p < 0.05$). Dietary rose petal supplementation also significantly upregulated intestinal expression of antioxidant (*HSP70*, *CYP1A*), growth (*IGF*, *TGF*), and immune (*LYZ*, *TNFα*) genes, primarily at 20–40 g/kg ($p < 0.05$). While rose petal significantly altered gut microbiota composition based on beta diversity (PERMANOVA $p = 0.017$) and specific taxon abundances (e.g., increased *Staphylococcus* at RP-40 and decreased *Alloprevotella* at RP-5; ANCOMBC2, $q < 0.05$), it did not significantly affect alpha diversity or exhibit strong correlations with host physiological parameters after FDR correction. Overall, the results of this study highlights rose petal as a natural functional additive for ornamental aquaculture.


# 1. Introduction

The ornamental fish trade has gained significant popularity in recent years, driven by the high aesthetic and commercial value of many species in the international market. Several ornamental fish are prized for their vivid coloration, diverse body shapes, and distinctive fin structures (Lau et al 2023). Among these, the goldfish (*Carassius auratus*) is one of the most famous and commercially valuable species (Yanar et al 2008). Goldfish have become widespread, with numerous farms and hatcheries established globally (Ota 2021). Their natural beauty and adaptability to a wide range of environmental conditions have contributed to their prominence in the ornamental fish industry (Martinez-Murcia et al 2008; Blanco and Unniappan 2022).

Coloration in goldfish is primarily determined by the distribution and type of pigment cells (Luo et al 2021). In teleosts, six types of pigment cells have been identified: cyanophores, leucophores, iridophores, xanthophores, and melanophores (Goda et al 2013; Zhang et al 2023). Variations in pigment cell types across different body regions lead to diverse color patterns. High carotenoid content, in particular, contributes to the distinctive red coloration of goldfish, enhancing their marketability and consumer acceptance (Gümüş et al 2022; Elshafey et al 2023). Consequently, maintaining vibrant natural pigmentation is critical for market demand and commercial success among fish farmers.

In recent years, there has been growing interest in using natural plant-based ingredients such as leaves, fruit peels, and flowers to enhance pigmentation in ornamental fish (Elshafey et al 2023). Successful intensive aquaculture requires nutritionally balanced diets that include essential nutrients and

carotenoids as dietary supplements (Ahilan et al 2013). Beyond pigmentation, carotenoids are also essential for growth, metabolism, reproduction, and overall health in goldfish (Ahilan et al 2013; Hilal and Duygu 2020; Sathyaruban et al 2021; Kautsar et al 2022; Elshafey et al 2023; Khieokhajonkhet et al 2023). Like other animals, fish cannot synthesize carotenoids de novo and must acquire them through their diet (Elshafey et al 2023). In addition to carotenoids, dietary sources of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are critical for the growth and survival of goldfish, as they cannot biosynthesize these essential fatty acids from shorter-chain precursors (Gümüş et al 2022). The level of dietary carotenoid intake directly influences pigmentation intensity in fish (Sathyaruban et al 2021). Consequently, a wide range of carotenoid sources has been incorporated into aquafeeds, including pure carotenoid extracts (Shahidi and Brown 1998; Yuangsoi et al 2011), animal-derived pigments (Swain et al 2020; Lim et al 2023), and plant-derived pigments (Wallat et al 2005; Harpaz and Padowicz 2007; Wagde et al 2018; Rana et al 2023). Plant-based sources offer dual advantages by providing essential nutrients (e.g., proteins, fats, vitamins) along with high carotenoid content (Ansari et al 2021). Given concerns regarding the cost and safety of synthetic additives, there is an increasing trend toward the use of natural carotenoids as alternatives in aquaculture (Elshafey et al 2023). Roses are recognized as rich natural sources of carotenoids (Wan et al 2018), and species such as China rose (*Hibiscus rosa-sinensis*), sweetbriar rose (*Rosa rubiginosa*), and cyme rose (*Rosa indica*) have been utilized in fish diets to promote growth, enhance immune function, and improve pigmentation (Sinha and Asimi 2007; Joseph et al 2011; Arulvasu et al 2013; Rintan et al 2019). These findings underscore the potential of natural carotenoid supplementation to enhance ornamental fish health and marketability.

Despite these promising outcomes, research specifically examining the effects of rose petal by-products as carotenoid sources in ornamental fish, particularly in goldfish, remains limited. This study seeks to address this knowledge gap by evaluating the effects of rose petal-derived carotenoids on key health indicators in goldfish, including growth performance, immune response, gut microbiota composition, and gene expression.

## 2. Materials and methods

# 2.1. Fish preparation and experimental design

Healthy goldfish (*Carassius auratus*) were obtained from a commercial farm in Chiang Mai Province, Thailand, and transported to the aquaculture research station at the Department of Animal and Aquatic Sciences, Faculty of Agriculture, Chiang Mai University. Upon arrival, fish were acclimated for 14 days in aerated tanks and fed a commercial pelleted diet containing 400 g/kg crude protein, 70 g/kg lipid, and 40 g/kg fiber, administered manually three times daily until apparent satiation (Khieokhajonkhet et al 2025). Prior to the feeding trial, all fish were fasted for 24 hours to standardize gastrointestinal clearance and metabolic conditions.

Following acclimation, 300 fish with an initial average body weight of 6.75–6.90 g was randomly assigned to 15 experimental tanks (50 × 100 × 40 cm; 180 L water volume), corresponding to five dietary

treatment groups with three replicates each. Fish were fed experimental diets twice daily at a rate of 3% body weight, adjusted weekly based on biomass measurements, for 8 weeks. Uneaten feed and waste were siphoned daily, and approximately 10% of the water was replaced with fresh dechlorinated tap water to maintain optimal conditions. Environmental parameters were closely monitored to reduce stress and mimic natural conditions. Tanks were exposed to a 12 h light/12 h dark photoperiod. Water temperature was maintained at 24 ± 1°C, dissolved oxygen levels > 5 mg/L, pH between 7.5–8.0, and ammonium concentrations below 1 mg/L.

## 2.2. Preparation of experimental diets

Five experimental diets were formulated by replacing fish meal with rose petal (RP) meal at inclusion levels of 0, 5, 10, 20, and 40 g/kg, designated as RP0, RP5, RP10, RP20, and RP40, respectively. All dry ingredients were sieved through a 250 μm mesh to ensure uniform particle size. Ingredients were then accurately weighed according to the formulation in Table 1 and homogenized in a mechanical mixer for 10 minutes to achieve a consistent blend (Linh et al 2025).

Table 1
Formulation of experimental diets containing graded levels of rose petal (RP) powder (g/kg dry matter basis).

| Ingredients | Rose petal concentration (g/kg diet) | | | | |
|---|---|---|---|---|---|
| | RP-0 (control) | RP-5 | RP-10 | RP-20 | RP-40 |
| Fish meal | 370 | 370 | 370 | 370 | 370 |
| Soybean meal | 400 | 395 | 390 | 380 | 360 |
| Corn meal | 20 | 20 | 20 | 20 | 20 |
| Rice bran | 80 | 80 | 80 | 80 | 80 |
| Wheat flour | 80 | 80 | 80 | 80 | 80 |
| Red yeast | 5 | 5 | 5 | 5 | 5 |
| Lecithin | 7 | 7 | 7 | 7 | 7 |
| Methionine | 5 | 5 | 5 | 5 | 5 |
| Lysine | 5 | 5 | 5 | 5 | 5 |
| Binder | 5 | 5 | 5 | 5 | 5 |
| Soybean oil | 5 | 5 | 5 | 5 | 5 |
| Rose petal | 0 | 5 | 10 | 20 | 40 |
| Premix | 10 | 10 | 10 | 10 | 10 |
| Vitamin C 98% | 10 | 10 | 10 | 10 | 10 |
| **Proximate (%)** | | | | | |
| Ash | 11.33 | 11.44 | 11.59 | 11.52 | 11.49 |
| Fiber | 3.98 | 3.96 | 3.88 | 3.95 | 3.93 |
| Crude lipid | 2.83 | 2.87 | 2.88 | 2.86 | 2.87 |
| Crude protein | 36.36 | 36.32 | 36.35 | 36.36 | 36.38 |
| Nitrogen-free extract | 45.50 | 45.41 | 45.3 | 45.31 | 45.33 |

Table 2
Gene-specific primers used for quantitative real-time PCR (qPCR) analysis in goldfish (*Carassius auratus*).

| Target gene | Sequence (5'–3') | | Genebank accession number |
|---|---|---|---|
| *β-actin 2* | Forward | TGCTGACCGTATGCAGAAAG | AB039726.2 |
| | Reversed | TGAGAGGTTTGGGTTGGTC | |
| *IL-1B* | Forward | TTCATTTGAAGGCAGTGACG | AJ249136.1 |
| | Reversed | TAAGCTGTGCCCGTCTCTTT | |
| *IL-10* | Forward | CAAGGAGCTCCGTTCTGCAT | HQ259106 |
| | Reversed | TCGAGTAATGGTGCCAAGTCATCA | |
| *TNF α* | Forward | TCATTCCTTACGACGGCATTT | EU069818.1 |
| | Reversed | CAGTCACGTCAGCCTTGCAG | |
| *TGF* | Forward | ACCATATGCCAAAGCCTCAC | EU086521.1 |
| | Reversed | TGATGCCTATACAGCGCAAG | |
| *IGF* | Forward | CAGGGGCATTGGTGTGA | GU583648 |
| | Reversed | GCAGCGTGTCTACAAGC | |
| *CYP1A* | Forward | TCACCGACTCGCTCATCAAC | DQ517445 |
| | Reversed | TTCAGCTCTGTACCGTCTGC | |
| *LYZ* | Forward | GCCGGAAATGTCCTGAATAA | KR092198.1 |
| | Reversed | GTGGTCCTGGCATCGATATT | |
| *HPS70* | Forward | GGCAGAAGGTGACAAATGCA | JN544930.1 |
| | Reversed | TGGGCTCGTTGATGTTCTCA | |

Table 3
Effects of dietary rose petal supplementation on survival rate, growth performance, and feed conversion ratio of goldfish (*Carassius auratus*) at weeks 4 and 8.

| Parameter | RP-0 | RP-5 | RP-10 | RP-20 | RP-40 |
|---|---|---|---|---|---|
| Initial weight (g) | 6.82 ± 0.06 | 6.85 ± 0.05 | 6.85 ± 0.09 | 6.85 ± 0.06 | 6.85 ± 0.09 |
| **Week 4** | | | | | |
| Survival rate (%) | 98.33 ± 2.89 | 98.33 ± 2.89 | 96.67 ± 5.77 | 98.33 ± 2.89 | 100.00 ± 0.00 |
| Final weight (g) | 12.53 ± 0.48$^{b}$ | 12.88 ± 0.16$^{b}$ | 12.96 ± 0.76$^{b}$ | 13.07 ± 0.26$^{b}$ | 14.38 ± 0.50$^{a}$ |
| Weight gain (g) | 5.72 ± 0.48$^{b}$ | 6.03 ± 0.20$^{b}$ | 6.11 ± 0.47$^{b}$ | 6.22 ± 0.23$^{b}$ | 7.53 ± 0.45$^{a}$ |
| Percent weight gain (%) | 83.88 ± 6.97$^{b}$ | 88.09 ± 3.51$^{b}$ | 89.30 ± 7.97$^{b}$ | 90.83 ± 3.19$^{b}$ | 109.96 ± 5.87$^{a}$ |
| Percentage specific growth rate (%.day$^{-1}$) | 2.03 ± 0.13 | 2.11 ± 0.63 | 2.13 ± 0.14 | 2.15 ± 0.06 | 2.47 ± 0.10 |
| Daily weight gain (g.day$^{-1}$) | 0.20 ± 0.02$^{b}$ | 0.22 ± 0.01$^{b}$ | 0.22 ± 0.02$^{b}$ | 0.22 ± 0.01$^{b}$ | 0.27 ± 0.01$^{a}$ |
| FCR | 0.89 ± 0.03 | 0.89 ± 0.03 | 0.91 ± 0.06 | 0.89 ± 0.03 | 0.88 ± 0.01 |
| **Week 8** | | | | | |
| Survival rate (%) | 98.33 ± 2.89 | 98.33 ± 2.89 | 96.67 ± 5.77 | 98.33 ± 2.89 | 100.00 ± 0.00 |
| Final weight (g) | 17.78 ± 0.32$^{b}$ | 18.02 ± 0.86$^{b}$ | 18.64 ± 0.68$^{b}$ | 18.70 ± 0.57$^{b}$ | 20.13 ± 0.41$^{a}$ |
| Weight gain (g) | 10.97 ± 0.37$^{b}$ | 11.17 ± 0.92$^{b}$ | 11.79 ± 0.62$^{b}$ | 11.85 ± 0.56$^{b}$ | 13.28 ± 0.49$^{a}$ |
| Percent weight gain (%) | 160.82 ± 6.24$^{b}$ | 163.09 ± 14.55$^{b}$ | 172.09 ± 7.87$^{b}$ | 172.98 ± 7.75$^{b}$ | 193.97 ± 9.71$^{a}$ |
| Percentage specific growth rate (%.day$^{-1}$) | 3.19 ± 0.09 | 3.71 ± 0.03 | 3.36 ± 0.16 | 3.60 ± 0.17 | 3.43 ± 0.22 |
| Daily weight gain (g.day$^{-1}$) | 0.20 ± 0.01$^{b}$ | 0.20 ± 0.02$^{b}$ | 0.21 ± 0.01$^{b}$ | 0.21 ± 0.01$^{b}$ | 0.24 ± 0.01$^{a}$ |
| FCR | 3.14 ± 1.10 | 3.71 ± 0.03 | 3.36 ± 0.16 | 3.60 ± 0.17 | 3.31 ± 0.22 |

*Data are presented as mean ± SD. Different letters within a row indicate significant differences ($p < 0.05$)*

The mixtures were pelleted using a 2 mm diameter mincer, selected to match the oro-pharyngeal dimensions of juvenile goldfish and facilitate optimal feed intake while reducing waste. The pellets were dried at 50°C overnight in a hot-air oven and subsequently stored in airtight polyethylene bags at 4°C until use in feeding trials and proximate composition analysis.

Proximate composition of the diets was determined following the Association of Official Analytical Chemists (AOAC 2016), standard procedures. Moisture content was assessed gravimetrically by drying triplicate samples at 105°C to constant weight using a Memmert UL50 incubator. Ash content was measured by incineration at 550°C for 8 hours in a Carbolite ELF 11/14 muffle furnace. Crude lipid content was determined via Soxhlet extraction with petroleum ether using a Gerhardt apparatus. Crude protein was analyzed using the Kjeldahl method (N × 6.25) with a semi-automatic Gerhardt Vapodest 45s system. All analyses were performed in triplicate to ensure accuracy and reproducibility.

## 2.3. Growth performance parameters

Fish were batch-weighed biweekly using a KERN analytical balance to monitor growth performance. After weighing, fish were promptly returned to their respective tanks to minimize handling stress. Growth performance indicators, including weight gain (WG), percent weight gain (PWG), specific growth rate (SGR), daily weight gain (DWG), feed conversion ratio (FCR), and survival rate (SR), were calculated using standard equations (LinhLubis et al 2024; LinhWannavijit et al 2024):

Survival rate (SR, %) = (Final number of fish / Initial number of fish) × 100

Weight gain (WG) = Final body weight − Initial body weight

Percent weight gain (PWG, %) = [(Final body weight − Initial body weight) / Initial body weight] × 100

Specific growth rate (SGR, %/day) = [(ln Final body weight − ln Initial body weight) / Culture days] × 100

Daily weight gain (DWG, g/day) = (Final body weight − Initial body weight) / Culture days

Feed conversion ratio (FCR) = Total dry feed intake / Live weight gain

## 2.4. Skin color measurement

To assess the effects of dietary treatments on skin pigmentation, five fish per tank were randomly netted and photographed at week 8 under standardized lighting conditions using a Panasonic digital camera (Panasonic, Japan). Immediately afterward, fish were gently blotted to remove excess moisture and subjected to colorimetric analysis before group weighing, thereby minimizing pigment distortion due to handling stress.

Skin color measurements were conducted on the left side of the head using a HunterLab MiniScan EZ 4500 L spectrocolorimeter (Hunter Associates, USA). The instrument was calibrated against standard black and white tiles according to the guidelines of the International Commission on Illumination (CIE 1976), prior to data collection. For each fish, three replicates of CIELAB color values were recorded: L*

(lightness), a* (red–green axis), and b* (yellow–blue axis). In this system, L* values range from 0 (black) to 100 (white), a* values from negative (green) to positive (red), and b* values from negative (blue) to positive (yellow) (Hunter and Harold 1987).

## 2.5. Blood biochemical analysis

Prior to biochemical analysis, six fish were randomly selected from each dietary treatment group. Blood was collected from the caudal vein using a heparinized 1 mL syringe and immediately transferred to enzyme-free 1.5 mL centrifuge tubes to prevent coagulation. Samples were centrifuged at 3,000 rpm for 10 minutes at 4°C to separate serum, which was carefully pipetted into clean microtubes and stored at −80°C until analysis.

Serum antioxidant capacity was assessed using the ABTS radical scavenging assay as described by Boonkong et al (2024). Briefly, 10 µL of thawed serum was mixed with deionized water and ABTS working solution, incubated in the dark for 10 minutes, and absorbance was measured at 734 nm. Radical scavenging activity (%) was calculated using a standard inhibition formula.

Superoxide dismutase (SOD) activity was measured using a commercial assay kit (CS0009, Sigma-Aldrich) following the protocol of Li et al (2020). Samples were incubated with kit reagents, and absorbance was recorded at 450 nm using a Varioskan LUX microplate reader (Thermo Scientific, Vantaa, Finland).

Lipid peroxidation was evaluated by measuring malondialdehyde (MDA) levels using an MDA assay kit (KTB1050, Abbkine). Serum samples were reacted with thiobarbituric acid to form MDA–TBA complexes, and absorbance was measured at 532 nm. Nonspecific turbidity was corrected by subtracting absorbance at 600 nm, following the method of Karatas and EM (2012).

## 2.6. Intestinal gene expression assays

To evaluate transcriptional responses of immune-related and antioxidant genes, six fish from each dietary group were anesthetized, and approximately 50 mg of mid-intestinal tissue was aseptically excised. Tissue samples were immediately immersed in 200 µL of TRIzol™ reagent (Invitrogen™, USA) in sterile microcentrifuge tubes and stored at − 80°C until RNA extraction.

Upon thawing, samples were homogenized using a Bullet Blender® Homogenizer to ensure complete cellular disruption. Total RNA was extracted following the TRIzol™ protocol, and the aqueous phase was further purified using a Total RNA Extraction Kit (Omega Bio-tek, USA). RNA concentration and purity were assessed using a NanoDrop™ One/OneC spectrophotometer (Thermo Fisher Scientific, USA), and only samples with an A260/280 ratio between 1.8 and 2.0 were used for downstream applications.

Complementary DNA (cDNA) was synthesized from 1 µg of total RNA using a commercial reverse transcription kit (Bio-Rad, USA), following the manufacturer's thermal cycling protocol. Quantitative real-time PCR (qRT-PCR) was then conducted using the CFX Connect™ Real-Time PCR Detection System (Bio-Rad, USA) in a 20 µL reaction volume, as described in previous studies (Linh et al 2023; Sintuprom et al

2024). Each reaction contained 1 µL of cDNA (100 ng), 0.4 µL of each gene-specific primer (10 µM), 10 µL of 2× iTaq™ Universal SYBR® Green Supermix (Bio-Rad, USA), and nuclease-free water to reach the final volume. The thermal cycling protocol included an initial denaturation at 95°C for 30 seconds, followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 30 seconds. A melt curve analysis (95°C for 15 seconds, 60°C for 60 seconds, and 95°C for 15 seconds) was performed to verify amplification specificity. Relative gene expression levels were calculated using the $2^{-\Delta\Delta ct}$ method (Livak and Schmittgen (2001), with *Actin-2* serving as the internal reference gene.

# 2.7. Gut microbiome analysis

At the end of the 8-week feeding trial, six fish per dietary group were randomly selected for gut microbiota analysis. Fish were euthanized, and the posterior intestine was aseptically excised. Samples were immediately stored at − 80°C until DNA extraction. Microbial genomic DNA was extracted using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. The V3–V4 hypervariable regions of the 16S rRNA gene were amplified using primers 341F (5'-CCTAYGGGRBGCASCAG-3') and 806R (5'-GGACTACNNGGGTATCTAAT-3'). PCR products were processed for library preparation and paired-end sequenced on an Illumina MiSeq platform by Macrogen Inc. (Seoul, Republic of Korea). Raw reads were processed using the QIIME 2 pipeline (v2022.2) (Bolyen et al 2019). After quality assessment, adapter trimming, and read filtering, denoising, merging of paired-end reads, and chimera removal were conducted using DADA2 (Callahan et al 2016) to generate amplicon sequence variants (ASVs). Taxonomic classification was performed using a Naive Bayes classifier trained on the SILVA database (v138, 99% similarity) (Quast et al 2012). Phylogenetic trees were constructed using MAFFT for alignment (Katoh and Standley 2013) and FastTree 2 for tree inference (Price et al 2010).

Downstream analyses and visualization were conducted in R (v4.4.3; R Core Team, 2024) using QIIME 2-exported data and the *phyloseq* (v1.48.0) (McMurdie and Holmes 2013) and *vegan* (v2.6.8) (Oksanen et al 2024) packages. Alpha diversity was assessed using Shannon entropy, Pielou's evenness, Chao1 richness, and Faith's phylogenetic diversity (PD), with group differences tested via Kruskal–Wallis tests. Beta diversity was calculated using Bray–Curtis dissimilarity, Jaccard distance, and weighted/unweighted UniFrac distances, with community structures visualized using Principal Coordinates Analysis (PCoA). Group differences were statistically evaluated using PERMANOVA with 999 permutations (QIIME 2 *beta-group-significance*), and pairwise p-values were adjusted using the False Discovery Rate (FDR) method. Differential abundance between groups was assessed using ANCOM-BC (v2.6.0) (Lin and Peddada 2020) applying a pseudo-count and Holm correction, with a significance threshold of $q < 0.05$. Spearman's rank correlation was used to examine associations between microbial ASV abundances (log1p-transformed after total sum scaling) and host growth parameters using the *Hmisc* package (v5.2.3) (Harrell and Dupont 2025), with FDR-adjusted significance at $q < 0.05$. Data visualization was performed using *ggplot2* (v3.5.2) (Wickham and Sievert 2016).Unless otherwise stated, statistical significance was set at $p < 0.05$ or $q < 0.05$, as appropriate.

# 2.8. Data analysis

All statistical analyses were conducted using OriginPro (v2021b, OriginLab Corporation, Northampton, MA, USA) and IBM SPSS Statistics (v29.0.2.0, IBM Corp., Armonk, NY, USA). Data normality was assessed using both the Kolmogorov–Smirnov and Shapiro–Wilk tests, while Levene's test was used to evaluate homogeneity of variances. For datasets meeting assumptions of normality and homogeneity, one-way analysis of variance (ANOVA) was employed to analyze growth performance, skin pigmentation, serum biochemical parameters, and gene expression data. Duncan's multiple range test was used for post hoc comparisons to identify significant differences among treatment means. When assumptions were not met, non-parametric alternatives such as the Kruskal–Wallis test were applied, followed by Dunn's post hoc test with Bonferroni correction where applicable. Pearson's and Spearman's correlation coefficients were calculated to assess relationships among growth performance, antioxidant activities, gene expression, and gut microbiota composition. All results are presented as mean ± standard deviation (SD), and statistical significance was set at $p < 0.05$.

# 3. Results

## 3.1. Growth performance, feed utilization, and survival

Growth performance, feed utilization, and survival of koi carp fed RP-supplemented diets were evaluated at weeks 4 and 8 (Table 1). Survival remained high across all groups, with no significant mortality observed in RP-0 to RP-20 (96.7–98.3%) and complete survival (100%) in the RP-40 group, indicating that dietary inclusion of RP up to 40 g/kg did not compromise fish viability.

By week 4, fish fed the RP-40 diet showed significantly higher FW, WG, and PWG compared to RP-0 ($p < 0.05$), while RP-5 to RP-20 groups showed only marginal, non-significant improvements. DWG in RP-40 (0.27 g/day) was ~ 18% higher than in other groups. Although SGR showed a supplementation-related increase, it was not statistically significant at this stage.

At week 8, the growth-promoting effect of high RP supplementation became more pronounced. RP-40-fed fish had significantly greater FW, WG, and PWG than RP-0 ($p < 0.05$), while RP-5 to RP-20 yielded moderate but non-significant improvements. SGR continued to exhibit a dose-dependent increase, though the differences remained statistically non-significant. FCR was unaffected by RP inclusion and remained stable across treatments (week 4: 0.88–0.91; week 8: 3.14–3.71), suggesting that improved growth was not associated with reduced feed efficiency.

Regression analysis revealed strong linear relationships between RP inclusion level and performance metrics (Fig. 1): FW increased from 17.8 g (RP-0) to 20.2 g (RP-40; Adj. $R^2 = 0.70$), SGR from 3.19%/day to 3.72%/day (Adj. $R^2 = 0.69$), FCR decreased from 3.71 to 3.29 (Adj. $R^2 = 0.70$), and WG increased from 11.0 g to 13.8 g (Adj. $R^2 = 0.77$).

Correlation analysis among growth parameters showed strong positive associations between FW, WG, PWG, DWG, and SGR ($r = 0.98–1.00$, $p < 0.05$), indicating consistent trends in weight-based growth indices (Fig. 2). In contrast, FCR was significantly negatively correlated with these parameters ($r = -0.57$

to − 0.62, $p < 0.05$), reflecting its inverse relationship with growth efficiency. Survival rate showed no significant correlation with other variables ($r = 0.00$, $p > 0.05$).

## 3.2. Skin pigmentation

After 60 days of feeding, skin color parameters (L*, a*, b*) were measured to evaluate the effects of dietary RP supplementation (Table 4). Skin lightness (L*) showed no significant differences among treatment groups, with values ranging from 61.10 ± 14.99 to 66.35 ± 13.00 ($p > 0.05$), and no significant correlation was observed between L* values and RP concentration ($r = 0.12$, $p = 0.072$; Fig. 3C).

Table 4
Effects of dietary rose petal supplementation on skin color parameters, luminosity (L*), redness (a*), and yellowness (b*), in goldfish (*Carassius auratus*) after 8 weeks.

| Parameter | Rose petal dietary (g/kg) | | | | |
|---|---|---|---|---|---|
| | RP-0 | RP-5 | RP-10 | RP-20 | RP-40 |
| Luminosity (L*) | 61.10 ± 14.99 | 63.72 ± 12.29 | 65.38 ± 14.38 | 65.63 ± 11.81 | 66.35 ± 13.00 |
| Redness (a*) | 20.54 ± 7.02$^{c}$ | 22.11 ± 5.82$^{bc}$ | 24.61 ± 7.32$^{ab}$ | 25.32 ± 5.69$^{a}$ | 26.14 ± 6.58$^{a}$ |
| Yellowness (b*) | 40.32 ± 14.04$^{b}$ | 41.02 ± 13.06$^{b}$ | 41.44 ± 14.46$^{b}$ | 43.84 ± 10.07$^{ab}$ | 47.73 ± 11.08$^{a}$ |

*Data are presented as mean ± SD. Different letters within a row indicate significant differences ($p < 0.05$)*

In contrast, skin redness (a*) increased significantly in a dose-dependent manner. Fish fed the RP-20 (25.32 ± 5.69) and RP-40 (26.14 ± 6.58) diets had significantly higher a* values compared to the RP-0 group (20.54 ± 7.02) ($p < 0.05$). This trend was supported by a significant positive correlation between RP concentration and a* values ($r = 0.30$, $p < 0.001$; Fig. 3A).

Similarly, skin yellowness (b*) was significantly enhanced at the highest supplementation level. The RP-40 group (47.73 ± 11.08) exhibited significantly higher b* values than the RP-0 group (40.32 ± 14.04) ($p < 0.05$), with a corresponding positive correlation between RP concentration and b* values ($r = 0.18$, $p = 0.0077$; Fig. 3B).

## 3.3. Antioxidant capacity and oxidative stress markers

Dietary RP supplementation significantly influenced serum antioxidant capacity and oxidative stress markers in goldfish (Fig. 4). Antioxidant capacity, measured by ABTS radical scavenging activity, increased progressively with rising RP levels (Fig. 4A). Fish fed RP-20 and RP-40 diets exhibited significantly higher ABTS activity than the RP-0 group ($p < 0.05$), with a strong positive correlation observed between RP concentration and ABTS activity ($r = 0.57$, $p < 0.001$; Fig. 5A).

Similarly, superoxide dismutase (SOD) activity showed a dose-dependent increase (Fig. 4B). While no significant differences were observed at lower RP levels (RP-5, RP-10), SOD activity in the RP-40 group was significantly higher than in RP-0 ($p < 0.05$). This was supported by a strong positive correlation between RP concentration and SOD activity ($r = 0.69$, $p < 0.001$; Fig. 5B).

Conversely, lipid peroxidation, indicated by malondialdehyde (MDA) content, declined with increasing RP inclusion (Fig. 4C). Fish in the RP-40 group exhibited significantly lower serum MDA levels compared to RP-0 ($p < 0.05$). A significant negative correlation was also observed between RP concentration and MDA levels ($r = -0.38$, $p = 0.037$; Fig. 5C).

## 3.4. Intestinal expression of antioxidant, growth, and immune-related genes

Dietary RP supplementation modulated the expression of multiple genes related to antioxidant defense, growth, and immune function in goldfish (Fig. 6).

Among antioxidant-related genes, *HSP70* expression remained unchanged at lower RP levels but was significantly upregulated in the RP-40 group compared to RP-0 ($p < 0.05$). *CYP1A* followed a similar trend, with moderate increases at RP-10 and RP-20 (not statistically significant), and a significant peak at RP-40 ($p < 0.05$). Both genes showed strong positive correlations with RP concentration (*HSP70*: $r = 0.68$, $p < 0.001$; *CYP1A*: $r = 0.61$, $p < 0.001$; Fig. 7A, D).

Growth-related transcripts were also significantly affected. *IGF* expression increased progressively from RP-5 to RP-40, reaching significance in RP-20 and RP-40 compared to RP-0 ($p < 0.05$). *TGF* showed a similar pattern, with significant upregulation at RP-20 and RP-40 ($p < 0.05$). Both genes exhibited strong positive correlations with RP inclusion (*IGF*: $r = 0.69$, $p < 0.001$; *TGF*: $r = 0.77$, $p < 0.001$; Fig. 7B, C).

Immune-related genes also responded positively to RP supplementation. *LYZ* expression increased in a dose-dependent manner, with significantly higher levels in RP-20 and RP-40 compared to RP-0 ($p < 0.05$). *TNFα* was significantly elevated from RP-10 onward, reaching its highest expression at RP-40 ($p < 0.05$). Both genes showed strong positive correlations with RP concentration (*LYZ*: $r = 0.92$, $p < 0.001$; *TNFα*: $r = 0.83$, $p < 0.001$; Fig. 7E, F).

In contrast, expression of the anti-inflammatory cytokines *IL10* and *IL1β* remained unchanged across treatments ($p > 0.05$), with no significant correlations with RP concentration (*IL10*: $p = 0.689$; *IL1β*: $p = 0.437$; Fig. 7G, H).

## 3.5. 16S rRNA sequencing and ASV characterization

High-throughput sequencing of the 16S rRNA gene V3–V4 region produced a total of 522,700 high-quality reads across 15 samples following quality filtering, with an average read length of 246 bp. Processing via the DADA2 pipeline yielded 8,525 unique Amplicon Sequence Variants (ASVs) across all samples.

Per-sample read counts ranged from 26,712 to 43,898, with a mean of 34,847 reads. The number of observed ASVs per sample ranged from 208 to 890, averaging approximately 626 ASVs. The distribution of total read counts across dietary treatments (RP-0, RP-5, RP-10, RP-20, RP-40) is presented in Fig. 8.

# 3.6. Gut microbiota composition

16S rRNA gene sequencing revealed diverse gut microbial communities across all treatment groups, with notable inter-sample variation, particularly in RP-0 (4) and RP-20 (5), which exhibited distinct taxonomic profiles (Fig.s 9 and 10).

At the phylum level, *Proteobacteria* was most abundant (~ 21.0%), followed by *Firmicutes* (10.3%), *Actinobacteriota* (9.7%), *Bacteroidota* (7.9%), and *Cyanobacteria* (5.1%), while unclassified taxa accounted for ~ 35.8% of sequences. Sample RP-0 (4) showed elevated *Proteobacteria* (~ 40%), unlike other RP-0 replicates with higher unclassified taxa and lower *Proteobacteria*. Similarly, RP-20 (5) was dominated by *Proteobacteria* (~ 49%).

At the genus level, *Chloroplast* (~ 4.7%) and *Escherichia-Shigella* (~ 3.8%) were among the most abundant classified genera, though unclassified genera (~ 50.8%) and low-abundance "Other" taxa (~ 20.7%) comprised the majority. RP-0 (4) was dominated by *Escherichia-Shigella* (> 33%), while RP-20 (5) showed elevated *Aeromonas* (~ 4.6%). Other genera like *Methyloversatilis*, *Vibrio*, *Staphylococcus*, and *Muribaculaceae* were present at low levels. Overall, the gut microbiota showed high diversity with distinct compositional outliers.

# 3.7. Microbial diversity

Alpha diversity, assessed using Shannon entropy, Pielou's evenness, Chao1 richness, and Faith's Phylogenetic Diversity (PD), showed no significant differences among dietary groups (Kruskal–Wallis test, $p > 0.05$; Fig. 11). Specifically, Shannon ($p \approx 0.60$), Chao1 ($p \approx 0.52$), Pielou's evenness ($p \approx 0.57$), and Faith's PD ($p \approx 0.23$) all indicated comparable within-sample diversity across treatments. Although exploratory pairwise comparisons for Faith's PD (RP-0 vs RP-10; RP-0 vs RP-40) yielded unadjusted $p < 0.05$, these were not significant after FDR correction ($q \approx 0.25$). Visual inspection showed substantial overlap among groups, and previously noted outlier samples (RP-0 [4], RP-20 [5]) did not differ markedly in alpha diversity.

Beta diversity analysis using Principal Coordinates Analysis (PCoA) based on Bray–Curtis dissimilarity revealed partial separation among groups, particularly between RP-10/RP-20 and the remaining treatments (Fig. 12). The first two axes explained 14.33% and 9.51% of the total variance. PERMANOVA confirmed significant differences in community composition among groups (Pseudo-F = 1.13, $p = 0.017$), though post hoc pairwise comparisons were non-significant after multiple testing correction (all $q > 0.05$). Analyses using Jaccard and UniFrac distances also yielded non-significant results (data not shown), suggesting the Bray–Curtis significance stemmed from subtle shifts in relative taxon abundances rather than major compositional restructuring.

## 3.8. Differential abundance of gut microbiota

Differentially abundant taxa across treatment groups were identified using ANCOM-BC2 with Holm-adjusted *q*-values (threshold: $q < 0.05$). Two taxa showed significant differences relative to the control (RP-0) group (Fig. 13): *Staphylococcus* (phylum *Firmicutes*) was significantly enriched in RP-40 (LFC ≈ 5.22, $q < 0.05$), while an uncultured *Alloprevotella* species (phylum *Bacteroidota*) was significantly depleted in RP-5 (LFC ≈ − 6.85, $q < 0.05$). No other taxa differed significantly from RP-0.

Pairwise comparisons among RP-treated groups revealed four additional differentially abundant taxa (Fig. 14). *Staphylococcus* abundance was higher in RP-40 than RP-20 and lower in RP-5 than RP-40. An uncultured *Muribaculaceae* species was significantly enriched in RP-40 compared to RP-10 and RP-20, and depleted in RP-5 relative to RP-40. A member of *Micrococcaceae* (phylum *Actinobacteriota*) was more abundant in RP-5 than RP-20. Lastly, *Chloroplast* sequences were significantly enriched in RP-5 and RP-40 compared to RP-20. These findings indicate that specific gut microbial taxa respond to distinct levels of dietary rose petal supplementation.

# 4. Discussion

Replacing fishmeal with sustainable, plant-derived ingredients is essential for reducing reliance on marine resources in aquaculture (Hossain et al 2024). This study demonstrated that partial replacement of fishmeal with RP powder up to 40 g/kg did not compromise growth performance or feed utilization in goldfish. RP is rich in carotenoids, amino acids, polyunsaturated fatty acids, and trace minerals, which likely acted synergistically to promote growth (Nowak et al 2014; dos Santos et al 2018; Nakano and Wiegertjes 2020; Lim et al 2023; Salamatullah et al 2024). These findings align with previous research in teleosts, including enhanced growth in orange swordtail and dwarf tilapia fed rose petal-enriched diets (Joseph et al 2011; Pailan et al 2012), and improvements observed in marine ornamental fish and goldfish fed natural pigment supplements such as rose or marigold powders (Sinha and Asimi 2007; Ezhil et al 2008; Ramamoorthy et al 2010).

Pigmentation enhancement is critical in ornamental aquaculture, where skin color influences consumer perception of quality, health, and value (de Carvalho and Caramujo 2017; Luo et al 2021; Sathyaruban et al 2021). Carotenoid-based pigmentation can be reliably quantified using CIE parameters (L*, a*, b*, chroma, and saturation) and must be supplied through the diet, as fish cannot synthesize carotenoids de novo (Kalinowski et al 2007). Once absorbed, carotenoids are transported via lipoproteins and deposited in chromatophores, xanthophores and erythrophores, responsible for yellow and red coloration, respectively (Barman et al 2024; Liao et al 2025). RP provides β-carotene, lutein, zeaxanthin, flavonoids, and phenolics that may enhance pigment deposition and stability (Wan et al., 2018, 2019). In this study, RP-40 yielded the greatest increases in redness (a*) and yellowness (b*), confirming a dose-dependent effect. Similar findings were reported in dwarf gourami, with progressive color enhancement observed at increasing RP levels (Pailan et al 2012).

Oxidative stress, caused by excess reactive oxygen species (ROS), damages lipids, proteins, and DNA (Di Giulio and Meyer 2008) (Amenyogbe et al 2024). MDA, a by-product of lipid peroxidation, is a widely used oxidative stress marker (Demirci-Cekic et al 2022). Organisms counteract ROS via enzymatic antioxidants like SOD and non-enzymatic scavengers such as ABTS activity (Chen et al 2021; F. Xu et al 2022). In this study, RP supplementation led to dose-dependent increases in ABTS and SOD activities and a corresponding reduction in serum MDA, indicating enhanced systemic antioxidant defense. These findings are consistent with ex vivo studies, where rose byproducts reduced lipid peroxidation in sea bass fillets (Giannakourou et al 2019), and in vivo studies, where *R. damascena* extracts upregulated antioxidant enzymes in rats (Hamza et al 2022). The potent radical-scavenging activity observed here supports previous reports of strong antioxidant potential in rose petals (Önder 2023), suggesting that RP contributes both direct and enzyme-mediated protection against oxidative stress in goldfish.

Dietary inclusion of RP powder elicited a clear, concentration-dependent upregulation of key antioxidant, growth, and immune-related genes, indicating activation of endogenous physiological pathways in goldfish. *HSP70*, a molecular chaperone involved in protein refolding under oxidative stress (Sen and Giri 2017), was significantly induced only at the highest RP dose (RP-40 vs. RP-0, $p < 0.05$). This response mirrors findings in pufferfish where dietary astaxanthin upregulated hepatic *HSP70* under thermal stress, highlighting the role of carotenoid antioxidants in stress adaptation (Eissa et al 2017). Similarly, *CYP1A*, an enzyme central to xenobiotic metabolism, was significantly elevated in RP-40, with non-significant trends at intermediate doses. Immune gene expression revealed selective activation of pro-inflammatory and innate immune markers. While *IL10* and *IL1β* levels remained unchanged ($p > 0.05$), *TNFα* and *LYZ* were significantly upregulated at RP-20 and RP-40 ($p < 0.05$), indicating that RP stimulates acute immune readiness without inducing chronic inflammatory responses.

RP supplementation also influenced the gut microbiota, though effects were modest and individualized. Alpha diversity indices (Shannon, Chao1, Pielou's evenness, Faith's PD) showed no significant differences among groups, suggesting the resilience of within-sample microbial diversity to dietary RP under these experimental conditions. This stability is noteworthy given that fish gut diversity is often sensitive to various factors including specific dietary interventions (Rabelo-Ruiz et al 2022), environmental stressors like mycotoxins or heavy metals (Zhang et al 2020; Spilsbury et al 2022), and disease state (Li et al 2017). However, PERMANOVA based on Bray–Curtis dissimilarity detected a significant difference in community structure ($p = 0.017$), whereas presence/absence (Jaccard) and phylogenetic (UniFrac) metrics, as well as pairwise comparisons, were non-significant after FDR correction. These findings suggest RP primarily induced subtle shifts in the relative abundance of existing taxa. Given that gut beta diversity is influenced by dietary composition(Silva et al 2011; Q. Xu et al 2022) and may correlate with host phenotypes such as coloration (Ahmed et al 2023), the observed compositional shift may reflect a biologically meaningful, though nuanced, response. The high proportion of unclassified taxa (~ 36% at the phylum and ~ 51% at the genus level) and strong inter-individual variability (e.g., samples RP-0 [4] and RP-20 [5]) likely reflect host-specific microbial assemblages and the presence of under-characterized aquatic microbiota (Spilsbury et al 2022; Li et al 2023).

Despite the overall subtlety of the community shift, differential abundance analysis (ANCOM-BC2, $q < 0.05$) identified several taxa that responded to RP supplementation. *Staphylococcus* was significantly enriched in the RP-40 group relative to RP-0. Although certain species (e.g., *S. warneri*) have been associated with disease in fish (Bunnoy et al 2019; Xiao Joe et al 2021), *Staphylococcus* is also part of the normal gut microbiota in healthy goldfish (Silva et al 2011) and shrimp. Its enrichment, coinciding with improved growth, pigmentation, and immune-antioxidant gene expression in RP-40, suggests a potentially neutral or even beneficial role in this context. RP-derived phenolic compounds, known to exert antimicrobial activity (Giannakourou et al 2019), may have selectively modulated gut microbial composition. Conversely, the depletion of *Alloprevotella* in RP-5 may reflect changes in fiber-related substrate availability, though its ecological role in fish remains unclear. Additional taxa showing dose-specific changes, including *Muribaculaceae*, *Micrococcaceae*, and *Chloroplast*, further suggest that microbial shifts were responsive to RP concentration.

Interestingly, no significant correlations were found between gut microbiota metrics (PCoA axes, key genera) and host parameters (growth, ABTS, SOD, MDA) after FDR correction. This may indicate that functionally important taxa shift, such as those identified by ANCOM-BC2, are more relevant than broader diversity indices, or that host-microbiota relationships in this system are non-linear or indirect. Similar dissociations between microbiota shifts and host phenotypes have been reported, such as in seabream fed Allium-derived supplements (Rabelo-Ruiz et al 2022)

Nevertheless, the established role of the gut microbiome in modulating fish immunity and metabolism (Butt and Volkoff 2019; Xiong et al 2019; Spilsbury et al 2022) supports the hypothesis that the RP-induced microbial changes may have contributed to the observed physiological improvements. For example, *Staphylococcus* has been associated with altered cytokine expression in grouper (Xiao Joe et al 2021), and other taxa have been linked to antioxidant responses in carp exposed to toxins (Zhang et al 2020; Xue et al 2023). Furthermore, studies in salmonids have linked gut microbiota composition, including potential carotenoid-associated genera like *Bacillaceae*, *Photobacterium*, and *Mycoplasma*, to pigmentation outcomes (Nguyen et al 2020; Ahmed et al 2023). While mechanisms remain to be clarified, it is plausible that RP-induced shifts in gut microbiota influenced the absorption, metabolism, or transport of dietary carotenoids, thereby contributing to the increased skin redness (a*) and yellowness (b*) observed in this study.

# 5. Conclusion

Dietary inclusion of rose petal powder at 20–40 g/kg significantly enhanced growth performance, feed efficiency, and skin pigmentation in goldfish. RP supplementation also improved systemic antioxidant capacity, evidenced by increased ABTS and SOD activity and decreased MDA levels. Expression of antioxidant (*HSP70*, *CYP1A*), growth (*IGF*, *TGF*), and immune (*TNFα*, *LYZ*) genes was upregulated in a dose-dependent manner. Although alpha diversity remained unchanged, RP induced a significant shift in gut microbial community structure. These findings highlight the potential of rose petal byproducts as a

sustainable, multifunctional alternative to synthetic additives in ornamental aquaculture, capable of promoting growth, enhancing coloration, and supporting fish health.

## Declarations

### CRediT authorship contribution statement

**Nutticha Nuntakad**: Methodology, Investigation, Conceptualization, Resources. **Luu Tang Phuc Khang**: Roles/Writing - Original draft, Formal analysis, Data curation, Software. **Suwanna Wisetkaew**: Methodology, Investigation. **Nguyen Dinh-Hung**: Writing-Review & Editing, Formal Analysis, Validation. **Cao Phuong Thao**: Roles/Writing - Original draft, Methodology, Software, Data Analysis. **Truong Anh Tu**: Roles/Writing - Original draft, Methodology, Software, Data Analysis. **Luu Phuc Loi**: Roles/Writing - Original draft, Methodology, Software, Validation, Supervision. **Papungkorn Sangsawad**: Methodology, Investigation, Writing-Review & Editing, Validation. **Mintra Seel-audom**: Conceptualization, Investigation, Methodology, Writing-Review & Editing, Validation, Formal analysis. **Patima Permpoonpatana**: Methodology, Investigation, Supervision, Validation, Writing-Review & Editing. **Nguyen Vu Linh**: Conceptualization, Methodology, Investigation, Roles/Writing - Original draft, Writing-Review & Editing, Data curation, Funding acquisition, and Project administration, Validation, Supervision.

# Declaration of Competing Interest

The authors declare no conflict of interest.

# Animal ethics

All experiments in this study were conducted in accordance with the relevant guidelines and regulations. The experimental protocols were approved by the Institutional Animal Care and Use Committee, Prince of Songkla University (Approval No. AG41/2025). All the procedure followed the ARRIVE guideline.

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# Data availability

The datasets used and/or analysed during the current study are available from the corresponding author upon reasonable request.

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# Figures

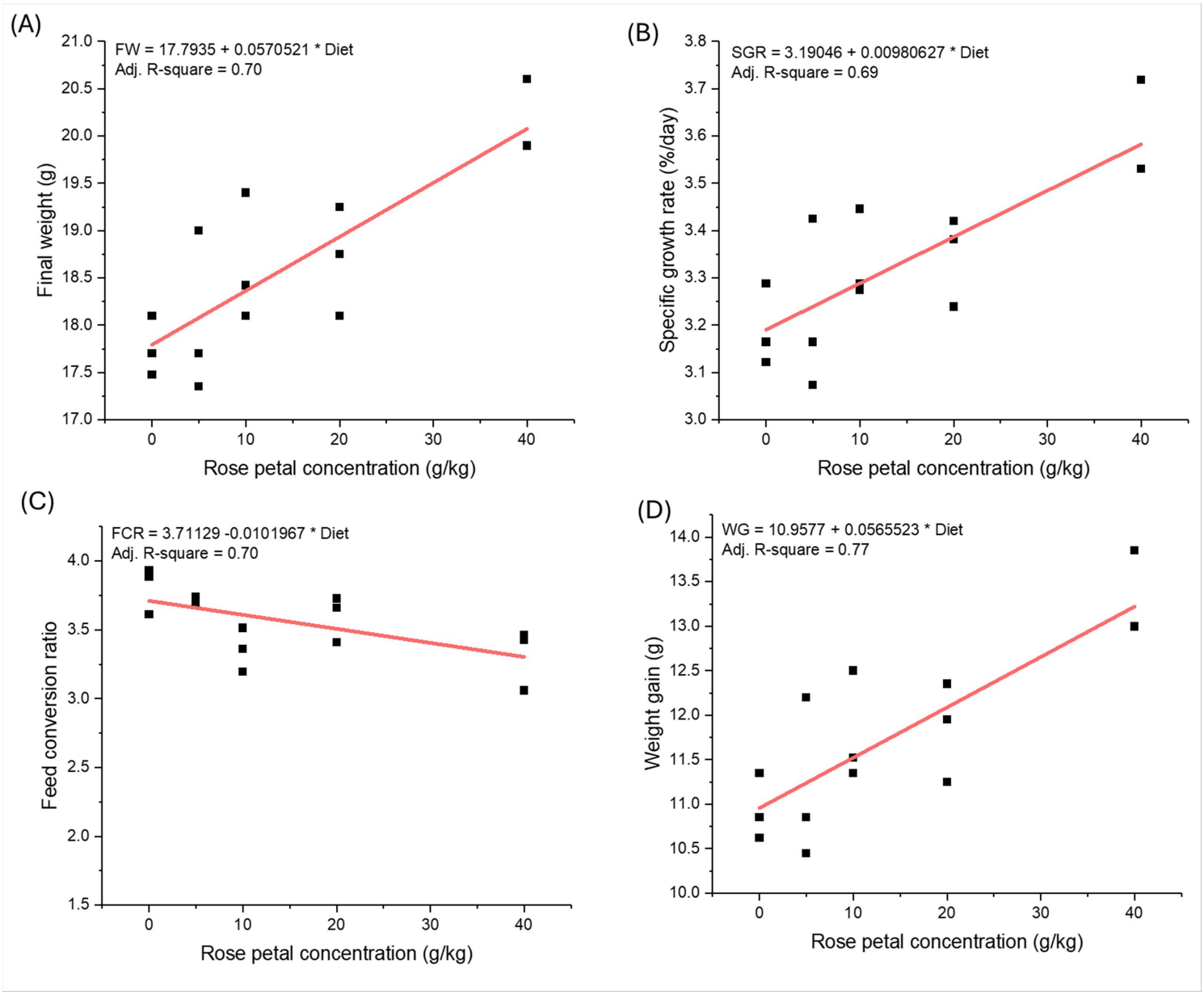
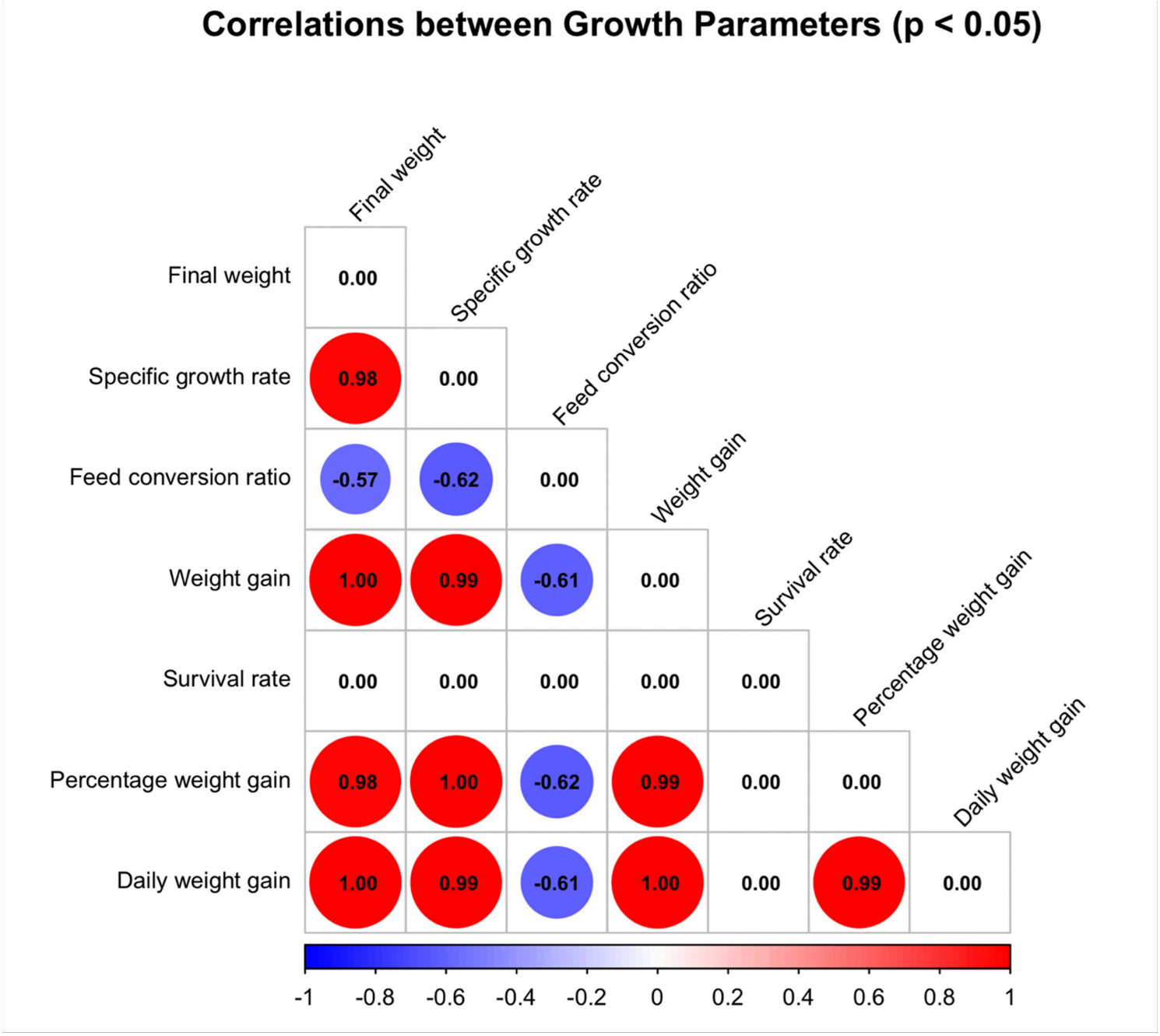


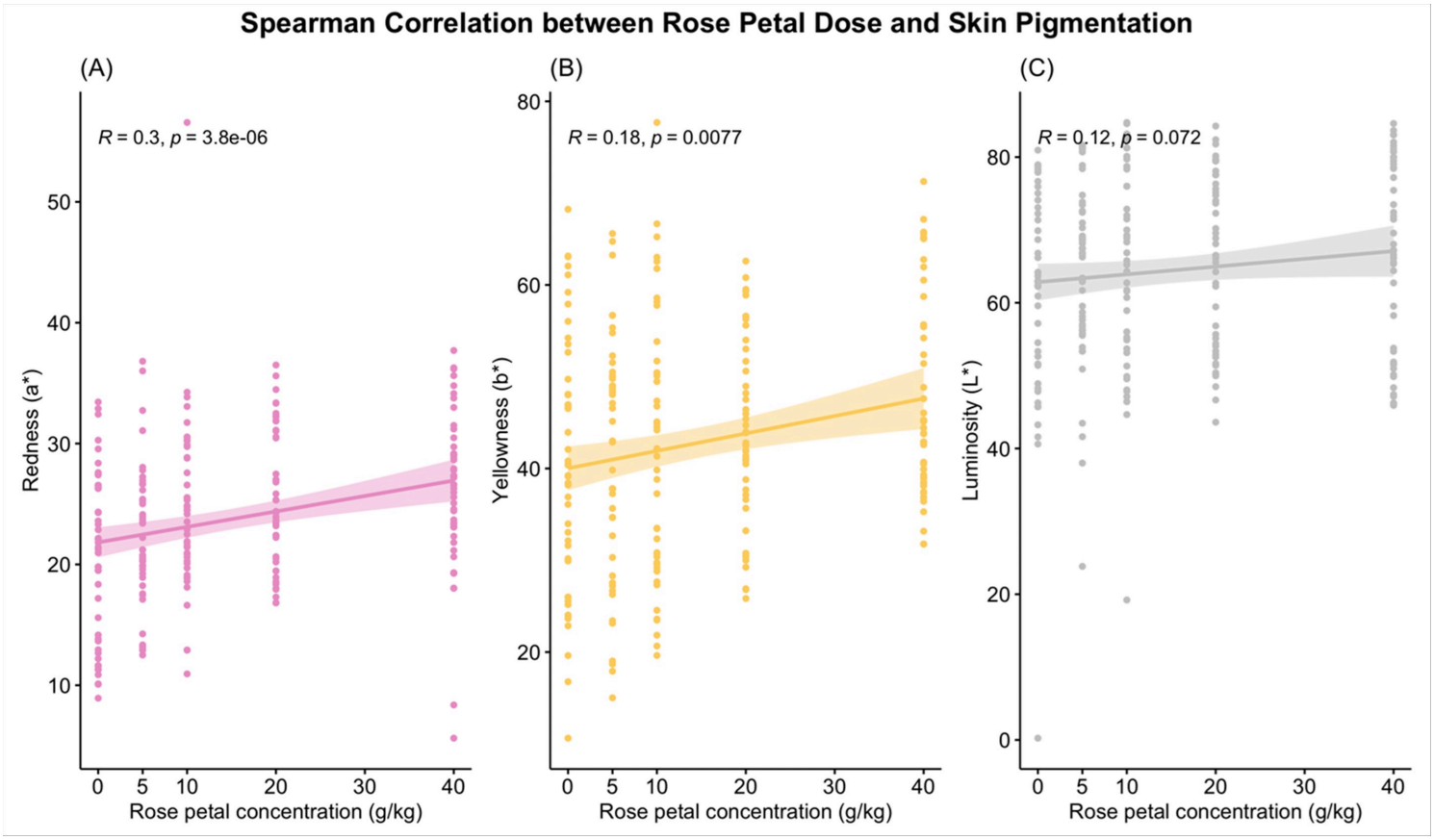

**Figure 1**

Growth performance of goldfish (*Carassius auratus*) after 8 weeks of dietary rose petal supplementation: (A) final weight, (B) specific growth rate, (C) feed conversion ratio, and (D) weight gain



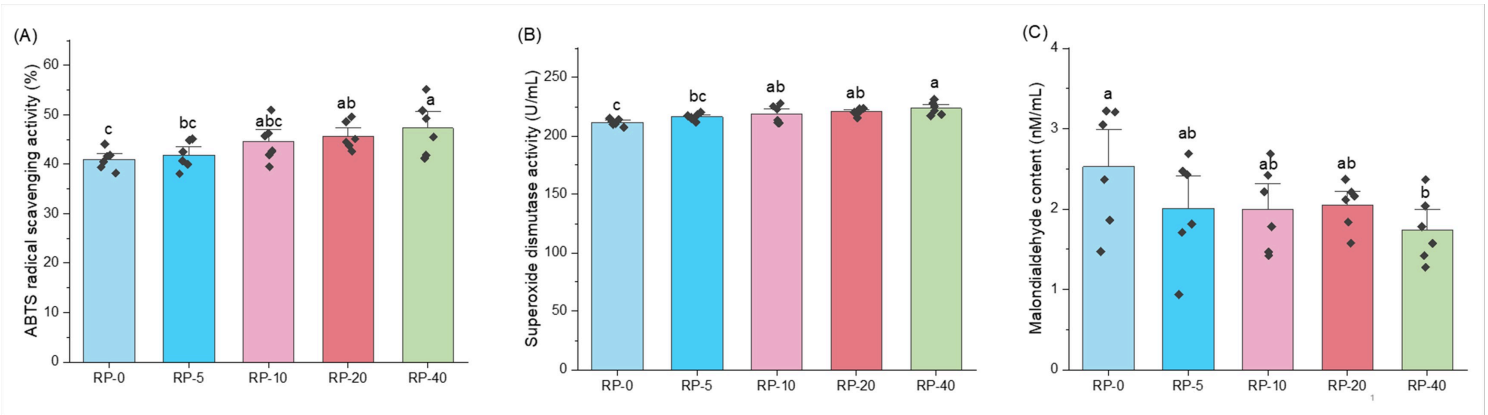

## Figure 2

Spearman correlation matrix showing significant correlations ($p < 0.05$) among growth performance parameters in goldfish (*Carassius auratus*) after 8 weeks. Circle size and color intensity indicate correlation strength and direction (red = positive; blue = negative). Correlation coefficients (r) are shown; non-significant correlations are omitted.




## Figure 3

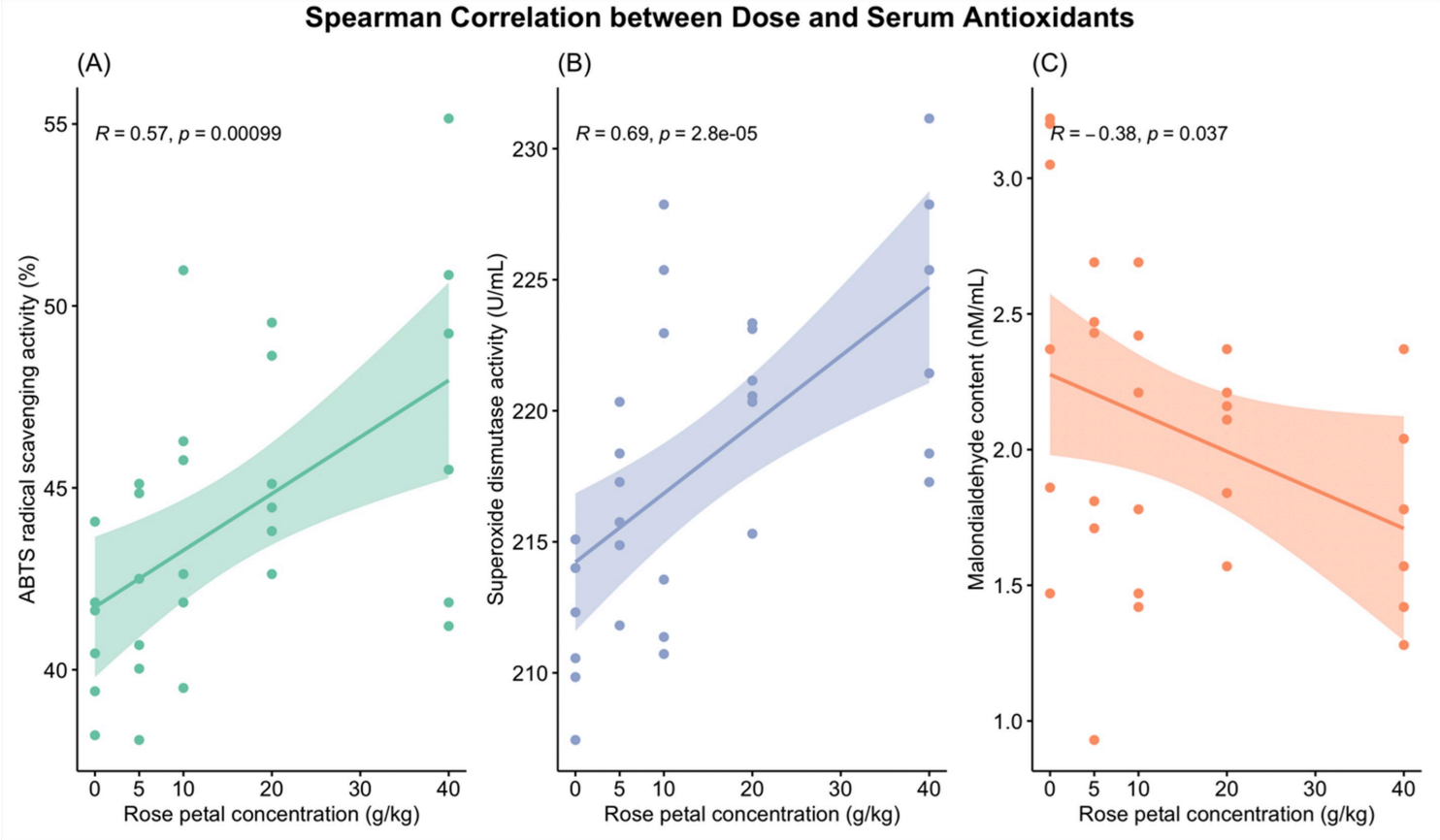
Spearman correlation between dietary rose petal concentration and skin pigmentation parameters in goldfish (*Carassius auratus*) after 8 weeks: (A) redness (a*), (B) yellowness (b*), and (C) luminosity (L*). Linear regression lines with 95% confidence intervals are shown. Correlation coefficients (r) and *p*-values are reported.




## Figure 4

Effects of 8-week dietary rose petal supplementation on serum antioxidant markers in goldfish (*Carassius auratus*): (A) ABTS radical scavenging activity, (B) superoxide dismutase (SOD) activity, and

(C) malondialdehyde (MDA) content. Bars represent mean ± SD; different letters indicate significant differences ($p < 0.05$).




## Figure 5

Spearman correlation between dietary rose petal concentration and serum antioxidant markers in goldfish (*Carassius auratus*): (A) ABTS radical scavenging activity, (B) SOD activity, and (C) MDA content. Linear regression lines with 95% confidence intervals are included. Correlation coefficients (r) and *p*-values are shown.

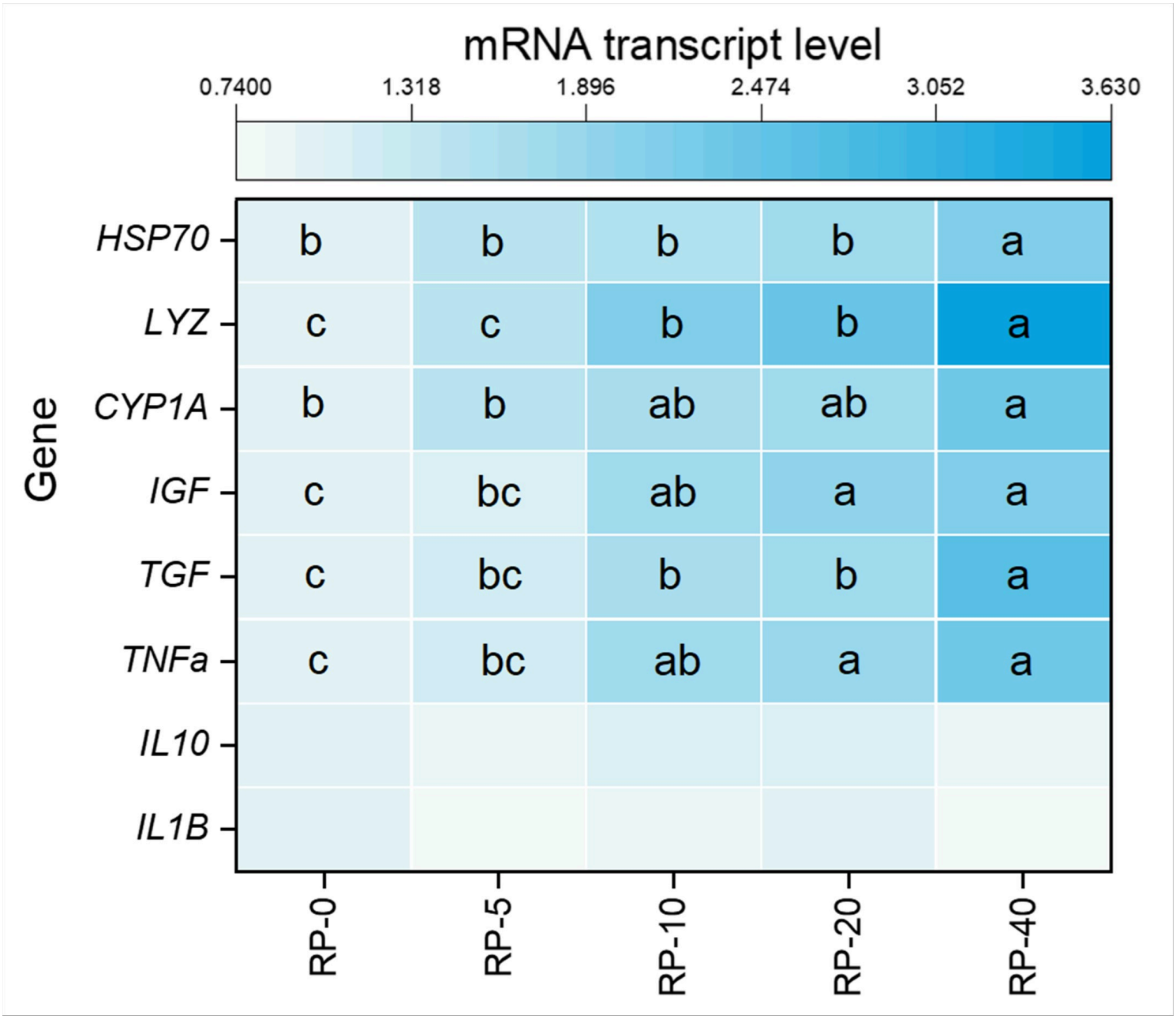
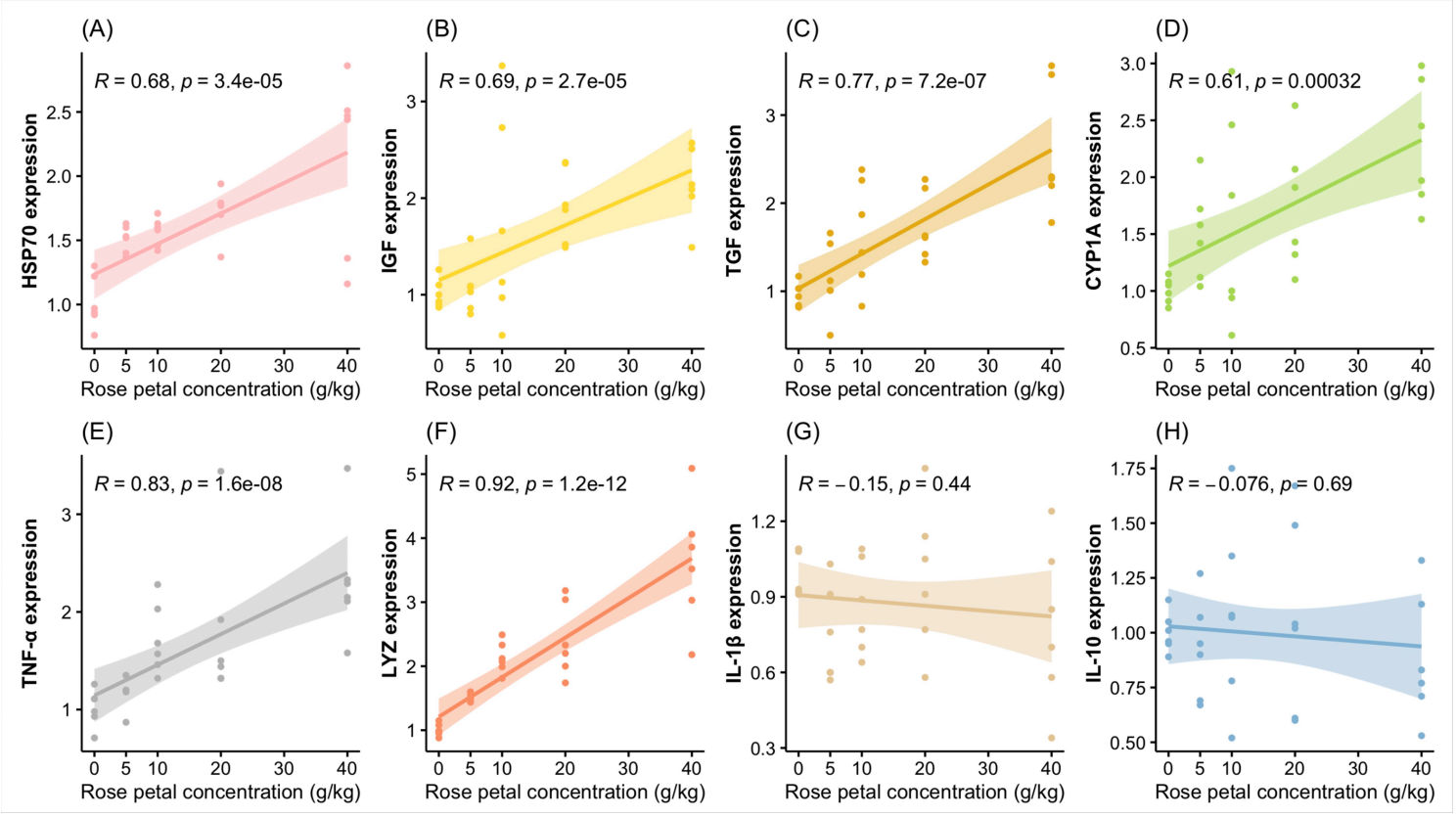


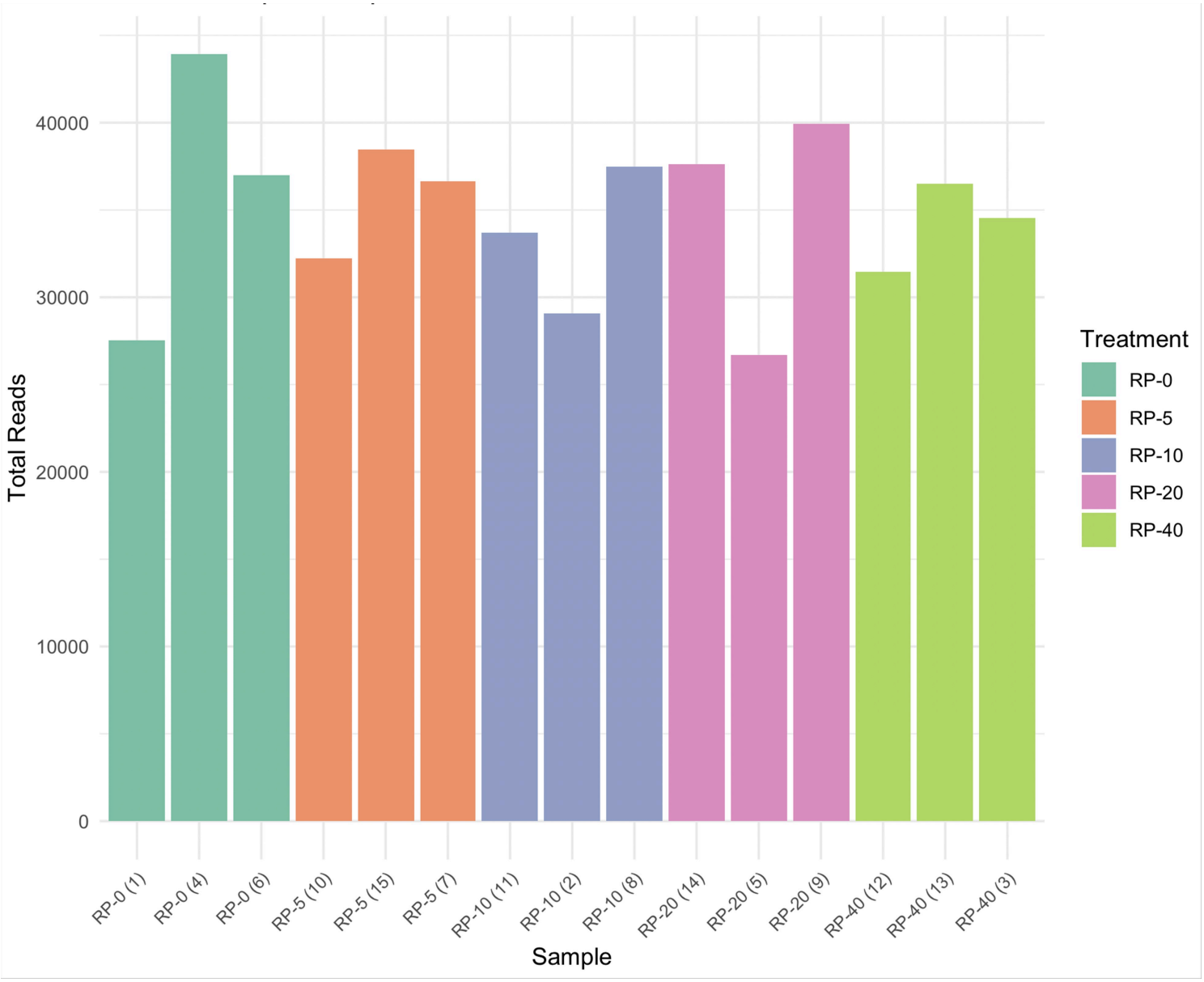

**Figure 6**

Dose-dependent effects of rose petal supplementation on intestinal mRNA expression of antioxidant (*HSP70*, *CYP1A*), growth (*IGF*, *TGF*), and immune-related (*LYZ*, *TNFα*, *IL10*, *IL1β*) genes in goldfish (*Carassius auratus*) after 8 weeks. Data are expressed as relative transcript levels. Different letters indicate significant differences ($p < 0.05$).



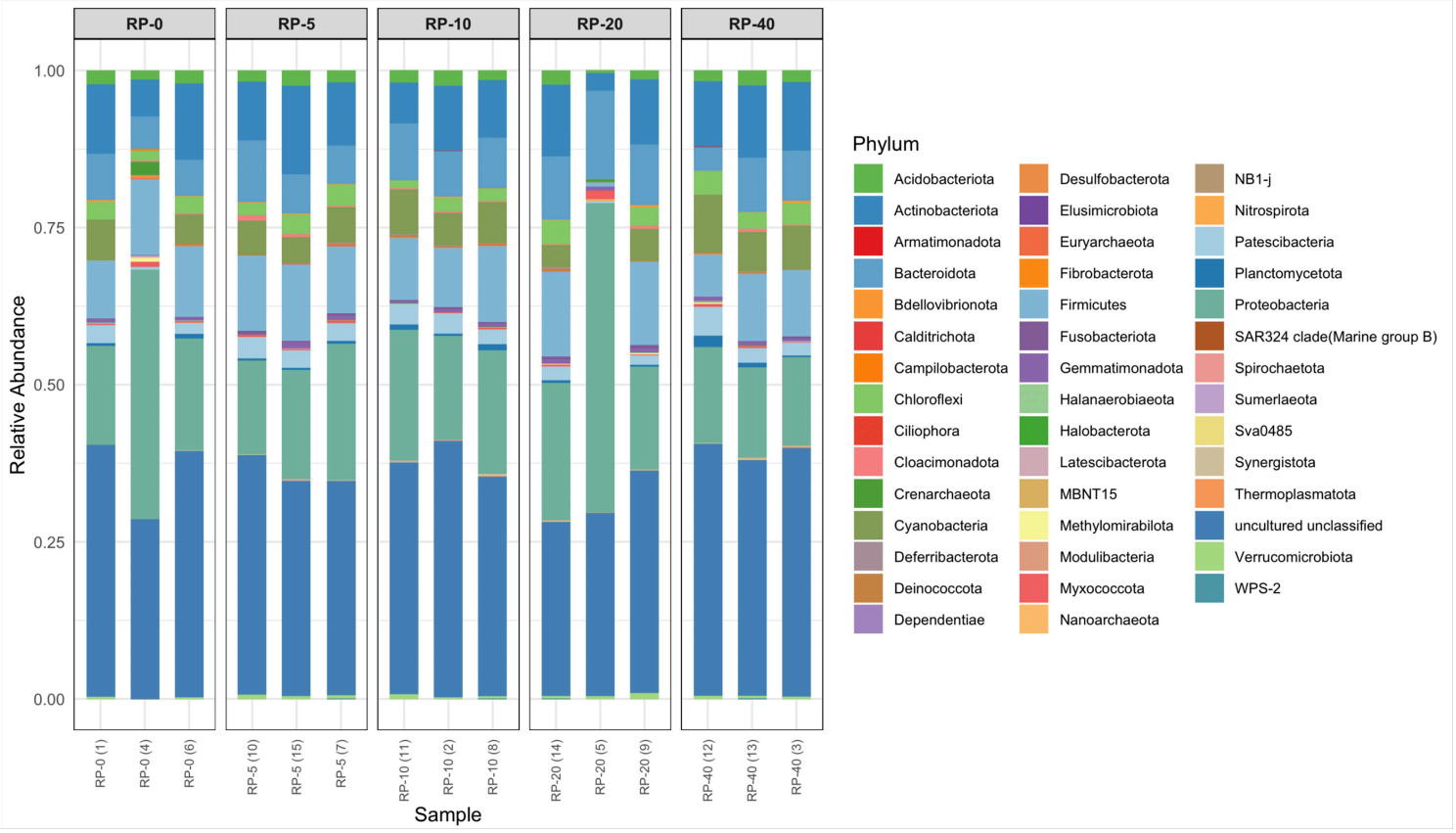

## Figure 7

Spearman correlation between dietary rose petal concentration and intestinal gene expression in goldfish (*Carassius auratus*): (A) HSP70, (B) IGF, (C) TGF, (D) CYP1A, (E) TNFα, (F) LYZ, (G) IL-1β, and (H) IL-10. Linear regression fits and 95% confidence intervals are shown. Correlation coefficients (r) and *p*-values are reported.



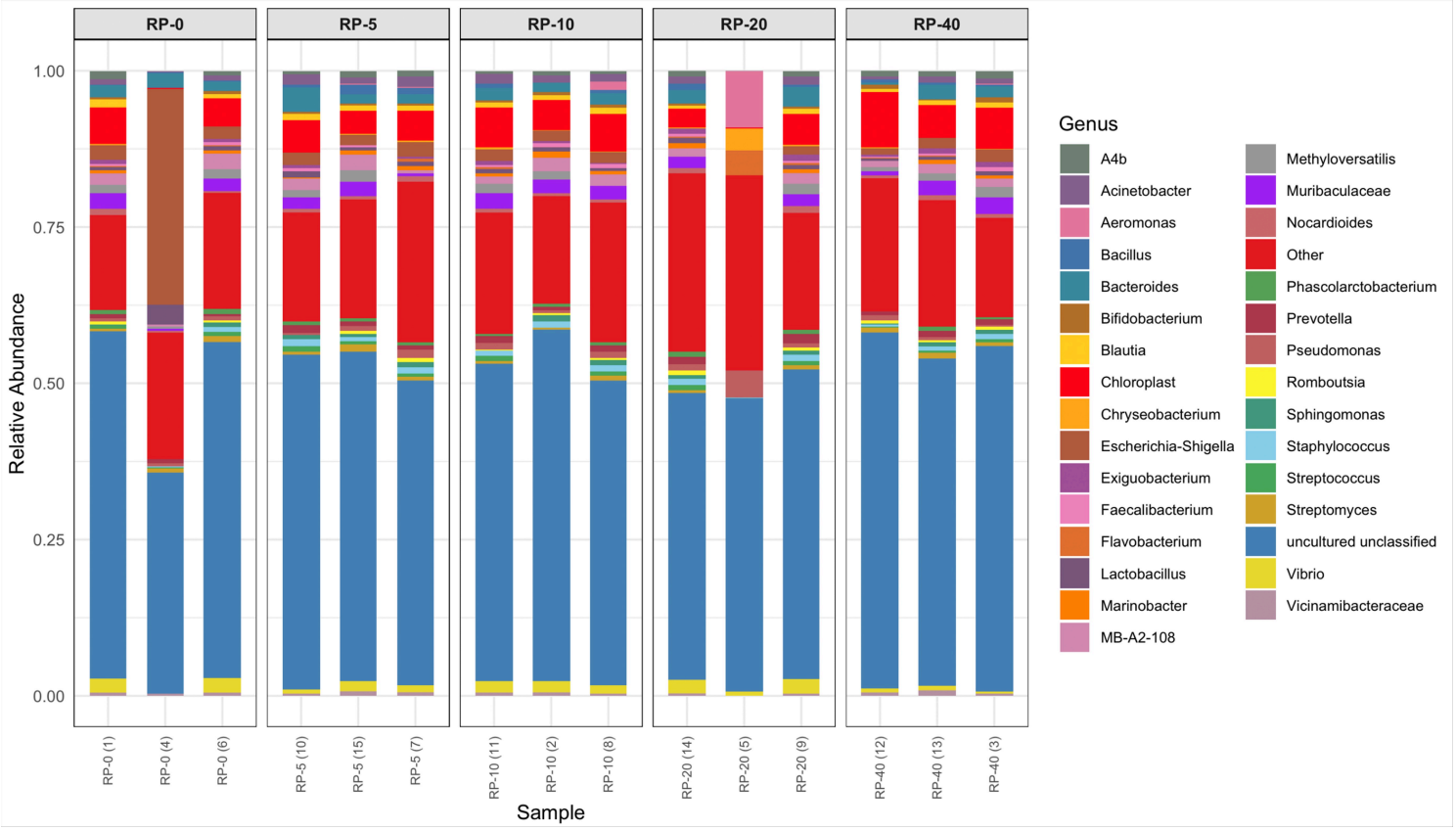

## Figure 8

Total number of high-quality 16S rRNA gene sequences obtained per sample from goldfish (*Carassius auratus*) gut microbiota across dietary treatments (RP-0, RP-5, RP-10, RP-20, RP-40).



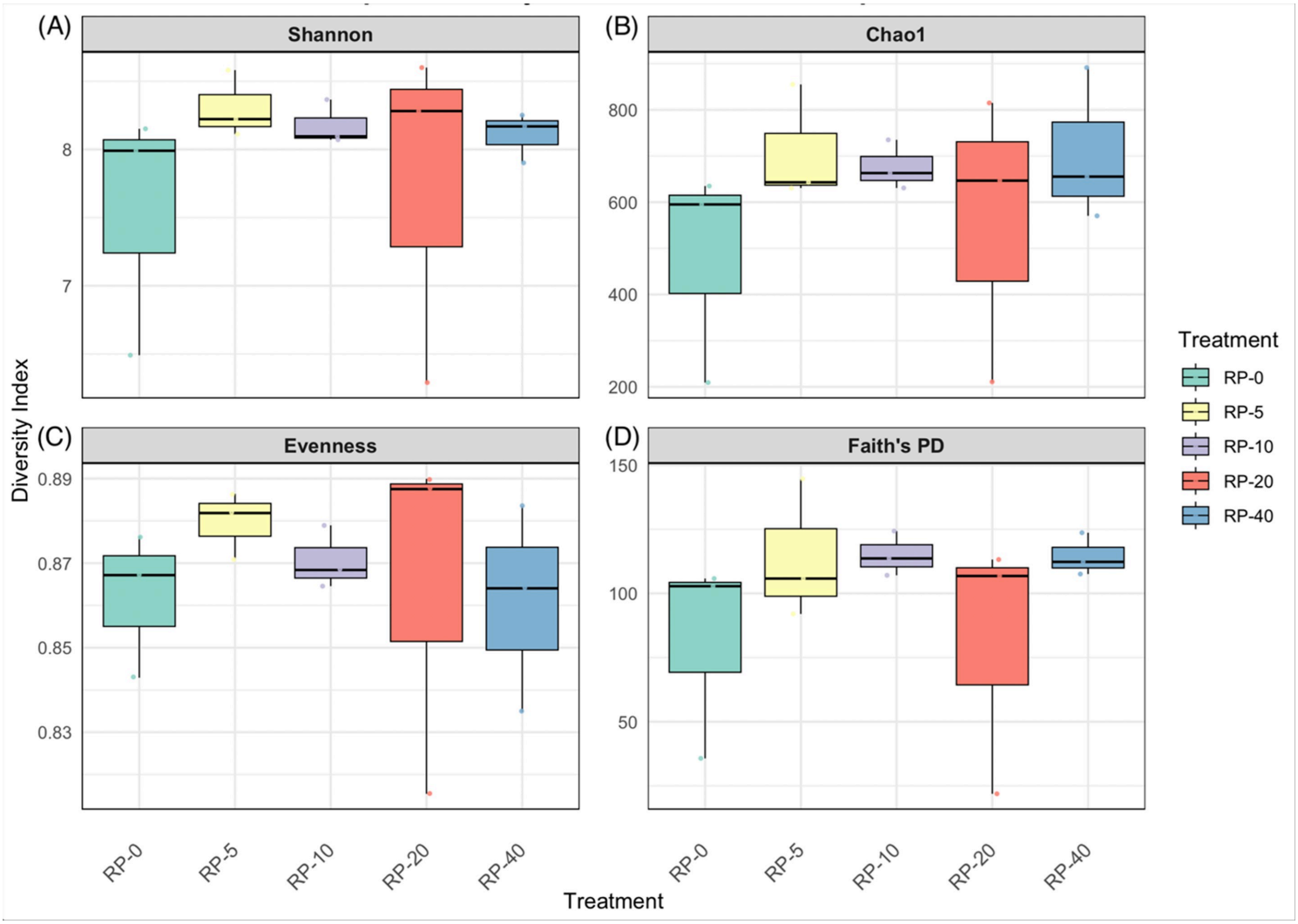

## Figure 9

Relative abundance of dominant bacterial phyla in the gut microbiota of individual goldfish (*Carassius auratus*) after 8 weeks of rose petal supplementation. Each bar represents one sample; phyla are color-coded as indicated in the legend.




## Figure 10

Relative abundance of dominant bacterial genera in the gut microbiota of individual goldfish (*Carassius auratus*) fed rose petal-supplemented diets. Genera not among the 30 most abundant are grouped as "Other". Sequences unclassified at the genus level are labeled "uncultured unclassified". Each bar represents one sample; colors correspond to the legend.




## Figure 11

Alpha diversity indices of the gut microbiota in goldfish (*Carassius auratus*) across dietary treatments (RP-0 to RP-40): (A) Shannon entropy, (B) Chao1 richness, (C) Pielou's evenness, and (D) Faith's Phylogenetic Diversity. Box plots show median, interquartile range, and outliers. No significant differences were found (Kruskal–Wallis, $p > 0.05$ for all).

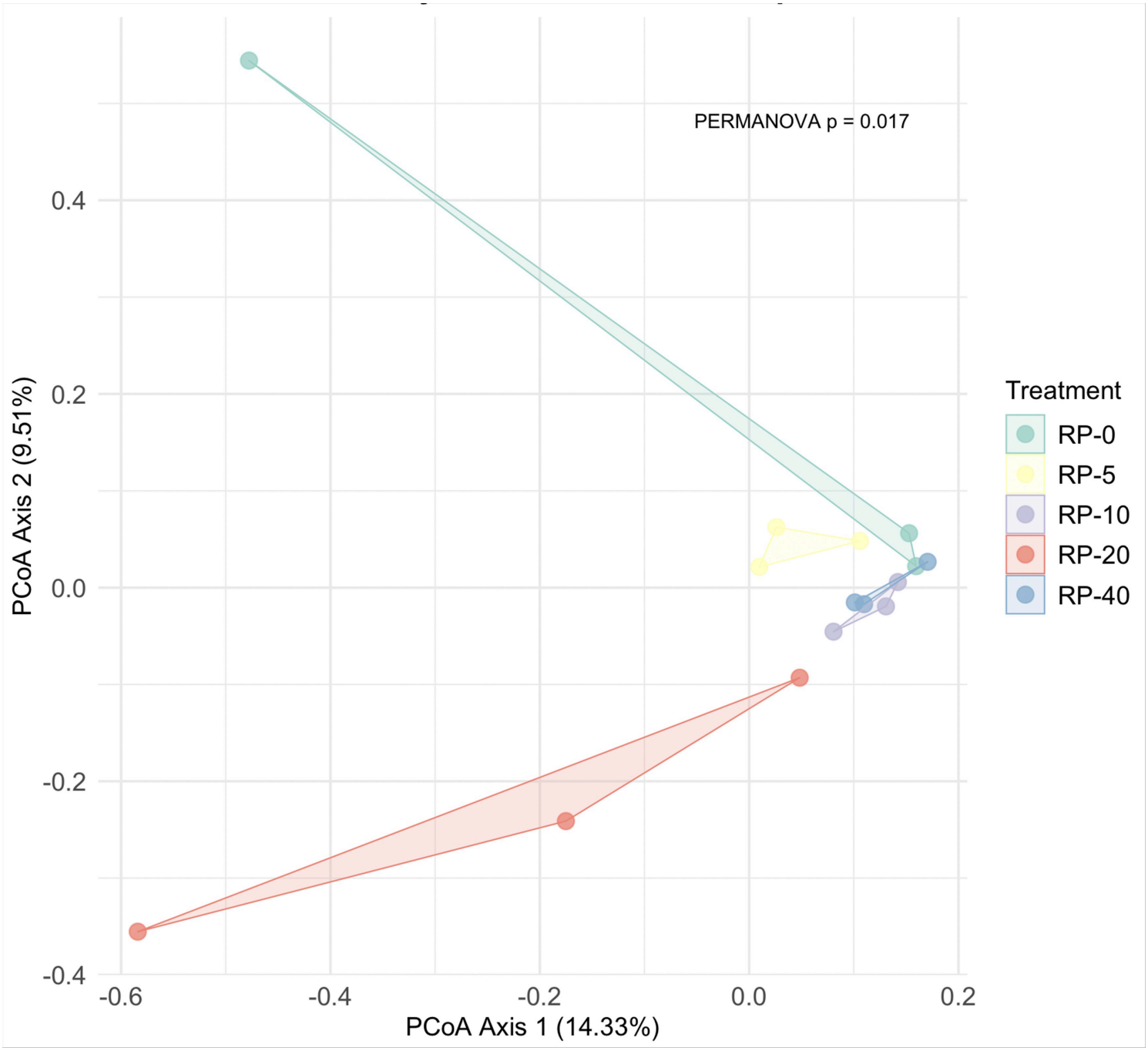



**Figure 12**

Principal Coordinates Analysis (PCoA) based on Bray–Curtis dissimilarity illustrating gut microbiota composition of goldfish (*Carassius auratus*) after 8 weeks of rose petal supplementation. Each point represents a sample colored by treatment group; convex hulls indicate group dispersion. Variance explained by each axis is shown. PERMANOVA revealed a significant group effect ($p$ = 0.017).

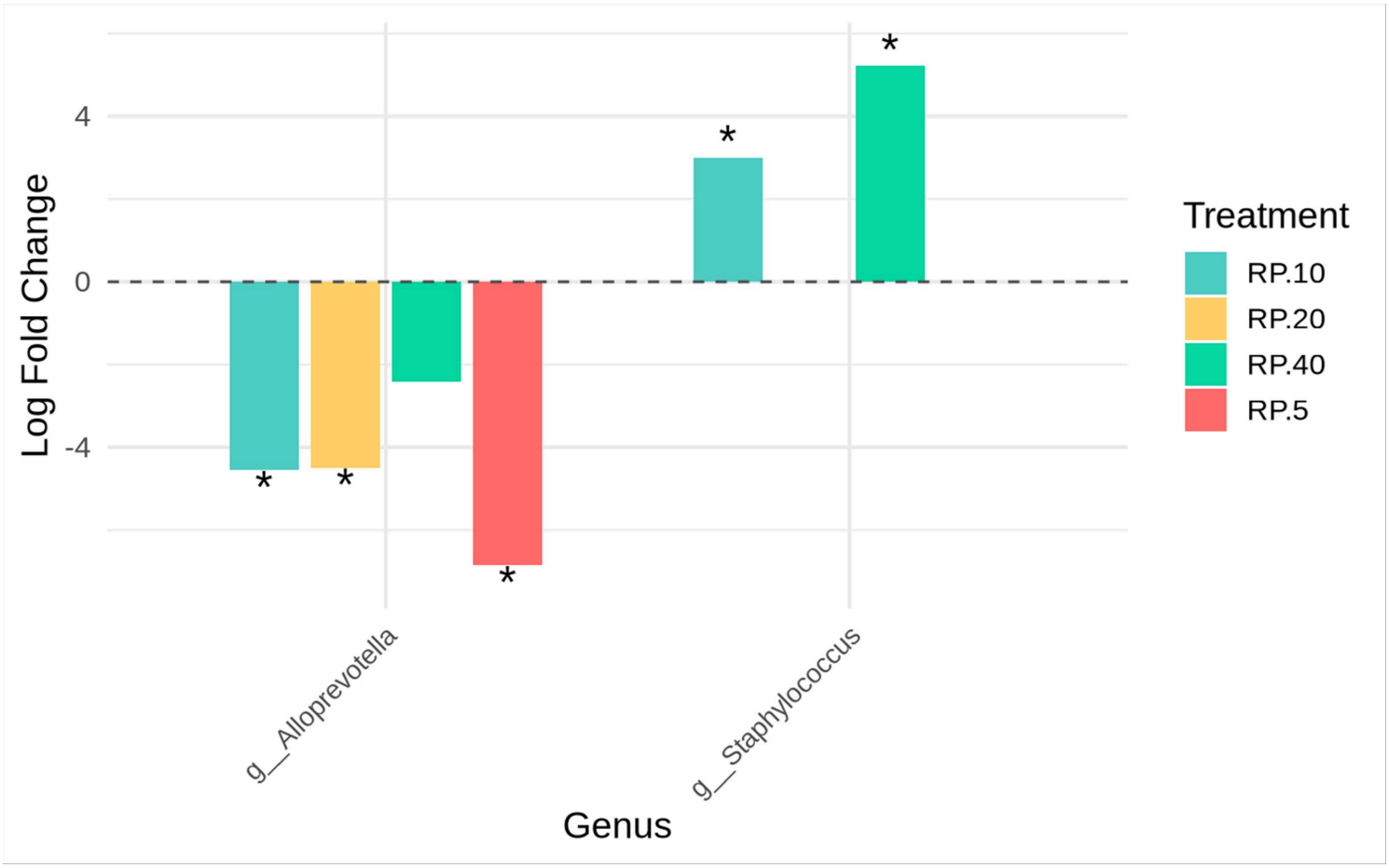
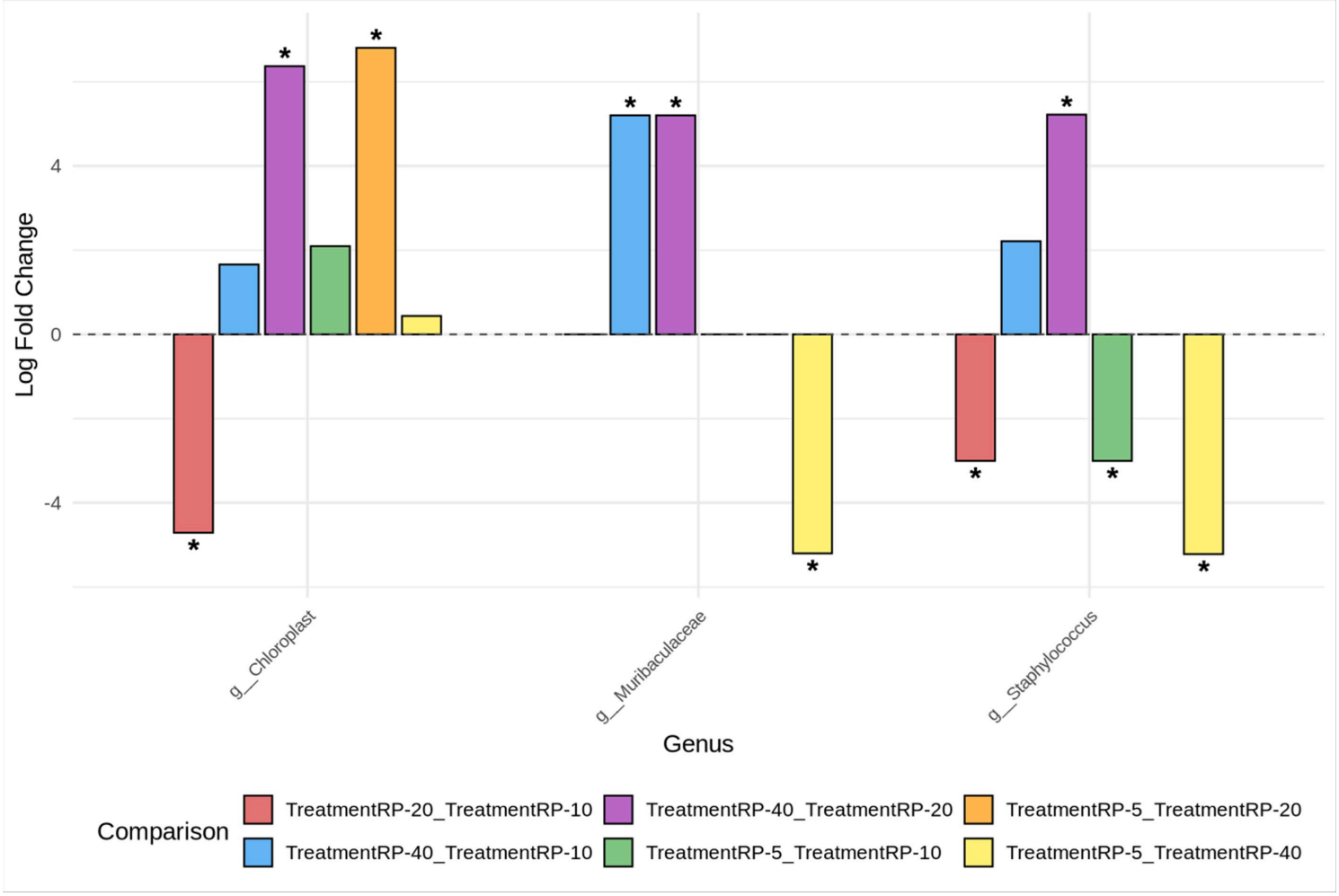



**Figure 13**

fold change (LFC) of differentially abundant genera (*Alloprevotella* sp., *Staphylococcus*) in goldfish (*Carassius auratus*) gut microbiota relative to the control (RP-0) group after 8 weeks. Significant taxa identified by ANCOM-BC2 ($q < 0.05$) are shown. Bars represent estimated LFC; * indicates significance.




## Figure 14

Log fold change (LFC) of differentially abundant genera (*Chloroplast*, *Muribaculaceae* sp., *Staphylococcus*) identified in pairwise comparisons between rose petal (RP) treatment groups in goldfish (*Carassius auratus*). Bars represent LFC estimates from ANCOM-BC2 analysis; * indicates significance ($q < 0.05$).