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Dietary lipid utilisation under different thermal conditions in flathead grey mullet (*Mugil cephalus*)

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Water temperature and dietary lipid levels are key factors influencing fish growth and metabolic homeostasis in aquaculture. This study aimed to understand the interactive effects on growth performance, metabolic response, gut microbiota, and gut evacuation dynamics in flathead grey mullet (*Mugil cephalus*). A 100-day feeding trial was carried out to assess the effects of two dietary lipid levels (10%, L10; 15%, L15) at 22 °C (LT) and 28 °C (HT). Specific growth rate (% day^{-1}) was higher ($p < 0.05$) at HT (0.90 ± 0.01 for both diets) than at LT (0.70 and 0.62 for L10 and L15, respectively). Feed intake at L15 diet was lower at both temperatures ($p < 0.05$) while FCR was reduced only by HT and not by lipid levels. Serum metabolic profiling indicated that higher temperatures accelerated energy turnover, as evidenced by elevated serum glucose and lactate at HT across both dietary groups ($p < 0.05$). Accordingly, fish at HT in both dietary treatments exhibited lower total protein and cholesterol ($p < 0.05$), suggesting a more efficient utilisation of protein reserves and lipid processing to meet increased metabolic demands. Gut evacuation dynamics were described here for the first time in this species by sampling multiple intestinal sections at different time points (1, 3, 5, 7, 9, and 12 hours post-prandial). The intestine was almost completely empty by approximately 8 h post-prandial, with the notable exception of fish fed the HL diet at LT, where a significant delay in evacuation (18 h) was observed. This delay may be attributed to the combined effects of higher dietary lipid levels and lower temperatures, which both prolong digestion and intestinal transit times. Gut microbiota composition was primarily shaped by temperature, with a shift toward a specialised profile dominated by *Cetobacterium* at HT in both dietary groups. Overall, the results highlight that the combination of a 15% lipid diet and 28 °C was associated with the most positive outcomes among the tested conditions, providing further insight into how dietary composition and temperature modulate digestive physiology in flathead grey mullet.

KEYWORDS
dietary lipids, flathead grey mullet, gut evacuation, gut microbiota, *Mugil cephalus*, temperature

1 Introduction

In the context of increasing environmental variability, the identification of low-trophic level (LTL) species capable of adapting to fluctuating thermal regimes has become a priority in aquaculture research (Krause et al., 2022; Maar et al., 2023). LTL species are positioned at the lower levels of the food chain and exhibit herbivorous, omnivorous, or detritivorous feeding habits (Tacon et al., 2011). Species such as tilapia (*Tilapia* spp.) and the flathead grey mullet (*Mugil cephalus*) represent key examples of these species, primarily consuming algae, aquatic plants, phytoplankton, and zooplankton (Soyinka, 2008). Flathead grey mullet possesses a unique digestive physiology, characterized by the absence of acidic digestion and an exceptionally long intestine, which facilitates the breakdown of complex organic matter (Bertini et al., 2023; Natale et al., 2025; Solovyev and Gisbert, 2022). Despite its potential, specific knowledge regarding its nutritional requirements and thermal physiology remains limited compared to other farmed fish. In particular, the interactive effects of temperature and dietary lipid levels on growth performance and feed utilization have yet to be fully elucidated. Temperature and diet composition are among the most influential environmental factors regulating fish physiology, performance, and health (Huang et al., 2021; Rountrey et al., 2014; Steiner et al., 2022; Yadav et al., 2024). As ectothermic organisms, fish depend on environmental temperature to control metabolic rate, feed intake, digestive processes, and energy allocation, all of which directly influence growth and body composition (Islam et al., 2019; Kumar et al., 2022; Volkoff and Rønnestad, 2020). However, the optimal thermal range for growth can be highly species-specific. For instance, Sanz-Latorre et al. (2025), identified an optimal temperature of 22 °C for *Chelon labrosus*, whereas Okamoto et al. (2006) observed a significantly higher optimum of 30 °C for *Mugil platanus*. While the FAO suggests an optimal growth range between 20 °C and 26 °C for *Mugil cephalus* (FAO - *Mugil cephalus*, 2009), the exact temperature required to achieve peak growth performance remains undetermined, leaving a critical gap in the understanding of its specific thermal limits. In parallel, diet composition, particularly lipid and protein levels, determines the availability of metabolic energy and influences nutrient partitioning between somatic growth and lipid storage (Li et al., 2023; Rahimnejad et al., 2021). Dietary lipids play a dual role in fish nutrition since lipids provide a concentrated source of non-protein energy and supply essential fatty acids, while also influencing feed intake, nutrient digestibility, and body fat deposition (Kim et al., 2012). Regarding the nutritional requirements of *Mugil cephalus*, Talukdar et al. (2020) established an optimal dietary protein level of 30%. Rosas et al. (2019) reported successful results with a 10% lipid inclusion; however, their study did not evaluate levels exceeding this threshold, leaving the potential benefits of higher energy densities unexplored. The balance between dietary lipid content and environmental temperature is especially important, as metabolic energy demand increases with temperature, potentially modifying the efficiency with which lipids and proteins are utilised (Badillo-Zapata et al., 2023). An inadequate ratio between dietary energy density and thermal conditions can lead to reduced growth efficiency and excessive lipid accumulation in visceral tissues. Furthermore, in recent years, the fish gut microbiota has emerged as a central component of host physiology, contributing to digestion, nutrient assimilation, immune modulation, and metabolic regulation (Egerton et al., 2018; Flint et al., 2012; Lee and Hase, 2014), representing an important indicator of health and well-being in aquatic organisms (Vargas-Albores et al., 2021). Both environmental temperature and diet composition are recognised as major factors shaping the structure and functional potential of the intestinal microbiota community (Domingo-Bretón et al., 2025). Temperature can directly affect microbial growth rates and community assembly, while dietary lipids modulate substrate availability and metabolic pathways (Domingo-Bretón et al., 2025; Neuman et al., 2016; Pelusio et al., 2021). Despite its ecological and aquaculture relevance, the gut microbiota of the flathead grey mullet remains poorly characterised, and the interaction between temperature and dietary lipid levels has not yet been investigated. In addition to microbiota dynamics, digestive kinetics such as gut evacuation rate, represent an overlooked key of fish nutritional physiology. Gut evacuation rate is known to be strongly affected by both temperature and diet composition (García-Meilán et al., 2014). Lipid-rich diets generally require longer digestion and evacuation times, while dietary fibre may accelerate intestinal passage (Le et al., 2021). Even though the gut evacuation has been studied in other farmed species as tilapia (de Oliveira et al., 2022; Moffatt et al., 2022), gilthead seabream (Busti et al., 2020; Pedro Andrade et al., 1996), trout (Dürrani and Seyhan, 2019; Liu et al., 2022), European seabass (Bonvini et al., 2018; Nikolopoulou et al., 2011), and salmon (Mock et al., 2022; Storebakken et al., 1999) has not yet been investigated for flathead grey mullet. The present study evaluated the combined effects of two isoproteic diet (30%) differing in lipid content (10% and 15%) and two temperatures (22 °C and 28 °C) in *Mugil cephalus*, selected to represent the lower limit of the optimal range and a value at the upper threshold for this species, respectively. This experimental design, reflecting both seasonal variations, was aimed to assessing metabolic responses, intestinal microbiota composition, and gut evacuation rates across multiple post-prandial time points (1, 3, 5, 7, 9, and 12 hours). This integrated approach provides a more comprehensive understanding of the relationship between nutrition, temperature, and digestive physiology in the flathead grey mullet, offering scientific knowledge useful for optimising feeding protocols.

2 Materials and methods

2.1 Experimental diets

The two experimental diets were produced via extrusion technology by SPAROS Lda (Olhão, Portugal). Diets were formulated to contain two lipid levels (10% and 15%, L10 and L5, respectively) and were tested at two temperatures: 22 °C and 28 °C. The diameter of the pellet was 1.5 mm. Ingredients and proximate composition are presented in Table 1.

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TABLE 1 Ingredients and proximate composition of the experimental diet (dry matter basis) administrated to flathead grey mullet (*Mugil cephalus*) specimens over 100 days.

Ingredients as % of the diet	Diet 10%	Diet 15%
Fishmeal super prime	3	3
Poultry meal	9	9
Feathermeal hydrolysate	4.8	4.8
Pea protein concentrate	7	7
Soybean meal (solvent extracted)	10.25	10.25
Sunflower meal	11	11
Wheat meal	39.84	39.84
Cellulose	5	0
Vitamin and mineral premix	1	1
Choline chloride 50%	0.2	0.2
MCP (Monocalcium phosphate)	0.2	0.2
L-Lysine HCl 99%	0.8	0.8
L-Threonine	0.1	0.1
L-Tryptophan	0.1	0.1
DL-Methionine	0.8	0.8
Yttrium oxide	0.01	0.01
Fish oil	3.45	5.95
Rapeseed oil	3.45	5.95
Composition	**Diet 10%**	**Diet 15%**
Crude protein, % feed	31	31
Crude fat, % feed	10	15
Fiber, % feed	6.9	2.5
Starch, % feed	26.5	26.5
Ash, % feed	5.1	5
Arg, % feed	2	2
His, % feed	0.6	0.6
Ile, % feed	1.1	1.1
Leu, % feed	2	2
Lys, % feed	2.1	2.1
Thr, % feed	1.2	1.2
Trp, % feed	0.3	0.3
Val, % feed	1.3	1.3
Met, % feed	1.2	1.2
Cys, % feed	0.4	0.4
Met + Cys, % feed	1.6	1.6
Phe, % feed	1.3	1.3
Tyr, % feed	0.9	0.9
Phe + Tyr, % feed	2.2	2.2
Asx, % feed	2.5	2.5
Glx, % feed	5.3	5.3
Ala, % feed	1.4	1.4
Gly, % feed	1.8	1.8
Pro, % feed	1.9	1.9
Ser, % feed	1.5	1.5
Tau, % feed	0.1	0.1
Total P, % feed	0.88	0.88
Ca, % feed	0.58	0.58
Na, % feed	0.5	0.5
Mg, % feed	0.2	0.2
K, % feed	0.6	0.6
Cu, mg/kg feed	12.6	12.6
Fe, mg/kg feed	100.1	100.1
I, mg/kg feed	3.2	3.2
Mn, mg/kg feed	31.2	31.2
Se, mg/kg feed	0.3	0.3
Zn, mg/kg feed	106	106
Vit A, IU/kg feed	26399.9	27116.4
Vit D3, IU/kg feed	1901.4	1901.4
Vit K3, mg/kg feed	31.4	31.4
Vit E, mg/kg feed	320.9	321.5
Vit B1, mg/kg feed	38.5	38.5
Vit B2, mg/kg feed	37.7	37.7
Vit B3, mg/kg feed	508.2	508.2
Vit B5, mg/kg feed	126.6	126.6
Vit B6, mg/kg feed	41.3	41.3
Vit B9, mg/kg feed	18.6	18.6
Vit B12, mg/kg feed	0.3	0.3
Vit C, mg/kg feed	227.7	227.7
Biotin, mg/kg feed	1.9	1.9
Choline, mg/kg feed	1254.6	1254.6
Inositol, mg/kg feed	618.9	618.9
Betaine, mg/kg feed	1238.7	1238.7
C14, % feed	0.3	0.5
C16, % feed	1.2	1.7
C18, % feed	0.3	0.4
C18:1n9, % feed	3.1	4.7
LNA (C18:2n6), % feed	1.2	1.7
ALA (C18:3n3), % feed	0.4	0.6
ARA, % feed	0.05	0.07
EPA, % feed	0.86	1.39
DHA, % feed	0.3	0.48
EPA+DHA, % feed	1.2	1.9
Proximate composition Ingredient (%) of feed	**Diet 10%**	**Diet 15%**
Crude protein	32.5	31.3
Crude fat	10.42	15.22
Moisture	6.55	10
Fiber	7.5	2.19
Ash	4.6	4.38

(Continued)

2.2 Fish rearing and feeding trial

Flathead grey mullet born in captivity at International Marine Centre (Oristano, Italy) were delivered to the Laboratory of Aquaculture at the University of Bologna (Cesenatico, Italy). At the beginning of the trial, fish (average body weight: 90.48 ± 19.21 g) were tagged and randomly distributed into 12 fiberglass square tanks (450 L each), with 30 fish per tank. These tanks were connected to a closed recirculation aquaculture system, which included a mechanical sand filter (PTK 1200; Astralpool, Barcelona, Spain), ultraviolet lights (UV PE 45; Sita Srl, Barcelona, Spain) and a biofilter (PTK 1200; Astralpool, Barcelona, Spain). Water consisted of a mixture of marine and freshwater (salinity 7 ± 2 ‰) (Cardona, 2006). The temperature was kept constant at 28 °C (HT) for tanks 1 to 6 and at 22 °C (LT) for tanks 7 to 12. Photoperiod was maintained at 12 h light/12 h dark through artificial light and oxygen saturation at 100% by an integrated control system. Ammonia (total ammonia nitrogen ≤ 0.1 mg L–1) and nitrite (≤ 0.2 mg L–1) were spectrophotometrically monitored once a day (Spectroquant Nova 60, Merck, Lab business, Darmstadt, Germany). Fish were fed to apparent satiation by over-supplying the feed (10% of the daily ration) by automatic feeders, once a day for six days a week (Bertini et al., 2023; Natale et al., 2025). Each meal lasted 6 hours to provide fish a continuous feed supply over daily hours. At the end of the 6-hour feeding period, any uneaten pellets were collected through siphoning and subsequently dried overnight at 105 °C. The uneaten feed was then deducted from the overall feed consumption calculations (Busti et al., 2020).

2.3 Sampling

Before each sampling, fish were either anaesthetised (100 mg L^{-1}) or euthanised (300 mg L^{-1}) by tricaine methanesulfonate MS-222 (Sigma Aldrich, Germany). On day 100, all fish were anesthetised (100 mg L^{-1}) and individually weighed to conclude the experiment. The proximate composition of the carcasses was determined at the start of the experiment using a pooled sample of 10 fish. Upon trial completion, analyses were performed on pooled samples of 3 fish per tank (9 fish per diet at each temperature: 28 °C and 22 °C). Additionally, viscera weight (g), liver weight (g), and blood were collected from 4 fish per tank (12 fish per diet) at each temperature. Blood was collected from the caudal vein and transferred to a clot activator tube (Vacutest Kima Srl, Padova, Italy). As soon as the blood clotted it was centrifuged at 3,000 x g for 10 min at 4 °C, serum aliquots removed and stored at -80 °C for chemical analysis. On the following day, the remaining fish were fed to satiety during a 6-hour feeding period before being sampled for gut microbiota analysis. Hindgut content (approximately 250 mg) was aseptically collected from 4 fish per tank (12 fish per diet at each temperature) by gently stripping the final 5 cm of the intestine into sterile tubes and stored at -80 °C for gut microbiota analysis. After microbiota sampling, the remaining fish were fasted for 72 hours, in accordance with Bonvini et al., 2018, to ensure that the stomach and gastrointestinal tract was empty before proceeding with sampling for gut evacuation analysis. Fish were fed during a single one-hour meal. Subsequently, samples for gut evacuation analysis were collected at 1, 3, 5, 7, 9, and 12 hours post-prandial. All experimental procedures were evaluated and approved by the Ethical-Scientific Committee for Animal Experimentation of the University of Bologna, in accordance with European directive 2010/63/UE on the protection of animals used for scientific purposes.

2.4 Calculations

Somatic growth was evaluated using the formulas listed below: Specific growth rate (SGR) (% day−1) = 100 ∗ (ln FBW − ln IBW)/days (where FBW and IBW represent the final and the initial body weights). Feed intake (FI) (% ABW−1 day−1) = ((100 * total ingestion)/(ABW))/days)) (where ABW = (IBW + FBW)/2). Feed conversion ratio (FCR) = feed intake/weight gain. Viscerosomatic index (VSI) (%) = 100 ∗ (viscera weight/body weight). Hepatosomatic index (HSI) (%) = 100 ∗ (liver weight/body weight). Perivisceral fat index (PFI) (%) = 100 ∗ (perivisceral fat weight/body weight). Relative gut length (RGL) = 100 * (gut length/body length). K, Fulton's condition Factor = 100 * (FBW/body length3). Protein efficiency rate (PER) = (FBW - IBW)/protein intake. Lipid efficiency rate (LER) = (FBW - IBW)/lipid intake. Gross protein efficiency (GPE) = 100 * ((% final body protein * FBW) - (% initial body protein * IBW))/total protein intake fish. Gross lipid efficiency (GLE) = 100 * ((% final body lipid * FBW) - (% initial body lipid * IBW))/total lipid intake fish.

2.5 Serum biochemistry

Metabolic blood parameters were determined in a 500 µl serum sample by using an automated analyser (AU 480; Beckman Coulter, Brea, CA, United States) with dedicated methods (Olympus system reagent, OSR, Beckman Coulter, Brea, CA, United States). The type of test used in the assay and the OSR identification number were reported in brackets following the reported parameters. Metabolic blood parameters include glucose (Enzymatic UV test, OSR6121), urea (Kinetic UV test, OSR6134), creatinine (CREA, kinetic colour test, Jaffè method, OSR6178), uric acid (Enzimatic colour test, OSR6198), bilirubin (photometric colour test, OSR6112), cholesterol (enzymatic method, OSR6116), high-density lipoprotein (HDL, direct clearance method, OSR6187), triglycerides (TG, enzymatic method, OSR61118), total protein (photometric colour test, OSR6132), albumin (ALB, photometric colour test, OSR6102), lactic acid (enzymatic colour test, OSR6193), aspartate aminotransferase (AST), alkaline phosphatase (ALP), creatine kinase (CK) and lactate dehydrogenase (LDH) (Kinetic colour test, OSR6009, OSR6170, OSR6004, OSR6179 and OSR6128, respectively), iron (Fe) (Photometric colour test, OSR 6186), sodium (Na+), potassium (K+), chloride (Cl) (ion selective electrode indirect method, 66317 ISE Low Serum Standard; 66316 ISE High Serum Standard) total calcium (Ca total, photometric colour test, OSR60117), phosphorus (P, photometric UV test, OSR6122) and magnesium (Mg, photometric colour test, OSR6189). The following ratios were also calculated: albumin/globulin and Na/K.

2.6 Gut evacuation

To assess gut evacuation analysis, fish were fasted for 72 hours according to several studies (Adamidou et al., 2009; Bonvini et al., 2018; Nikolopoulou et al., 2011) to ensure that gastrointestinal tract

were empty before proceeding. Each fish was individually weighed and then carefully dissected. The gastrointestinal tract was carefully uncoiled, and samples were collected from the anterior, mid, and posterior sections of the intestine. Since the flathead grey mullet's intestine can reach up to twice the body length (Crosetti and Blaber, 2015; Natale et al., 2025), each intestine was measured and divided into three equal sections to ensure consistent sampling. The contents of the anterior, middle, and posterior sections of the intestine were collected into pre-weighed dishes. Each sample was weighed with the wet content for each tissue and individual fish, then dried and reweighed. as described in Adamidou et al., 2009 and Nikolopoulou et al., 2011. The measured weights were used to carry out the subsequent calculation. For each diet at each temperature, the geometric means of the dry digesta content in the intestinal sections, normalised to body weight, were individually regressed against time, in order to fit the data to a model for calculating the anterior, middle and posterior filling times and evacuation times (AET, MET and PET), respectively. The Elliott regression is one of the most widely used models for describing stomach evacuation patterns following feeding (Elliott, 1972; Nikolopoulou et al., 2011). Following Busti et al., 2022, in order to assess the effect of diet on evacuation time, the Elliott model was applied and extended by introducing an additional component that allows the model to depend on a shape parameter, *s*:

$$ln\ W_{ti} = ln\ A_i - r_i t + s_i ln\ t$$

Where *Wt* represents the mean normalized stomach content at time *t*, *A* is the intercept estimated from the model regression, *r* is the gastric evacuation rate, and *i* denotes the diet, with $i = 1,2,3,4$ corresponding to the four experimental treatments (10 and 15 at 28 °C, and 10 and 15 at 22 °C) for a specific gut segment. When $s = 0$, the model reduces to the classical Elliott exponential curve, whereas for $s > 0$ the curve can assume different non-monotonic shapes. A non-monotonic curve implies that the time at which the segment reaches its maximum load does not necessarily coincide with time zero, as instead assumed by the Elliott exponential model, as reported by Busti et al., 2022 in Figure 2. This shape rate formulation is essentially equivalent to fitting a Gamma probabilistic model to the normalized gut content data as a function of time.

2.7 Gut microbiota analysis

DNA for microbiome analysis was extracted from 200 mg of intestinal content, using the DNeasy PowerSoil Pro Kit (Qiagen). Samples were mechanically lysed using tubes containing ceramic beads provided in the kit and processed in a FastPrep-24 homogenizer (MP Biomedicals) at 6 m/s for 40 seconds. Subsequent steps were carried out according to the manufacturer's instructions. DNA concentration and quality were assessed using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific) and further evaluated by agarose gel electrophoresis (1% w/v in TAE buffer). All extracted DNA samples were stored at −20 °C until sequencing. Amplification of the full-length 16S rRNA gene (V1-V9 region) was performed using 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-CGGTTACCTTGTTACGACTT-3') primer pairs following the PCR conditions described in Domingo-Bretón et al., 2024. The resulting PCR products were purified using the Agencourt AMPure XP beads (Beckman Coulter, USA) at a sample/beads ratio of 0.4×. Purified amplicons were then visualized by agarose gel electrophoresis (1.5% w/v in TAE buffer) to verify the presence of the ~1.5 kb band. Amplicons were then quantified using Quant-iT™ PicoGreen™ dsDNA Assay Kit and all the samples were normalized at 200 fmol. Library construction for third-generation sequencing on the Oxford Nanopore Technologies (ONT) platform was performed using the ligation-based kit Native Barcoding Kit 96 V14 (SQK-NBD114.96, Oxford Nanopore Technologies). The ONT protocol version NBA_9170_v114_revP_30Jan2025 was followed. Resulting libraries were loaded at a concentration of 50 fmol and sequenced in a PromethION P2 Solo device (Oxford Nanopore Technologies, UK) using a FLO-PRO114M flow cell (Oxford Nanopore Technologies). Sequencing data acquisition was performed with MinKNOW v6.4.12 (Oxford Nanopore Technologies). Sequencing was continued until approximately 200,000 reads per sample were obtained. Resulting pod5 files were basecalled with Dorado v.1.0.1 basecaller (Oxford Nanopore Technologies; https://github.com/nanoporetech/dorado), using the most accurate available model (SUP). Demultiplexing and barcode/adapter trimming were performed using the same tool. Resulting files were converted to FASTQ format using samtools v1.19.2 (Danecek et al., 2021). These obtained raw sequenced data were lodged in the Sequence Read Archive (SRA) under the Bioproject accession number PRJNA1438441 (BioSample accession numbers: SAMN56537492-544). Sequences were then filtered by length (1200–1800 nt) and quality (Qscore > 14) using Chopper v0.10.0 (De Coster and Rademakers, 2023). Overall run quality was assessed with NanoPlot v1.44.1 (De Coster and Rademakers, 2023). Finally, resulting reads were taxonomically assigned using minimap2 v2.29-r1283 (Li, 2021) against the SILVA NR99 (16S/18S) v138.2 reference database (Chuvochina et al., 2026).

2.8 Statistical analysis

All data are presented as mean ± standard deviation (SD). Data of growth performance and metabolic serum parameters were analysed by a two-way analysis of variance (ANOVA) and in case of significance ($p \leq 0.05$) the Tukey's *post hoc* test was performed. The normality and homogeneity of variance assumptions were validated for all data preceding ANOVA using Shapiro-Wilk and Levene's (or Brown-Forsythe) tests. If these tests were not passed, growth performance data were analysed via Kruskal-Wallis test and Dunn's multiple comparison test while serum parameters were analysed using an Aligned Rank Transformation (ART) ANOVA. Graph Pad 8.0 were used to perform all statistical for growth performance analysis while R (base 'stats' and 'car' packages) was employed for serum metabolic parameters.

Gastrointestinal evacuation data analyses were performed using the function lm in R to estimate the parameter of the three models (corresponding to the three gut segments: anterior, middle and posterior intestine) and the uniroot function to calculate the corresponding evacuation times.

Microbiota data results, including rarefaction curves, were obtained using phyloseq package for R (McMurdie and Holmes,

2013). The normality and homogeneity of variance of microbiota data were assessed with Shapiro–Wilk test (base R 'stats' package), while homogeneity of variances was evaluated using Levene's test implemented in the 'car' package. Differences between experimental groups for alpha diversity indices and bacterial relative abundances were assessed using Aligned Rank Transform (ART) ANOVA implemented in the ARTool R package and considering Temperature and lipids as fixed factors, including their interaction significance threshold was set for $p < 0.05$. *Post hoc* multiple comparisons were performed using estimated marginal means with Tukey adjustment through the emmeans R package. Simple effects were tested by comparing Temperature levels within each lipids level and lipids levels within each Temperature level, and pairwise contrasts were also computed for the interaction terms. Beta diversity among groups was tested with multivariate analysis of variance (PERMANOVA) using the non-parametric method adonis and 10.000 random permutations, also testing the homogeneity of multivariate dispersion using the R package vegan (Oksanen et al., 2015). To further investigate microbiota differences between groups, supervised partial least-squares discriminant analysis (PLS-DA) using EZinfo v3.0 (Umetrics, Umea, Sweden). The quality of the model was evaluated by the parameters, R2Y (cum) and Q2 (cum) and validated through the test performed with the Bioconductor R package ropls, consisting of 7-fold cross-validation and 500 random permutations to prevent overfitting. From the obtained model, the taxa contributing to group separation were determined by the minimum variable importance in the projection (VIP) values, using a VIP threshold ≥ 1.2. Bacterial markers for each variable were also investigated through the linear discriminant analysis effect size (LEfSe). The analysis was performed with the microbiomeMarker R package at the genus level. Counts were normalized using counts per million (CPM). Statistical significance among groups was assessed using the Kruskal–Wallis test ($\alpha = 0.05$), followed by pairwise Wilcoxon tests ($\alpha = 0.05$) with multi-group strategy enabled. Taxa with a Linear Discriminant Analysis (LDA) score ≥ 4 were considered significant biomarkers. To explore the putative functional potential of the microbiota profiles, inferred metagenomic profiles were obtained from 16S rRNA data using the PICRUSt2 protocol with the Kyoto Encyclopaedia of Genes and Genomes database (KEGG) as reference (Kanehisa et al., 2023). Differences between groups based on Temperature and lipid variables were performed using (ART) ANOVA following the already reported evaluations.

3 Results

3.1 Growth performance and feed utilization

Growth performance results are presented in Table 2, while normality and homogeneity results are reported in Supplementary Table 1. At the end of the trial, fish reared at HT showed significantly higher final body weight (FBW) and specific growth rate (SGR) compared to those reared at LT (temperature effect, $p < 0.05$). Feed conversion ratio (FCR) was lower in the HT groups (temperature effect, $p < 0.05$). Feed intake (FI) was significantly lower in fish fed diet L15 compared to those fed L10 (diet effect, $p < 0.05$). No diet × temperature interaction was observed for any of the parameters considered.

3.2 Body composition, nutritional indices and somatometric indices

Data on body composition, nutritional indices, and somatometric indices are reported in Table 3. Whole body protein content was higher in fish fed the L10 diet (diet effect, $p < 0.05$), whereas lipid content was higher in fish reared at LT (temperature effect, $p < 0.05$). Fish reared at LT also showed significantly higher moisture content (temperature effect, $p < 0.05$). However, a significant diet × temperature interaction was observed, with lower moisture levels in fish fed L10 at HT. Ash content did not differ significantly among treatments ($p > 0.05$). PER, LER, GPE, and GLE were higher in HT (temperature effect, $p < 0.05$). Additionally, PER was higher in fish fed the L15 diet (diet effect, $p < 0.05$), whereas LER and GLE were significantly higher in fish fed L10 (diet effect, $p < 0.05$). VSI and PFI were higher in LT (temperature effect, $p < 0.05$). HSI showed a significant diet × temperature interaction

TABLE 2 Growth performance and feed utilisation of flathead grey mullet fed the experimental diets at two temperature (28 and 22 °C).

	Temperature 28 °C		*Temperature 22 °C*		*P value*		
	Diet 10 %	*Diet 15%*	*Diet 10 %*	*Diet 15%*	*Interaction*	*Diet*	*Temp.°C*
Growth performance							
IBW (g)	90.47 ± 0.31	90.69 ± 0.20	90.37 ± 0.20	90.36 ± 0.37	0.500	0.538	0.214
FBW (g)	221.48 ± 9.48	223.63 ± 3.78	181.31 ± 15.96	168.60 ± 12.99	0.266	0.409	< 0.05
SGR (% day^{-1}g)	0.89 ± 0.04	0.90 ± 0.01	0.70 ± 0.09	0.62 ± 0.08	0.306	0.385	< 0.05
FCR	2.99 ± 0.39	2.38 ± 0.17	3.97 ± 0.72	3.60 ± 0.29	0.647	0.092	< 0.05
FI (g feed/fish day^{-1})	2.45 ± 0.09	2.05 ± 0.13	2.63 ± 0.13	2.13 ± 0.11	0.529	< 0.05	0.105
Survival (%)	98.89 ± 1.92	98.89 ± 1.92	98.89 ± 1.92	100 ± 0.00	0.5796	0.5796	0.5796

Data are given as the mean (n = 3) ± SD.
IBW, Initial body weight; FBW, Final body weight.
SGR = Specific growth rate (% day^{-1}) = 100 ∗ (ln FBW − ln IBW)/days.
FI = Feed Intake = (feed intake/ABW/days); ABW = Average body weight (g) = (FBW + IBW)/2.
FCR = Feed conversion rate = feed intake/weight gain.

TABLE 3 Body composition, nutritional indices and somatometric indices of flathead grey mullet fed the experimental diets at two temperature (28 and 22 °C).

	Temperature 28 °C		Temperature 22 °C		P value		
	Diet 10 %	Diet 15%	Diet 10 %	Diet 15%	Interaction	Diet	Temp.°C
Whole body composition, %							
Protein	17.33 ± 0.39	16.48 ± 0.52	17.03 ± 0.54	16.56 ± 0.23	0.468	< 0.05	0.685
Lipid	18.90 ± 0.53	17.36 ± 0.32	20.12 ± 2.02	19.05 ± 0.22	0.713	0.066	0.045
Ash	3.64 ± 0.11	3.78 ± 0.41	3.39 ± 0.21	3.56 ± 0.33	0.908	0.377	0.195
Moisture	58.43 ± 0.16	60.21 ± 0.36	61.12 ± 0.90	60.38 ± 0.72	< 0.05	0.177	< 0.05
Nutritional indices							
PER	1.05 ± 0.08	1.32 ± 0.07	0.79 ± 0.13	0.9 ± 0.08	0.187	<0.05	<0.05
LER	3.29 ± 0.26	2.72 ± 0.15	2.47 ± 0.41	1.85 ± 0.17	0.891	<0.05	<0.05
GPE	18.1 ± 1.41	21.65 ± 1.19	14.08 ± 2.26	14.48 ± 1.36	0.127	0.066	<0.05
GLE	74.96 ± 5.22	57.02 ± 3.23	53.04 ± 7.61	34.58 ± 2.77	0.932	<0.05	<0.05
Somatometric indices							
VSI (%)	5.59 ± 0.83	6.16 ± 0.53	7.85 ± 1.26	8.39 ± 1.27	0.960	0.113	< 0.05
HSI (%)	0.84 ± 0.15	1.14 ± 0.55	1.03 ± 0.24	0.83 ± 0.16	< 0.05	0.624	0.547
PFI (%)	1.13 ± 0.35	0.83 ± 0.53	2.88 ± 0.84	2.71 ± 0.55	0.797	0.577	< 0.05
K	1.12 ± 0.09	1.16 ± 0.90	1.16 ± 0.07	1.14 ± 0.06	0.277	0.822	0.762

Data are given as the mean (n = 3; n = 9 for VSI, HSI, RGL and K value) ± SD.
PER, Protein efficiency rate = (FBW - IBW)/protein intake.
LER, Lipid efficiency rate = (FBW - IBW)/lipid intake.
GPE, Gross protein efficiency = 100 * ((% final body protein * FBW) - (% initial body protein * IBW))/total protein intake fish.
GLE, Gross lipid efficiency = 100 * ((% final body lipid * FBW) - (% initial body lipid * IBW))/total lipid intake fish.
VSI, Viscerosomatic index (%) = 100 * (viscera weight/FBW).
HSI, Hepatosomatic index (%) = 100 * (liver weight/FBW).
RGL, Relative gut length = 100 * (gut length/body length).
K, Fulton's condition Factor = 100 * (FBW/body length3).

($p < 0.05$): at HT, HSI was higher in fish fed L15 compared to L10, whereas at LT the opposite trend was observed, with higher values in fish fed L10 than L15. The Fulton's condition factor (K) did not show significant differences ($p > 0.05$).

3.3 Serum biochemistry

Data on serum variables are reported in Table 4, while normality and homogeneity results are reported in Supplementary Table 2. Among the metabolites analysed, glucose, urea, albumin/globulin ratio, and lactate serum concentrations were significantly affected by temperature ($p < 0.05$), showing lower concentrations in fish reared at LT compared with HT. In contrast, uric acid, total bilirubin, cholesterol, total protein, and albumin serum concentrations displayed higher values in LT than in HT ($p < 0.05$). Creatinine and uric acid serum concentrations were significantly influenced by diet ($p < 0.05$), with higher values observed in fish fed the L10 diet compared with those fed L15. A significant diet × temperature interaction ($p < 0.05$) was detected for cholesterol, HDL, triglycerides, total protein serum concentration, and the albumin/globulin ratio. In particular, cholesterol, triglycerides, and total protein were higher in fish fed L15 than L10 at LT, whereas the opposite pattern was observed at HT, with higher values in fish fed L10. HDL levels were higher in fish fed L10 at LT, while the albumin/globulin ratio showed higher values in fish fed L15 at HT. Among serum enzymes, alkaline phosphatase (ALP) was significantly affected by the diet × temperature interaction ($p < 0.05$), with higher values observed in fish fed L15 at HT. Serum electrolytes were primarily influenced by temperature. Na/K ratio, iron, sodium, chloride, calcium, and phosphorus showed significantly higher concentrations in fish reared at LT compared with HT ($p < 0.05$), whereas magnesium exhibited the opposite trend, with higher values at HT ($p < 0.05$). Additionally, Na/K ratio and potassium were significantly affected by the diet × temperature interaction ($p < 0.05$): the Na/K ratio was higher in fish fed L10 at LT, while potassium concentrations were higher in fish fed L10 at HT.

3.4 Gut evacuation

Gut evacuation analysis was performed at the end of the trial (day 100) at 1, 3, 5, 7, 9 and 12 h postprandial in the anterior, middle and posterior intestine of *Mugil cephalus* (Figure 1). Under all experimental conditions, gut content increased markedly immediately after feeding. Specifically, the peak is reached within the first three 3 hours post-prandial, after which gut content rapidly decreased over time in all segments. Following this peak, gut content progressively decreased over time in all intestinal segments. By 8 h post-prandial, the intestine was almost empty in all sections. A significant effect of the treatment was observed. In particular, as illustrated in Table 5, the combination of L15 diet and LT exhibited consistently lower evacuation rates (r) compared to the other treatments, while no significant differences were observed among the remaining groups across all three intestinal segments as illustrated in Table 5. This reduced evacuation rate corresponded to

TABLE 4 Serum biochemistry of flathead grey mullet fed the experimental diets at two temperature (28 and 22 °C).

	Temperature 28 °C		*Temperature 22 °C*		*P value*		
	Diet 10%	*Diet 15%*	*Diet 10%*	*Diet 15%*	*Interaction*	*Diet*	*Temperature*
Serum metabolites							
Glucose mg/dl	117.67 ± 12.72	119.56 ± 38.05	90.56 ± 9.71	105.67 ± 20.31	0.56	0.3	< 0.05
UREA mg/dl	1.35 ± 0.12	1.32 ± 0.07	1.19 ± 0.12	1.33 ± 0.2	0.48	0.47	0.05
Creatinine mg/dl	0.15 ± 0.01	0.17 ± 0.02	0.14 ± 0	0.17 ± 0.01	0.79	< 0.05	0.82
Uric Acid mg/dl	0.13 ± 0.02	0.16 ± 0.02	0.16 ± 0.01	0.23 ± 0.05	0.13	< 0.05	< 0.05
Total Bilirubin mg/dl	0.07 ± 0.01	0.07 ± 0.01	0.1 ± 0.01	0.09 ± 0.01	0.21	0.53	< 0.05
Cholesterol mg/dl	241.44 ± 25.62	302.89 ± 29.57	325.06 ± 28.62	301.22 ± 49.85	< 0.05	0.31	< 0.05
HDL mg/dl	33.44 ± 2.69	32.22 ± 1.07	30 ± 1.67	36.89 ± 6.5	< 0.05	0.08	0.92
Triglycerides mg/dl	370.89 ± 26.93	449.44 ± 22.95	541.33 ± 50.98	375 ± 44.79	< 0.05	0.2	0.19
Total Proteins g/dl	3.18 ± 0.21	3.4 ± 0.13	4.07 ± 0.14	3.78 ± 0.18	< 0.05	0.9	< 0.05
Albumin g/dl	1.01 ± 0.06	1.02 ± 0	1.2 ± 0.05	1.12 ± 0.04	0.25	0.57	< 0.05
Albumin/Globulin	0.47 ± 0.02	0.43 ± 0.02	0.42 ± 0.01	0.43 ± 0.01	< 0.05	< 0.05	< 0.05
Lactate mg/dl	89.2 ± 7.15	91.92 ± 16.5	61.96 ± 5.15	61.98 ± 13.41	0.97	0.89	< 0.05
Serum enzymes							
AST U/L	11.89 ± 6.74	11.44 ± 3.72	13.44 ± 5.32	16.22 ± 0.84	0.95	0.48	0.24
ALP U/L	57.89 ± 17.93	76.22 ± 7.72	99.44 ± 22.42	59.22 ± 9.92	< 0.05	0.32	0.45
CK U/L	223.89 ± 212.47	161.44 ± 74.55	221.22 ± 113.96	207.5 ± 101.08	0.34	0.44	0.24
LDH U/L	194.22 ± 130.9	123.11 ± 66.82	255.5 ± 132.04	272.67 ± 61.44	0.39	0.71	0.26
Serum electrolytes							
Na/K	70.67 ± 10.58	57.11 ± 5.59	75.67 ± 7.8	88.67 ± 8.19	< 0.05	0.95	< 0.05
Iron µg/dl	131.33 ± 12.84	99.89 ± 9.14	137.72 ± 13.92	159.56 ± 20.62	0.05	0.6	< 0.05
Na mEq/l	144 ± 2.33	146.44 ± 0.69	160.44 ± 2.34	159 ± 7.97	0.78	0.94	< 0.05
K mEq/l	2.1 ± 0.38	2.62 ± 0.24	2.24 ± 0.29	1.84 ± 0.23	< 0.05	0.47	0.06
Cl mEq/l	123.44 ± 1.35	125.78 ± 3.4	136.11 ± 2.67	135.11 ± 8.55	0.82	0.93	< 0.05
Calcium mg/dl	10.62 ± 0.77	11.27 ± 0.32	11.91 ± 0.47	11.59 ± 0.74	0.15	0.33	< 0.05
P mg/dl	6.06 ± 0.42	6.62 ± 0.63	8.29 ± 0.9	8.36 ± 0.59	0.69	0.11	< 0.05
MG mg/dl	2.42 ± 0.16	2.58 ± 0.09	2.27 ± 0.08	2.16 ± 0.24	0.18	0.97	< 0.05

longer evacuation times, as reflected in the estimated time required for 50%, 75%, and 90% of the feed to be evacuated (Table 6). In contrast, no significant differences were detected among the remaining treatments. According to the Table 5, the shape parameter (s) were significantly different from zero only in the first intestinal segment, whereas in the second and third segments, they did not differ significantly from zero. Consequently, the evacuation curves in these latter segments were decreasing and broadly resembled exponential decay patterns, with the notable exception of the L15 diet at LT, in which a slight peak was observed at 3h.

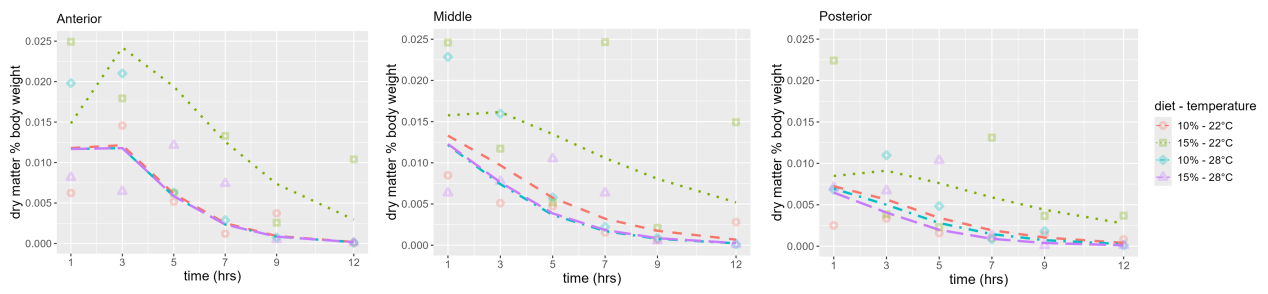

FIGURE 1
Gut evacuation analysis of flathead grey mullet fed the experimental diets (10% lipid; 15% lipid) at two temperature (28 and 22 °C) at 1, 3, 5, 7, 9 and 12 hours post-prandial in the anterior, middle and posterior intestine.

TABLE 5 Estimated model parameters for the different intestinal traits.

	Temperature 28 °C		Temperature 22 °C				
	(r) Diet 10%	(r) Diet 15%	(r) Diet 10%	(r) Diet 15%	log A	S	R^2
Anterior	0.66[a]	0.66[a]	0.65[a]	0.42[b]	-3.79	1.21*	0.82
Middle	0.44[a]	0.43[a]	0.35[ab]	0.18[b]	-3.97	0.35	0.71
Posterior	0.40[a†]	0.47[a]	0.36[ab]	0.20[b]	-4.57	0.43	0.69

Different superscripts indicate significant differences between rates ($p < 0.05$), or marginally significant differences ($0.05 \leq p < 0.1$) if followed by "†". For the shapes, a superscript "*" indicates a significant difference from 0 ($p < 0.05$).

3.5 Gut microbiota

Nanopore sequencing generated a total of 10,386,623 reads, with a mean sequencing depth of 225,769.152 reads per sample. These reads were assigned to a total of 53,639 unique sequence variants classified up to genus level (84%), and more than 99% to the phylum level. Rarefaction curves approached a plateau (horizontal asymptote), indicating that the number of assigned sequences provided adequate coverage of the bacterial community (Supplementary Figure 1). The analysis of alpha diversity data exhibited a greater effect of temperature than of the quantity of lipids in the diet, as shown in Figure 2. In fact, although there were no differences for the richness indices (Chao1, ACE), the diversity metrics (Shannon and Simpson) shown higher values for the two groups at 22 °C. This trend was further explored with the two-way ART analysis (Supplementary Table 3) which confirmed the importance of temperature in influencing the microbial community, although highlighting how the amount of lipids in the diet also significantly affected the Simpson index, mainly influenced by the low value recorded in the group at HT and L15. Phylum- and genus-level microbiota compositions are presented in Figure 3. For both taxonomic levels, statistical analysis revealed significant differences among groups, as reported in the Supplementary Tables 4 and 5. At the phylum level, among the most abundant, such as Pseudomonadota, Fusobacteriota and Actinomycetota, the results indicated that the observed bacterial variation was mainly determined by temperature, while dietary treatment had only a minor effect on Bacillota and Fusobacteriota. At the genus level, focusing on the most abundant in all groups, *Cetobacterium* and *Shewanella* showed significant responses to both temperature and diet, with a higher relative abundance observed at the HT and in fish fed the L15 diet. In contrast, *Shinella* and *Agromyces* were significantly influenced only by temperature, exhibiting greater abundance at LT and no significant response to dietary lipid level. Although less abundant, four different taxa belonging to Lactobacillales, including *Leuconostoc*, *Lactococcus*, *Streptococcus* and *Pediococcus* showed a similar pattern among groups. In particular they exhibited higher values associated with LT and strong significative influence of dietary lipid content. Furthermore, LEfSe analysis corroborated these findings, identified distinct microbial taxa enriched under different temperature and dietary lipid conditions, as shown in Figures 4A, B. At HT, *Cetobacterium*, *Gemmobacter*, and *Shewanella* exhibited significantly higher relative abundances and were identified as biomarkers of the 28 °C condition. In contrast, at LT, *Shinella*, *Agromyces*, *Paracoccus*, *Nocardioides*, *Rubripirellula*, *Corynebacterium*, *Hyphomicrobium*, and *Tessaracoccus* were more abundant and characterized this temperature condition. Regarding dietary lipid levels, *Cetobacterium* was the only biomarker associated with the L15 diet, whereas *Streptococcus*, *Pediococcus*, *Lactococcus*, *Leuconostoc*, *Nocardioides*, *Roseitalea*, and *Lactiplantibacillus* were identified as biomarkers of the L10 diet. The combination of the two variables (temperature and lipids) was also analysed from a general perspective. Permutational multivariate analysis of variance highlighted statistically significant differences among the four experimental groups (PERMANOVA, F = 6.4429, R2 = 0.3157, P<0.001), also supported by the not significant result (P>0.05) of the dispersion homogeneity. To go deeper in the evaluation, a PLS-DA model was constructed and validated (R2Y = 91%, Q2 = 69, p=0.002) (Supplementary Figure 2), confirming a clear separation of sample profiles according to temperature (Figure 5A) as the main factor. Fish reared at HT clustered together within the same quadrant regardless of dietary lipid level and were clearly separated from fish reared at LT. In contrast, samples from fish reared at LT exhibited separation between dietary treatments (10% and 15% lipid levels), as also reported by the heatmap showing the hierarchical distribution of samples based on VIPs (Figure 5B). Similar to what was previously reported, the predicted functional profiles revealed a greater influence of the temperature rather than dietary lipids inclusions

TABLE 6 Estimated evacuation times (h) for the different intestinal segments under each experimental diet at both temperatures (22 °C and 28 °C) in flathead grey mullet.

	Temperature 28 °C		Temperature 22 °C	
	Diet 10%	Diet 15%	Diet 10%	Diet 15%
Anterior intestine				
AET (50%)	4.55	4.55	4.62	7.15
AET (75%)	6.15	6.15	6.25	9.67
AET (90%)	8.03	8.03	8.15	12.62
Middle intestine				
MET (50%)	3.56	3.65	4.48	8.71
MET (75%)	5.48	5.61	6.89	13.40
MET (90%)	7.85	8.03	9.87	19.19
Posterior intestine				
PET (50%)	4.30	3.66	4.78	8.60
PET (75%)	6.47	5.51	7.19	12.94
PET (90%)	9.13	7.77	10.15	18.26

Values correspond to the time required to evacuate 50%, 75% and 90% of gut contents in the anterior (AET), middle (MET) and posterior (PET) intestine.

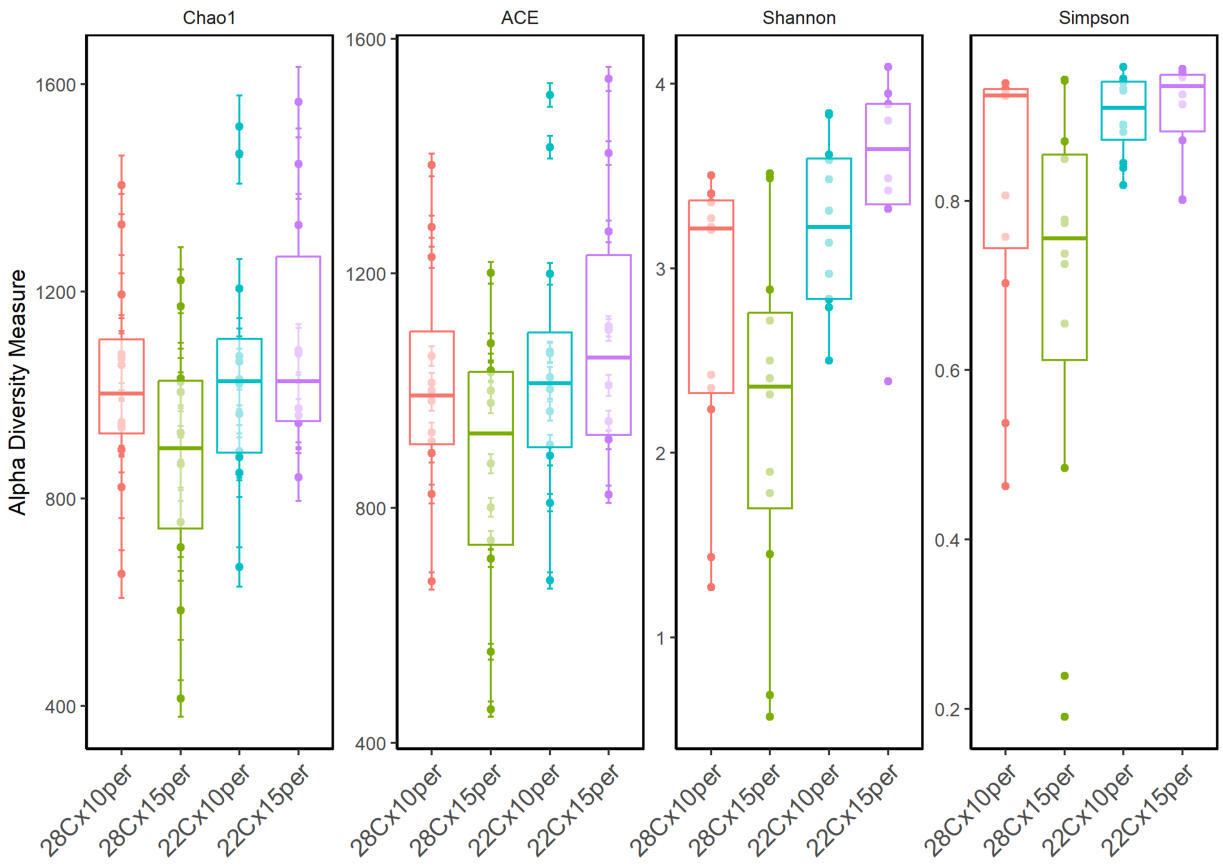

FIGURE 2
Boxplots representing richness estimators (Chao1 and ACE) and diversity indexes (Shannon and Simpson) of gut microbiota populations of flathead grey mullet fed experimental diets at two temperatures (28 and 22 °C).

(Supplementary Table 6). These findings can be organized in three different blocks according to the response. Inferred functions associated with LT included bacterial chemotaxis, ABC transporters, valine, leucine, and isoleucine biosynthesis, as well as ketone body synthesis and degradation (Figure 6). In contrast, samples at HT showed higher predicted abundance of pathways related to fatty acid metabolism, aminoacyl-tRNA biosynthesis, peptidoglycan biosynthesis, lysine biosynthesis, D-alanine metabolism, D-glutamine

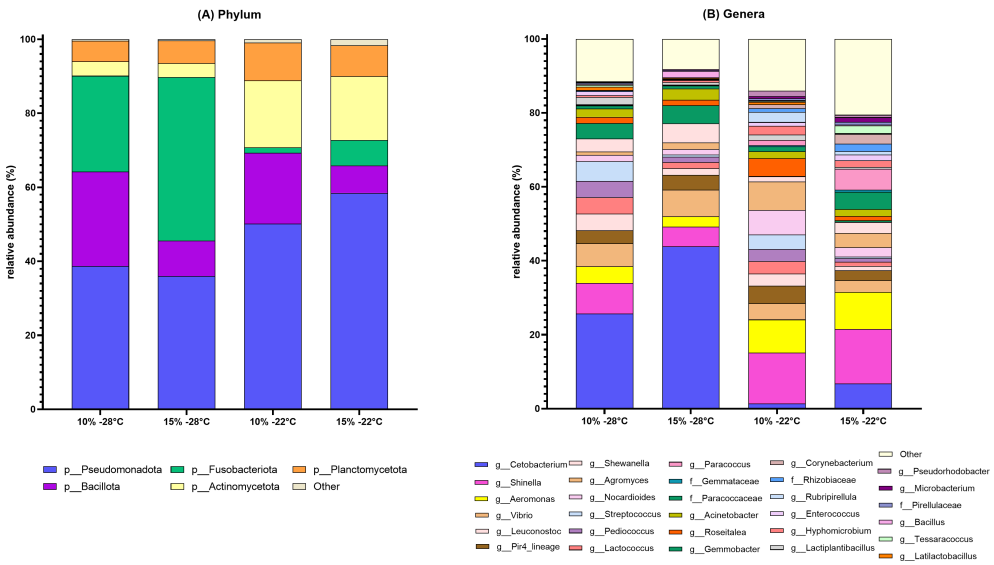

FIGURE 3
Profiles of the gut microbiota of the flathead grey mullet fed the experimental diets at two temperatures (28 and 22 °C) at the phylum **(A)** and genus **(B)** levels. The main five phyla and the 30 most abundant bacterial genera were reported, the rest of the profiles were grouped as "other".

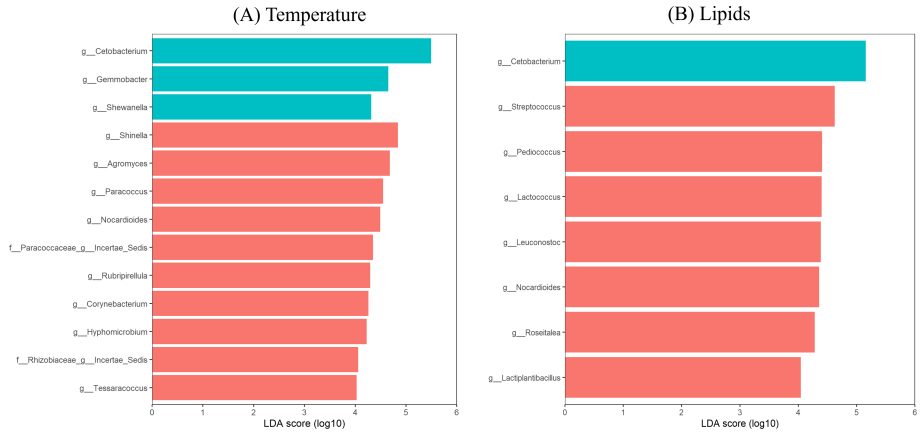

FIGURE 4
LEfSE analysis of the gut microbiota of flathead grey mullet considering the two experimental variables of the study separately, temperature **(A)** and lipid content **(B)**.

and D-glutamate metabolism, alanine, aspartate and glutamate metabolism, and the pentose phosphate pathway. Finally, inferred pathways involved in biotin, pantothenate/CoA, streptomycin, vancomycin, and ansamycin biosynthesis can be grouped together as highlighted a combined diet- and temperature-dependent effects.

4 Discussion

4.1 Growth performance indicators

This study provides novel insights into the interactive effects of dietary lipid levels and temperature on growth performance, gut microbiota composition, and gut evacuation dynamics in *Mugil cephalus*. To date, information on the nutritional requirements and thermal physiology of this species remains limited, highlighting the need for a deeper understanding to optimise rearing strategies and feed formulation in flathead grey mullet aquaculture. Among environmental factors influencing fish performance, temperature plays a key role. As ectothermic organisms, fish rely on environmental temperature to regulate metabolic rate, feed intake, digestive processes, and overall growth performance (Islam et al., 2019; Volkoff and Rønnestad, 2020). At the end of the experimental period, fish fed diets containing 10% and 15% lipids at 28 °C exhibited significantly higher final body weights compared with fish receiving the same diets at 22 °C. These results indicate that rearing flathead grey mullet at 28 °C promotes enhanced growth performance, likely because this temperature approaches the species' optimal thermal range (26–27 °C) previously reported (Crosetti and Blaber, 2015). Under these conditions, fish presumably utilised dietary nutrients more efficiently to satisfy metabolic energy demands,

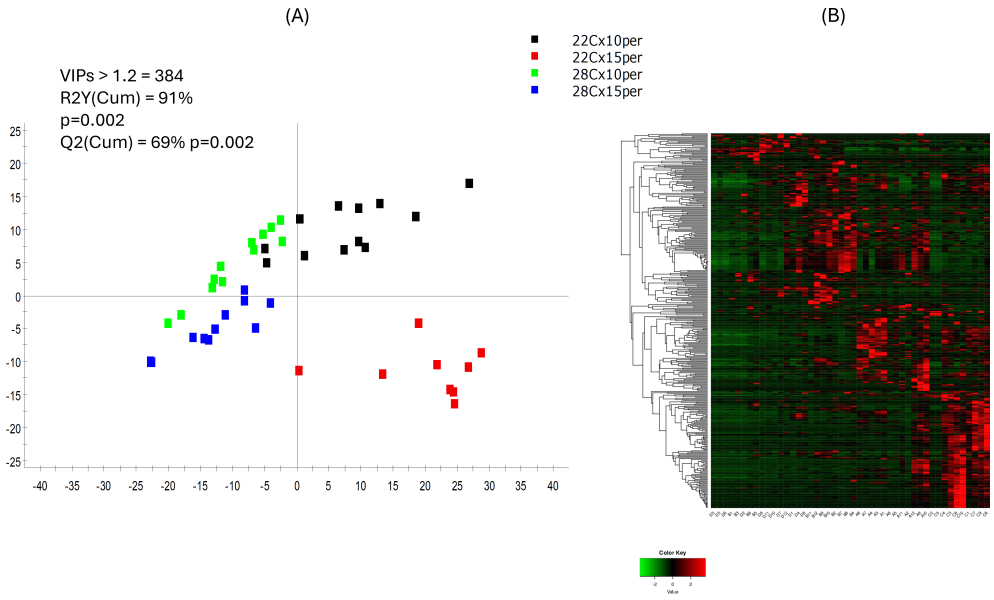

FIGURE 5
The two-dimensional PLS-DA score plot for the first two components of the model representing the separation of the microbiota samples of flathead grey mullet fed the experimental diets at two temperatures (28 and 22 °C) **(A)**. Heatmap showing the hierarchical clustering of the identified VIPs (VIP value > 1.2) **(B)**.

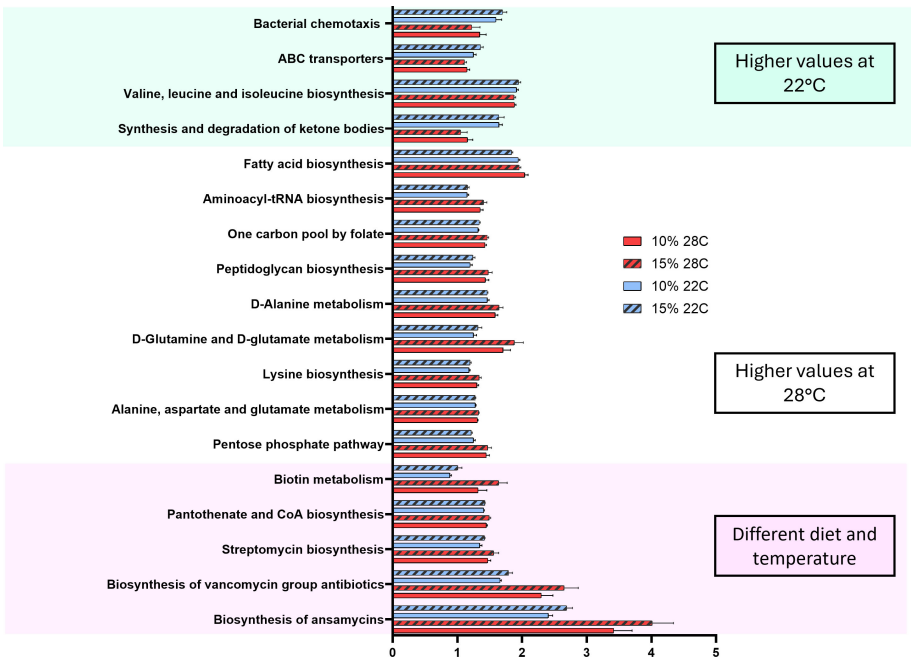

FIGURE 6
Inferred functional enrichment analysis of gut microbiota of flathead grey mullet fed the experimental diets at two temperature (28 and 22 °C). KEGG metabolic pathways were grouped based on the different response they exhibited with respect to the experimental variables.

thereby supporting increased growth. These findings are consistent with the general pattern described in the literature, whereby fish growth increases with temperature up to a species-specific optimum (Ashaf-Ud-Doulah et al., 2020; Islam et al., 2019; Khieokhajonkhet et al., 2022; van Denderen et al., 2020). However, growth responses are also modulated by diet composition and feed energy density (El-Sharkawy et al., 2023; Nahida et al., 2025; Quirós-Pozo et al., 2025). Supporting this interpretation, both feed conversion ratio (FCR) and specific growth rate (SGR) confirmed improved growth efficiency at higher temperature, as demonstrated by lower FCR and higher SGR values irrespective of dietary lipid level. This suggests that temperature exerted a stronger influence on growth performance than moderate variations in dietary lipid inclusion. Considering the limited available data for flathead grey mullet, these results are particularly relevant, indicating that optimal growth occurs at approximately 28 °C rather than at cooler conditions (22 °C). Interestingly, feed intake was significantly lower in fish fed the 15% lipid diet at both temperatures compared with those receiving the 10% lipid diet, consistent with FCR results. The reduced feed intake observed with the higher lipid diet is likely related to its greater dietary energy density, enabling fish to satisfy energetic requirements with lower feed consumption. Conversely, fish fed the 10% lipid diet may have compensated for the lower dietary energy supply by increasing feed intake (Guo et al., 2019; Targett and Targett, 1990). The improved FCR observed in fish fed the 15% lipid diet at the same temperature may also be explained by the protein-sparing effect. When dietary lipids provide sufficient non-protein energy, amino acid catabolism for energy production is reduced, allowing dietary protein to be preferentially allocated to tissue synthesis and growth (Jia et al., 2017; Kim et al., 2012; Santinha et al., 1996). In the present study, the 15% lipid diet had a higher estimated gross energy content (21.99 vs. 20.32 MJ kg^{-1}), which likely enabled fish to meet metabolic energy requirements with reduced feed intake, thereby improving growth efficiency. Moreover, the combined effect of elevated temperature (28 °C) and increased dietary energy availability appears to have enhanced nutrient utilisation. Higher temperatures generally accelerate metabolic processes and increase energy demand, favouring the utilisation of readily available energy sources (Badillo-Zapata et al., 2023; Perez Velazquez et al., 2015). This interpretation is supported by the protein efficiency ratio (PER), which was significantly higher at 28 °C and further improved with 15% dietary lipid inclusion, indicating a greater efficiency in converting dietary protein into biomass. In contrast, lipid efficiency ratio (LER) increased at 28 °C but declined at higher dietary lipid levels, suggesting reduced marginal efficiency of lipid utilisation when supplied in excess. In agreement with these findings, fish reared at 22 °C under both dietary treatments exhibited a reduced capacity to efficiently utilise dietary energy, resulting in an imbalance between energy intake and metabolic demand. This interpretation is supported by the observation that, at 22 °C, dietary lipids were likely diverted towards storage rather than somatic growth. Fish maintained at this temperature showed significantly higher perivisceral fat index and viscero-somatic index (VSI) values compared with those reared at 28 °C, indicating increased lipid deposition and reduced conversion of dietary energy into growth (Ibarz et al., 2010; Mininni et al., 2014).

4.2 Serum biochemistry

Analysis of serum parameters reveals that temperature and dietary lipid levels exert a significant and often combined influence on the flathead grey mullet's physiology. Regarding energy metabolism, both glucose and lactate levels were significantly higher in fish maintained at 28 °C compared to those at 22 °C. Lactate plays a central role in energy

dynamics by supporting ATP production (Marikar et al., 2021) and can be reconverted into glucose, thereby contributing to the restoration of glycogen reserves (Keihani et al., 2024; Li et al., 2022; Marikar et al., 2021; Sheridan, 1988). Since elevated temperatures generally accelerate metabolic processes and necessitate higher energy expenditure, the simultaneous increase in serum glucose and lactate at 28 °C likely reflects an adaptive metabolic response to meet these heightened demands (Badillo-Zapata et al., 2023; Perez Velazquez et al., 2015). A crucial aspect of this study involves the interaction between diet and temperature in lipid metabolism, as significant interactions were observed for both cholesterol and triglyceride levels. At 22 °C, fish fed the 10% lipid diet exhibited the highest concentrations of cholesterol (325.06 mg/dl) and triglycerides (541.33 mg/dl). Conversely, at 28 °C, these levels were lower in the same dietary group (241.44 mg/dl and 370 mg/dl, respectively). Such patterns indicate that at lower temperatures, lipid metabolism may be less efficient, potentially leading to an accumulation of lipids in the blood. In contrast, at 28 °C, the animals appear to possess a superior capacity to process the lipid load (Krasnov et al., 1999; Preston et al., 2017; Tocher, 2003). Furthermore, as demonstrated by the nutritional results, the increased energy demand at 28 °C likely necessitated higher lipid utilisation. Given that the 10% lipid diet provided lower gross energy (20.32 MJ kg^{-1}) compared to the 15% diet (21.99 MJ kg^{-1}), the available lipids were likely prioritised for energy production, resulting in the lower circulating levels observed in the serum analyses. Supporting these observations, total protein (TP) and albumin levels provide further insight into the metabolic state of the fish (Kaneko et al., 1997; Sala-Rabanal et al., 2003). Total protein reflects the concentration of circulating proteins and is generally regarded as an indicator of nutritional and physiological status (Domingo-Roura et al., 2001; Ihuţ et al., 2020). In this study, TP levels were significantly higher in fish maintained at 22 °C. This elevation likely results from a lower rate of protein utilisation at colder temperatures, whereas at 28 °C, the lower circulating TP suggests an increased metabolic demand and more rapid consumption of protein reserves. This hypothesis is further supported by the nutritional indices, specifically the Protein Efficiency Ratio (PER), which was higher at 28 °C than at 22 °C, indicating more efficient protein assimilation and utilization at higher temperatures. Both albumin and globulin are recognised as reliable indicators of the general health status in fish (Sala-Rabanal et al., 2003). Furthermore, these proteins act as essential carriers for fatty acids (Metcalf and Gemmell, 2005). Since these proteins are synthesised in the liver, their circulating levels are closely tied to hepatic metabolic activity. In this study, the Albumin/Globulin (A/G) ratio was significantly influenced by both temperature and diet. Specifically, lower temperatures resulted in a decrease in the A/G ratio, likely reflecting a temperature-induced metabolic slowdown that affects lipid absorption and protein synthesis. At 28 °C, the A/G ratio was 0.47 (10% diet) vs. 0.43 (15% diet). At 22 °C, the values shifted to 0.42 (10% diet) and 0.43 (15% diet). These fluctuations in the A/G ratio are consistent with the observed changes in the Hepatosomatic Index (HSI). Notably, the groups with the lowest A/G ratios (15% diet at 28 °C and 10% diet at 22 °C) corresponded to those with the highest HSI values (1.14 and 1.03, respectively). This inverse relationship suggests that lipid accumulation, there may be a concomitant shift in the synthesis or utilization of transport proteins, highlighting a complex interaction between thermal stress, dietary intake, and liver function (Sala-Rabanal et al., 2003). Statistically significant differences in uric acid levels were observed between the dietary groups. Generally, elevated uric acid is associated with increased feed intake and the subsequent catabolism of dietary proteins (Rodrigues et al., 2018); however, in the present study, the highest uric acid levels did not correspond to the highest feed intake values. This discrepancy can be explained by the metabolic utilization of macronutrients, as previously discussed regarding growth performance. In fish fed the 10% lipid diet at both temperatures, the relative scarcity of dietary lipids likely necessitated the diversion of proteins toward energy production, resulting in lower circulating protein levels. Conversely, in the 15% lipid group, the greater availability of lipids allowed these to be prioritised as the primary energy source. In this scenario, lipids were prioritised as the primary energy source, thereby reducing the need for protein oxidation for energy and leading to higher uric acid levels compared to the 10% lipid group at both experimental temperatures. Despite the significant differences observed in uric acid levels, serum urea concentrations, which represent the primary byproduct of nitrogen metabolism derived from protein catabolism (Miruna et al., 2009), did not show significant variation. This stability in urea levels suggests that, while the metabolic pathways for protein processing and energy partitioning shifted between dietary groups, the overall rate of nitrogenous waste production remained balanced across the experimental conditions. Another serum metabolite that exhibited significant thermal variation was bilirubin, with lower levels recorded at 28 °C. Generally, an increase in bilirubin levels can be associated with an imbalance between heme production and the liver's capacity to produce conjugated bilirubin (Abbas et al., 2016; Hastuti et al., 2019; Tsai and Tarng, 2018). Such a condition is often linked to hepatic dysfunction (Hastuti et al., 2019); therefore, the lower concentrations observed at 28 °C across both dietary groups may suggest a better physiological state and enhanced liver function for fish maintained at the higher temperature. Serum enzymes, including ALT, ALP, LDH, and CK, are widely utilized as reliable indicators of physiological condition in fish, as elevated serum levels typically signal tissue damage in organs such as the liver, muscle, spleen, and kidney (Guardiola et al., 2018; Kumar et al., 2018a, 2018; Parma et al., 2020; Pelusio et al., 2022; Peres et al., 2014). In the present study, the lack of significant differences in ALT, LDH, and CK levels suggests the absence of such systemic or organ-specific damage across the experimental groups. In contrast, alkaline phosphatase (ALP) exhibited significant variation. Beyond its application as a health biomarker, ALP is frequently associated with the transport and metabolism of fatty acids (Buchet et al., 2013; Lallès, 2019). The observation of the highest ALP values in fish fed the 10% lipid diet at 22 °C suggests a metabolic shift rather than a pathological condition. These results indicate that, within the context of this study, temperature acts as a primary driver of metabolic rate, subsequently modulating lipid processing and transport mechanisms. Regarding osmoregulation, electrolytes such as iron (Fe), sodium (Na), chloride (Cl), and calcium (Ca), along with phosphorus (P), were all significantly higher at 22 °C than at 28 °C. Such variation suggests that cold temperatures influence gill membrane permeability and the efficiency of ion pumps, triggering an adaptive response to maintain plasma homeostasis under conditions of reduced active transport (Ibarz et al., 2010; Kumar et al., 2016, 2019; Metz et al., 2003). These elements play vital physiological roles: while calcium and

phosphorus are essential for the development and maintenance of the skeletal system and acid-base balance, iron is crucial for regulating cardiovascular systems, blood flow, and oxygen transport to support thermal resistance (Dallman, 1986; Kumar et al., 2024). The absorption of these minerals occurs through both the gills and the gastrointestinal tract (Cooper and Bury, 2007; Grosell, 2010; Lall and Kaushik, 2021). Specifically, the gills are a major site for Ca uptake (Evans and Claiborne, 2008) and Fe acquisition (Bury and Grosell, 2003), particularly when the digestive tract is not fully functional (Andersen et al., 1997). However, the gastrointestinal tract also serves as an important site for the absorption of all the mentioned minerals (Cooper and Bury, 2007; Grosell, 2010). In this study, the higher levels at lower temperatures could indicate either that gill permeability functioned more efficiently at 22 °C or that the lack of acidic stomach digestion in the flathead grey mullet modified the intestinal absorption of these elements. These dynamics are highly species-specific and vary between freshwater and marine environments (Z. Crivelenti et al., 2011; Lall and Kaushik, 2021). In fact, while this results contrast with findings in gilthead seabream, where lower values were recorded at lower temperatures (Ibarz et al., 2010; Sala-Rabanal et al., 2003), they align with observations in carp, where higher levels were found at higher temperatures (Metz et al., 2003). This might represent a species-specific adaptive response and a more energetically efficient condition for maintaining homeostasis. Conversely, magnesium (Mg) exhibited the opposite trend, with significantly higher levels recorded at 28 °C than at 22 °C. As a fundamental intracellular divalent cation, magnesium plays an essential physiological role by forming a key complex with ATP and acting as a cofactor in vital biological processes, including protein synthesis, cellular replication, and energy metabolism (Lall and Kaushik, 2021). This increase at higher temperatures likely reflects the heightened metabolic demands and greater energy requirements of the animals compared to those maintained at 22 °C, necessitating an increased availability of Mg to support accelerated physiological activities.

4.3 Gut evacuation

To our knowledge, this is the first study describing gut evacuation dynamics in *Mugil cephalus* by separately analysing the anterior, middle, and posterior intestinal regions at multiple time points (1, 3, 5, 7, 9, and 12 h post-prandial). A key finding concerns the overall evacuation pattern observed across intestinal sections. Immediately after feeding, the shape parameter (*s*) was significantly different from zero in the anterior intestine compared with the mid and posterior sections. This likely reflects a physiological process, as ingested material first enters and accumulates in the anterior portion of the intestine immediately after feeding. Nevertheless, a consistent evacuation pattern was observed across all treatments, characterised by a rapid increase in intestinal content, with peak levels reached within the first 3 hours postprandially, followed by a gradual decline. By approximately 8 hours after feeding, the intestine was nearly empty in all sections. Interestingly, although gut evacuation would be expected to proceed more slowly at 22 °C due to reduced metabolic and digestive activity, a pronounced and significant delay was observed specifically in fish fed the 15% lipid diet at this temperature, as indicated by lower evacuation rates (*r*). As reported in Table 6, approximately 90% evacuation in this group was estimated to occur only after 18 h, compared with about 9 h in fish receiving the 10% lipid diet at the same temperature. This substantial difference may be attributed to the higher dietary lipid content, as lipids generally require longer digestion and intestinal transit times (García-Meilán et al., 2014). In contrast, the 10% lipid diet included additional cellulose, a component known to increase passage rate and promote intestinal evacuation compared with protein- or lipid-rich fractions (Le et al., 2021; Spannhof and Plantikow, 1983). Taken together, these findings suggest that under lower temperature conditions, the combined effects of elevated dietary lipid levels and reduced digestive activity contributed to prolonged feed retention within the gastrointestinal tract, potentially limiting nutrient utilisation efficiency and favouring lipid deposition. However, when considering the standard evacuation rate of approximately 8 hours observed in this study, *Mugil cephalus* displays a remarkably rapid digestive transit compared to other farmed species. While evacuation dynamics are influenced by diet, temperature, and feeding frequency (García-Meilán et al., 2014; Gilannejad et al., 2019; Mazumder et al., 2020), these processes are primarily shaped by a species' specific trophic adaptations. For instance, carnivorous species such as European sea bass (*Dicentrarchus labrax*) or Atlantic salmon (*Salmo salar*) exhibit much slower evacuation times, ranging from 22 to 30 hours, respectively (Bonvini et al., 2018; Storebakken et al., 1999). Omnivorous species like gilthead seabream (*Sparus aurata*) show intermediate rates of approximately 17–18 hours (Busti et al., 2022). In contrast, species with strong herbivorous tendencies, such as tilapia (*Oreochromis niloticus*), exhibit rapid transit times of about 7 hours (Uscanga et al., 2010). Given that the flathead grey mullet is generally considered an omnivorous detritivorous species, and considering that this represents the first study investigating gut evacuation in this species, the observed evacuation time of approximately 8 hours suggests a pattern more similar to species with omnivorous diets and a strong herbivorous component, rather than to strictly carnivorous fish.

4.4 Gut microbiota

Integrating microbiota data into the broader interpretation of our findings, the results provide partial but meaningful support for our initial hypothesis. Alpha diversity analyses indicated that microbial richness and evenness were significantly influenced by temperature, with thermal variation exerting a stronger effect than dietary lipid levels (Pelusio et al., 2021; Soriano et al., 2018). Although the effect of dietary lipid content was less pronounced, it remained detectable and should not be considered negligible. This pattern was also confirmed by beta diversity analysis. The PLS-DA model, in fact, revealed a clear separation of microbial communities primarily according to temperature, however, underlining that in the 22 °C condition, where the distribution between taxa was more homogeneous, the differences due to diet, although minor, between the two microbial profiles showed a significant spatial difference in the model (Neuman et al., 2016). The predominance of temperature in shaping gut microbiota aligns with previous observations in other fish species. For example, Pelusio et al. (2021) in gilthead seabream (*Sparus aurata*) and Soriano et al. (2018)

in yellowtail kingfish (*Seriola lalandi*) reported that temperature drove the most pronounced microbial shifts, while dietary lipid variations had a smaller effect. Similarly, Neuman et al. (2016) found that gut microbial profiles in Atlantic salmon (*Salmo salar*) changed primarily with seasonal temperature fluctuations, despite minimal differences in diet lipid content. At the phylum level, notable shifts were observed in *Pseudomonadota*, *Bacillota*, *Fusobacteriota*, *Actinomycetota*, and *Planctomycetota*, reflecting both temperature-dependent changes and, to a lesser extent, dietary effects. Among these, *Fusobacteriota* members are generally considered beneficial intestinal bacteria due to their roles in vitamin production and gut metabolic regulation, contributing to intestinal homeostasis. Specifically, descending to lower taxonomic levels, *Cetobacterium* has been associated with enhanced digestive efficiency, metabolic performance, and endogenous vitamin B12 synthesis (Bertini et al., 2023; Tsuchiya et al., 2008; Xie et al., 2021). Additionally, *Cetobacterium* functions as an acetate producer and has been implicated in regulating glucose metabolism, potentially improving glucose utilization and maintaining glucose homeostasis (Wang et al., 2022, 2023). These functional traits may explain the increased abundance of *Cetobacterium* in fish fed the 15% lipid diet, particularly at the higher rearing temperature of 28 °C. In this study, taxonomic results showed a marked increase in *Cetobacterium* relative abundance in fish reared at 28 °C compared with those maintained at 22 °C. Consistent with previous reports, *Cetobacterium* is a common component of freshwater fish microbiota (Tsuchiya et al., 2008; van Kessel et al., 2011), and Bertini et al. (2023) also identified it as the dominant genus in flathead grey mullet at ~27 °C. Apart from the genus *Cetobacterium*, identified as the primary biomarker at 28 °C, LEfSe analysis also highlighted other taxa associated with the thermal variable. Other high-temperature markers included *Gemmobacter*, a genus that has been reported in previous studies to display probiotic traits and to contribute to vitamin and amino acid metabolism (Wang et al., 2023), and *Shewanella*, which has been previously reported in mullet at similar temperatures and linked to fatty acid synthesis and energy metabolism (Dailey et al., 2016; Yazawa, 1996). Conversely, a greater diversity of biomarkers was associated with 22 °C, including *Agromyces*, a genus reported to participate in carbon cycling and degradation of cellulose and chitin (Li et al., 2023), and *Shinella*, a genus which has often been linked to stress-related conditions such as phosphate limitation (Poehlein et al., 2016) or heavy metal exposure (Lai et al., 2020). Their prevalence suggests that lower temperatures may impose greater physiological or metabolic constraints on flathead grey mullet. Predicted functional profiles of the intestinal microbiota revealed enrichment of fatty acid biosynthesis and aminoacyl-tRNA biosynthesis pathways at 28 °C, processes typically associated with active cellular growth, lipid synthesis, and protein production, hallmarks of a rapidly growing bacteria (Dailey et al., 2016; Wang et al., 2009). In this context, the inferred enrichment related to fatty acid biosynthesis, including omega 3 polyunsaturated fatty acid production, has been linked to *Shewanella* activity (Wang et al., 2009), and may provide a putative functional association between this genus and elevated temperature. Warm conditions were also associated with higher predicted representation of peptidoglycan biosynthesis and D-glutamine/D-glutamate metabolism, key pathways for protein synthesis, bacterial cell wall construction and D amino acid metabolism that typically scale with cell proliferation and cell wall remodelling (da Silva Carneiro et al., 2025; Wu, 2010). Altogether, these results suggest that higher temperatures may induce distinct energetic and metabolic demands, potentially shaping both gut microbiota composition and functional potential. Furthermore, although these results require experimental confirmation, which the technique used cannot provide, they also indicate that the combination of elevated temperature and high dietary lipid profile may favour several metabolic pathways. These inferred functional responses include biotin metabolism and pantothenate/CoA biosynthesis, alongside streptomycin, vancomycin and ansamycin biosynthesis pathways. Considering the over–representation of vitamin, cofactor and antibiotic biosynthesis, this pattern generates the hypothesis that such condition could increase competitive interactions within the gut environment through the production of antimicrobial secondary metabolites (Kokou et al., 2020; Tyagi et al., 2019). This scenario is consistent with the aforementioned reduction in bacterial diversity and the expansion of dominant taxa such as *Cetobacterium*, which could indicate a shift towards a more functionally specialised gut microbiota, adapted to intensified energetic and metabolic demands (Chen et al., 2025; Zhang et al., 2023). Overall, the microbiota data generated in this study provide a comprehensive view of the taxonomic and functional diversity of the flathead grey mullet gut microbiota, highlighting the interactive roles of temperature and diet in modulating the intestinal microbial ecosystem.

5 Conclusion

The present study provides evidence that rearing temperature plays a primary role in determining growth performance in flathead grey mullet (*Mugil cephalus*). Fish reared at 28 °C consistently exhibited superior growth compared to those maintained at 22 °C, confirming that warmer conditions within this thermal range better support somatic growth and overall production efficiency. At the same rearing temperature, dietary lipid level influenced feeding dynamics without markedly altering growth outcomes. Fish fed the L15 diet showed lower feed intake compared to those receiving the L10 diet, despite achieving comparable final body weight and specific growth rate. This pattern, combined with the absence of statistically significant differences in feed conversion ratio (FCR), suggests that the higher-lipid diet provided sufficient energy density to sustain growth with reduced feed consumption. Although FCR values were not significantly different between diets, their tendency towards improved efficiency under the L15 treatment may indicate a more favourable utilisation of dietary energy. Overall, the results indicate that increasing dietary lipid levels can reduce feed intake without compromising growth performance, which may have practical implications for feed formulation and feeding strategies. From a production perspective, the L15 diet appears to offer advantages in terms of energy supply and potential feed utilisation efficiency, particularly under warmer rearing conditions while the combination of low temperature (22 °C) and high lipid intake (15%) represents the condition of greatest physiological load for the flathead grey mullet. From the intestinal microbiota perspective, the present results highlighted a clear community shift primarily driven by temperature, with dietary lipid intake exerting a

secondary but still relevant effect. The combination of these two factors promoted a transition toward a more specialised microbiota profile, characterised by the increased dominance of taxa such as *Cetobacterium*. These outcomes contribute to defining baseline nutritional knowledge for flathead grey mullet and support the optimisation of feeding protocols for this species within diversified and climate adaptive aquaculture systems.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was approved by Ethical-Scientific Committee for Animal Experimentation of the University of Bologna. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

SN: Writing – review & editing, Conceptualization, Methodology, Investigation, Writing – original draft. FM: Methodology, Writing – review & editing, Investigation. RD-B: Writing – review & editing, Methodology, Investigation. JP-S: Writing – review & editing, Investigation, Methodology. MB: Methodology, Investigation, Writing – review & editing. MF: Methodology, Writing – review & editing. FD: Methodology, Writing – review & editing. DC: Writing – review & editing. DV: Writing – review & editing. SC: Writing – review & editing. TP: Writing – review & editing. PG: Methodology, Conceptualization, Investigation, Writing – review & editing. EB: Writing – review & editing, Methodology. AB: Methodology, Conceptualization, Supervision, Investigation, Writing – review & editing. LP: Supervision, Writing – review & editing, Investigation, Conceptualization, Methodology.

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Conflict of interest

The author(s) declared that this work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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