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Intermittent fasting driven different adaptive strategies in *Eothenomys miletus* (Red-backed vole) at different altitudes: based on the patterns of variations in intestinal microbiota

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Abstract

In the face of global warming, the *Eothenomys miletus* (Red - backed vole), a species dwelling in highland mountainous regions, is likely to encounter difficulties. Given its restricted mobility, it may struggle with the uncertainty of food resources. In such circumstances, it becomes increasingly crucial for this species to adjust its diverse responses to fulfill its energy requirements. *E. miletus* specimens were gathered from different altitudes for intermittent fasting (IF) experiments. In these experiments, the specimens underwent random fasting for 3 days within a seven - day cycle. 16 S rDNA sequencing technology, combined with physiological and biochemical assessment methods, was employed to analyze the impacts of IF on gut microorganisms, physiological and biochemical indicators, and the interactions among them. By exploring the adaptive responses of *E. miletus* to uncertain food resources, which provides novel perspectives on the adaptive strategies of small rodents in the wild during food-scarce periods. The results showed that IF significantly reduced the body mass of *E. miletus*. Significant correlations were found between various gut microbes and physiological indicators. Under IF conditions, *E. miletus* at high altitudes experienced a smaller reduction in body mass compared to those at low altitudes. Moreover, the diversity of gut microbes and endemic bacteria in *E. miletus* at high altitudes varied more than that of low altitudes. The differential response in body mass reduction between high-altitude and low-altitude *E. miletus* under IF conditions indicated that altitude is an important factor influencing the physiological adaptation of this species to dietary changes. High-altitude *E. miletus* showed a relatively smaller decrease in body mass, potentially reflecting their better adaptation to environmental stressors over time. Additionally, the greater variation in gut microbe diversity and endemic bacteria in high-altitude *E. miletus* implied that altitude may shape the gut microbiota, which in turn could be related to their unique physiological adaptations at high altitudes. Overall, *E. miletus* at high altitude may possess more stable regulatory mechanisms, demonstrating better adaptation under IF conditions.

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These findings provide valuable insights into the complex interplay between diet, altitude, and gut microbiota in the context of *E. miletus* physiology, highlighting the importance of considering both environmental and microbial factors in understanding the species' responses to nutritional challenges..

Keywords *Eothenomys miletus*, Altitudinal differences, Intermittent fasting, Adaptation, Host-gut microbiota symbiosis

Introduction

Under the impact of climate warming, the increasing frequency of extreme temperature events and droughts can cause rapid and substantial declines in biodiversity within high-altitude ecosystems over a short time span, resulting in significant species loss [1]. Wildlife frequently encounters difficulties due to the unpredictability of food resources, and such fluctuations have a direct bearing on their survival, growth, and reproductive success [2–3]. Unstable climatic conditions and natural disasters further exacerbate the uncertainty regarding food resources for small herbivorous mammals [4]. In response to food shortages stemming from changes in the natural environment, numerous small mammals have developed storage behaviors. Conversely, rodents devoid of such behaviors have adjusted the molecular mechanisms of their energy metabolism through cellular responses activated by altered biological rhythms. This adaptation enables them to meet the energy demands for survival, as manifested by reduced body mass, body temperature, and energy expenditure [5–6]. Uncertain food resources involve aspects such as food composition, quantity, and fasting duration, among which fasting duration is a crucial factor [7].

Gut microorganisms are highly diverse and form a mutually beneficial symbiotic relationship with their hosts. They supply essential nutrients for normal physiological functions and boost the hosts' capacity to endure extreme environments [8–10]. Microorganisms modulate host metabolism and a variety of physiological responses by generating bioactive compounds that interact with the endocrine and immune systems [11]. Intermittent fasting (IF) represents an eating pattern characterized by alternating between intervals of energy restriction and food intake, during which no calorie-bearing foods are ingested [8]. Research has demonstrated that intermittent fasting modifies the composition and diversity of gut microbiota, and these alterations align with the cyclical fluctuations in fasting rhythms [11–14]. IF has been demonstrated to augment the population of beneficial microorganisms, such as *Lactobacillaceae*, *Mycobacteriaceae*, and *Prevotella*. Simultaneously, it enhances the metabolic pathways of antioxidant microorganisms, thus safeguarding the host from the damage inflicted by oxidative stress [11]. Moreover, intermittent fasting ameliorates obesity, insulin resistance, and hepatic steatosis. It does so by modifying the composition of gut flora and the content of its metabolites, and by upregulating the expression of transporter proteins in beige adipocytes [15]. Nevertheless, prolonged or excessive periodic fasting might impede the protective response against pathogens and could even be associated with the exacerbation of inflammatory bowel disease [16–18]. Currently, the mechanisms through which intermittent fasting influences host gut microbiology and overall health remain incompletely understood.

Eothenomys miletus (Red backed vole) is an endemic species inhabiting the Hengduan Mountains, with a diet mainly consisting of grasses [19]. Research has shown that food, temperature, and photoperiod are key environmental factors affecting the energy metabolism of *E. miletus*. Notably, *E. miletus* populations from different regions display remarkable physiological plasticity. They adapt to environmental changes by modulating their body composition, hormone expression, and gut microbial composition [20–23]. When on high-fiber and high-fat diets, the gut microbiota of *E. miletus* becomes enriched with bacteria capable of degrading fiber and fat. These bacteria interact synergistically with physiological indices, aiding the host in adapting to diverse dietary environments. Moreover, high - altitude *E. miletus* populations have been found to possess enhanced adaptive abilities [24–25]. Under 80% food restriction (FR) conditions, the gut microbiota of *E. miletus* helps it adapt to food scarcity by enriching genera such as *Bacterroides*, *Ruminococcus*, and *Turicibacter* [26]. These findings suggest that the gut microbiota is essential for the survival and adaptation of *E. miletus*.

In the face of a warming climate, plants at higher altitudes are potentially at risk of survival, and herbivorous animals may migrate to these elevated areas. However, *E. miletus*, a small, relatively immobile mammal, tends to meet its energy demands by regulating its physiological responses within an environment featuring uncertain food resources [4, 27, 28]. In winter, *E. miletus* may find it difficult to secure food because of low temperatures and the presence of predators. This leads toIF, during which the gut microbiome likely has to adapt to aid the animal's survival. Moreover, during winter at higher altitudes, the quantity and quality of food are inferior. Under such circumstances, *E. miletus* may be better equipped to survive in IF conditions. Consequently, the present study concentrated on exploring how IF influences the survival adaptation of *E. miletus* at different altitudes. Are there

regional differences in body mass regulation among *E. miletus* under IF conditions? To address this scientific question, the present study integrated gut microbiota analysis with physiological indicator measurements. It examined changes in the composition and diversity of the gut microbiota. Additionally, the study investigated the correlation between dominant bacterial flora and the physiological indicators of *E. miletus* at different altitudes under an IF regimen of random fasting for 3 days within a 7-day period. The goal was to clarify the adaptive strategies of *E. miletus* to IF at various altitudes. This research aims to provide a basis for understanding the adaptation of small rodents in the context of climate warming. We hypothesized that: (1) IF would boost the metabolism of *E. miletus*, enhance the inflammatory response, increase gut microbiota diversity, and promote the enrichment of probiotics; and (2) *E. miletus* at different altitudes would display distinct adaptive responses to IF, with those at lower altitudes being more adaptable to IF conditions.

Materials and methods

Experimental samples

Animals were captured during the winter of 2023 in Jindong County (JD, at a lower altitude) and Xianggelila County (XGLL, at a higher altitude). All the captured specimens were non-breeding, healthy adult E. miletus. The details of the collection sites and the animals are presented in Table 1.

Experimental design

E. miletus individuals were captured from two regions. After disinfection and flea elimination, they were transported to the animal room at Yunnan Normal University. Each specimen was housed separately in a mouse box with dimensions of 260 mm × 160 mm × 150 mm. The room temperature was maintained at 25 ± 1 °C, and a photoperiod of 12 L:12D (Light: Dark) was set.

Following a 4-day acclimatization period, a two - factor experimental design (region × IF) was carried out. Day 0 of the experiment was designated as the control group. Three random fasting days were chosen from the 7-day experimental period to form the IF group. After the fasting period, a refeeding group was established, where the animals were returned to a normal diet for 14 days. *E. miletus* from the two regions were divided into six groups: Jingdong control group (JDC, $n = 6$), Jingdong IF group (JDIF, $n = 7$), Jingdong refeeding group (JDR, $n = 7$), Xianggelila control group (XGC, $n = 5$), Xianggelila IF group (XGIF, $n = 7$), and Xianggelila refeeding group (XGR, $n = 6$).

E. miletus were fed a standard rat chow provided by Kunming Medical University. Throughout the experimental period, the animals had free access to food and water. The experimental period spanned 21 days. Body mass and resting metabolic rate (RMR) were measured on days 0, 7, and 21. Body mass was measured using an LT502 electronic balance (with an accuracy of 0.01 g), and RMR was measured with a portable respirometer. After recording the relevant indexes, the animals were euthanized by carbon dioxide anesthesia, and serum and rectal fecal samples were collected.

Measurement of body components

After the *E. miletus* specimens were anesthetized and euthanized, rectal feces and brown adipose tissue (BAT) were promptly collected and placed into cryopreservation tubes. These were then weighed in liquid nitrogen and subsequently transferred to a -80 °C refrigerator for storage. The heart, lungs, liver, spleen, and kidneys were also rapidly harvested. Tissues adhering to these organs were removed. After blotting the blood from their surfaces with filter paper, the organs were weighed to the nearest 0.001 g. The digestive tracts of the animals were excised, with the small intestines and cecum separated, and other tissues discarded. The lengths of the intestines were measured using a straightedge. All white adipose tissue (WAT) was dissected out and weighed.

Measurement of physiological and biochemical indicators

After blood collection, the samples were stored in a refrigerator at 4 °C for 1 h. Subsequently, they were centrifuged at 4 °C at a speed of 4000 revolutions per minute (r/min) for 30 min. The separated serum was then stored in a freezer at -80 °C. Serum levels of leptin, short - chain fatty acids (SCFAs), lipopolysaccharide - binding protein (LBP), fasting - induced adipocyte factor (FIAF), and tumor necrosis factor - α (TNF - α) were measured using enzyme - linked immunosorbent assay (ELISA). Serum triglyceride (TG), total cholesterol (TC), and blood glucose levels were evaluated using biochemical test kits. ELISA kits were also used to determine the level of uncoupling protein 1 (UCP1) in brown adipose tissue (BAT). All kits were purchased from Shanghai Preferred Biotechnology Co., Ltd., and their kit numbers are listed

Table 1 Information on collection sites of *E. miletus*

Regions	Sample number	Longitude and latitude	Altitude/m	Mean Temperature/°C	Precipitation/mm	Vegetation type
JD	21	100°42′49″E, 24°90′30″N	2217	16.1	597.0	Savannah shrubs
XGLL	19	99°83′16″E, 27°90′30″N	3321	6.9	984.2	Subalpine meadows

in Table 2. The experiments were carried out in strict accordance with the provided instructions.

16sRNA high-throughput sequencing and biosignature analysis

0.1 g of rectal feces was collected. The DNA was extracted using a centrifugal column genome extraction kit (DNeasy PowerSoil kit, Germany), with enrichment on a filter membrane. The extracted DNA was diluted to a concentration of 10 ng/μL and subsequently amplified via polymerase chain reaction (PCR).

PCR amplification was carried out using universal primers 515F (5'-GTGYCAGCMGCCGCGGTA-3') and 909R (5'-CCCGYCAATTCMTTTRAGT-3'), which target the highly variable V4 - V5 region of the 16S rRNA gene. Notably, the 5' end of the 515 F primer contained a specific 12-bp barcode [29–30].

The PCR reaction system had a total volume of 30 μL, comprising 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1.0 μM of each primer, 0.25 U of DNA polymerase (TaKaRa, Dalian), and 10 ng of genomic DNA template. The volume was adjusted to 30 μL with sterile deionized water.

The thermocycling program consisted of the following steps: pre - denaturation at 94 °C for 3 min, followed by 32 cycles of amplification. Each amplification cycle included denaturation at 94 °C for 40 s, annealing at 56 °C for 60 s, and extension at 72 °C for 60 s. The final extension step was performed at 72 °C for 10 min, and the samples were stored at 4 °C. Two replicate reactions were conducted for each sample [31].

PCR products were separated by 1.2% agarose gel electrophoresis. The concentration of the purified PCR products was measured using a Nanodrop 2000 spectrophotometer. A valid sample was defined as having a nucleic acid concentration greater than 10 ng/μL and a purity ratio (A260/A280) exceeding 1.8. Purified DNA samples were then combined at equimolar concentrations and sequenced on the Illumina MiSeq platform (Illumina, San Diego, CA, USA).

The 2 × 250 bp paired-end sequences were generated by sequencing on the Illumina MiSeq platform. Raw data processing and analysis were carried out using the QIIME platform. Sequences were assembled, and low-quality sequences, chimeras, monomers, and chloroplast sequences were removed following the method described by Li et al. Subsequently, the sequences were clustered at a 97% similarity threshold. The sequences with the longest lengths were chosen as representative sequences and annotated with taxonomic information using the Ribosomal Database Project database [22–24, 30]. Finally, the sequences from all samples were standardized using the "Daisychopper" script, and each sample was rarefied to 15,209 sequences.

Table 2 Reagent kit information

Name of kit	Product Number
Leptin ELISA Kit	JM-11498M1
SCFA ELISA Kit	JM-11498M1
LBP ELISA Kit	M-12488M1
FIAF ELISA Kit	JM-12613M1
TNF-α ELISA Kit	JM-02415M1
UCP1 ELISA Kit	JM-12185M1
TG ELISA Kit	S0140O-1
TC ELISA Kit	S05042-1
Blood Glucose ELISA Kit	S0104F-1

Data analysis

Chao1 and Shannon metrics were utilized to characterize alpha diversity. In SPSS 26, the Kruskal - Wallis test was applied to evaluate differences in Chao1 and Shannon's indices among groups. By means of QIIME, beta diversity was described using unweighted and weighted UniFrac distance matrices [21]. Furthermore, Permutational Multivariate Analysis of Variance (PERMANOVA) was employed to assess the impacts of region and IF on beta diversity. The diversity results were visualized with Origin 2024, and percentage stacked bar graphs were constructed to depict microbial composition. Inter - group shared and endemic bacteria were analyzed using Venn diagrams generated by Venn 2.1 (https://bioinfogp.cnb.csic.es/tools/venny/index.html). Heat maps were produced using the "pheatmap" package in R 4.3.3, which demonstrated differences among the top 20 OTUs for heat mapping ($P < 0.05$). Additionally, the "psych" package in R 4.3.3 was used to analyze the correlation between the abundance of the top 20 OTUs and various physiological indicators. The correlations between dominant genera (relative abundance ≥ 0.01) and physiological indicators were evaluated using Canoco 5.0 through redundancy analysis (RDA). The results from the "psych" package in R 4.3.3 were visualized with Gephi 0.10.1 to generate network analyses ($P < 0.05, |r| > 0.4$). SPSS 26.0 was used for data statistical analysis. A Two - way ANOVA was used to assess the body mass of groups in the two regions, and other indicators were analyzed through a Two - way ANCOVA with body mass as a covariate. Duncan's multiple comparisons were carried out for indicators within the same region, along with independent T - tests. The results are presented as mean ± standard error (mean ± SE), with a significance level set at $P < 0.05$.

Results

Effect of IF on body mass, body composition, and physiological and biochemical indices

Body mass was significantly affected by both region and intermittent fasting (IF), as revealed by two-way ANCOVA (Region: $F = 33.41$, $P < 0.01$; IF: $F = 23.31$,

$P < 0.01$, Table 3). IF significantly reduced body mass. Across all groups, body mass was significantly higher in the JD region than in the XGLL region. Moreover, the reduction in body mass was more substantial in the JD region than in the XGLL region (Fig. 1a). Resting metabolic rate (RMR) was significantly influenced by region (Two-way ANCOVA: Region: $F = 31.87$, $P < 0.01$, Table 3), with RMR being markedly lower in the JD region compared to the XGLL region (Fig. 1b).

Splenic mass was significantly affected by region and the interaction between region and IF (Two-way ANCOVA: Region: $F = 4.05$, $P = 0.05$; Region×IF: $F = 5.35$, $P = 0.01$, Table 3). In the JD region, IF significantly decreased splenic mass, while in the XGLL region, it significantly increased splenic mass. Additionally, within the IF group, splenic mass was significantly higher in the XGLL group than in the JD group (Fig. 1c). Kidney mass was significantly influenced by region (Two-way ANCOVA: Region: $F = 42.92$, $P < 0.01$, Table 3). In all groups, kidney mass was significantly higher in the XGLL region than in the JD region. After heavy feeding, kidney mass in the JD region was significantly greater than that in the IF group (Fig. 1d).

Brown adipose tissue (BAT) mass was significantly affected by region and the interaction (Two-way ANCOVA: Region: $F = 55.27$, $P = 0.05$; Region×IF: $F = 6.02$, $P = 0.01$, Table 3). Across all groups, the XGLL region had significantly higher BAT mass than the JD region. Additionally, in the XGLL region, BAT mass increased significantly after refeeding compared to the IF group (Fig. 1f). The content of short-chain fatty acids (SCFAs) was significantly influenced by both region and IF (Two-way ANCOVA: Region: $F = 6.98$, $P < 0.01$; IF: $F = 3.07$, $P = 0.03$, Table 3). In the re-feeding group, SCFA content was significantly higher in the JD region than in the XGLL region (Fig. 1h). In the re-feeding group, white adipose tissue (WAT) mass and stomach length were significantly greater in the JD region than in the XGLL region (Fig. 1e and g).

Effects of IF on gut microbiota

The diversity of gut microbiota

In the JD region, the Chao1 index of gut microbiota decreased, with the value in the JDIF group being significantly lower than that in the JDR group. In the XGLL region, the Chao1 index showed a trend of increasing from the control group to the IF group and then to the heavy - feeding group. In the IF group, the Chao1 index in the JD area was significantly lower than that in the XGLL area (Fig. 2a). The Shannon index of gut microbiota displayed a similar trend to the Chao1 index (Fig. 2b).

Regarding the β - diversity of gut microbiota, there was no significant trend in the aggregation among different groups on the PCoA plot (Fig. 2c and d). The PERMANOVA test indicated a significant difference in the β - diversity (unmassed matrix) of gut microbiota between the XGIF and XGR groups in *E. miletus* (Table 4).

Composition of gut microbiota

At the phylum level, across all regions, the dominant phyla in the fecal microbiota were Firmicutes, Bacteroidetes, and Spirochaetes, with mean relative abundances of 77.27%, 19.75%, and 1.23% respectively (Fig. 3a). At the genus level, the predominant genera in the fecal microbiota were *Lactobacillus*, S24–7(UG), and Clostridiales(UG), with mean relative abundances of 57.08%, 12.05%, and 6.90% (Fig. 3b). The mean relative abundance of the dominant genus *Lactobacillus* showed a decreasing trend during the experimental period in the XGLL group, while no significant changes were observed in the JD group (Fig. 3b).

In the JD region, the total number of gut microorganism genera was 96. This included 25 genera exclusive to the JDC group, 21 genera exclusive to the JDIF group, and 44 genera exclusive to the JDR group (Fig. 3c). Compared with the control group, the IF group exhibited a 16% reduction in genera, while the Re group showed a 76% increase. In the XGLL region, there were 94 genera of gut microorganisms. Specifically, 9 genera were unique to the XGC group, 130 genera were unique to the XGIF group, and 23 genera were unique to the XGR group (Fig. 3d). Relative to the control group, the IF group in the XGLL region had a 1,344% increase, and the Re group had a 155% increase. A total of 82 genera were shared among different groups of gut microorganisms. In the IF group, the prevalence of genera endemic to XGLL was 3.4 times that of genera endemic to the JD region (Fig. 3e).

In the JDIF group, *g_Lactobacillus* was significantly enriched ($P < 0.05$), while in the JDR group, f_S24-7 was significantly enriched ($P < 0.05$) (Fig. 3f). In the XGC group, *g_Lactobacillus* was significantly enriched ($P < 0.05$), and in the XGIF group, f_S24-7 was significantly enriched ($P < 0.05$). In the XGRe group, f_Rikenellaceae and *g_Odoribacter* were significantly enriched ($P < 0.05$) (Fig. 3f).

Correlation analysis of gut microbiota with body composition and biochemical indices in different regions

In the JD region, body mass and BAT mass were significantly positively correlated ($P < 0.05$) with the abundance of Rikenellaceae(UG) and Clostridiales(UG), respectively (Fig. 4a). Conversely, lung mass and stomach length were significantly negatively correlated ($P < 0.05$) with the abundance of *Bacteroides* and *Treponema*, respectively (Fig. 4a). FLAF was significantly negatively correlated with the abundance of Clostridiales(UG) and *Odoribacter*, while it was significantly positively correlated with

Table 3 Effect of IF on physiological and biochemical indices of *E. miletus*

Parameter	JD			XGLL			Region		IF		Region*IF	
	Con group *n* = 6	IF group *n* = 7	Re group *n* = 7	Con group *n* = 6	IF group *n* = 7	Re group *n* = 6	F	*P*	F	*P*	F	*P*
Body mass(g)	43.22 ± 3.27	37.95 ± 1.10	42.96 ± 3.11	37.85 ± 1.63	34.43 ± 1.09	39.95 ± 1.82	33.41	< 0.01	23.31	< 0.01	1.05	0.36
RMR (mLO_2/g.h)	1.92 ± 0.33	1.75 ± 0.02	1.92 ± 0.45	2.59 ± 0.18	2.37 ± 0.32	2.62 ± 0.32	31.87	< 0.01	0.06	0.95	0.09	0.91
Heart mass(g)	0.27 ± 0.06	0.28 ± 0.02	0.27 ± 0.02	0.34 ± 0.06	0.31 ± 0.04	0.31 ± 0.03	5.13	0.03	0.75	0.48	0.51	0.61
Liver mass(g)	2.02 ± 0.29	2.18 ± 0.29	2.13 ± 0.32	2.35 ± 0.26	2.27 ± 0.26	2.34 ± 0.10	6.55	0.02	0.40	0.68	1.02	0.37
Spleen mass(g)	0.1 ± 0.02	0.08 ± 0.02	0.11 ± 0.01	0.12 ± 0.02	0.15 ± 0.06	0.10 ± 0.02	4.05	0.05	0.50	0.61	5.35	0.01
Kidney mass(g)	0.56 ± 0.05	0.54 ± 0.06	0.63 ± 0.07	0.78 ± 0.12	0.72 ± 0.05	0.72 ± 0.06	42.92	< 0.01	0.01	0.99	3.42	0.04
Lung mass(g)	0.29 ± 0.04	0.27 ± 0.03	0.30 ± 0.04	0.33 ± 0.05	0.32 ± 0.07	0.34 ± 0.05	6.30	0.02	0.15	0.86	0.02	0.98
Stomach length(cm)	2.00 ± 0.25	2.09 ± 0.26	2.20 ± 0.12	1.97 ± 0.33	1.94 ± 0.34	1.96 ± 0.17	1.37	0.25	0.40	0.67	0.51	0.61
Small intestine length(cm)	39.57 ± 4.83	41.13 ± 2.27	41.16 ± 2.45	41.25 ± 3.85	41.67 ± 3.07	41.63 ± 8.43	0.94	0.34	0.42	0.66	0.14	0.87
Caecum length(cm)	15.80 ± 0.40	15.84 ± 0.41	16.03 ± 0.48	16.03 ± 0.67	16.08 ± 0.43	16.40 ± 0.45	0.14	0.71	2.46	0.10	0.23	0.79
WAT mass(g)	3.49 ± 0.05	3.48 ± 0.14	3.59 ± 0.06	3.45 ± 0.10	3.40 ± 0.16	3.44 ± 0.09	3.23	0.08	0.91	0.41	0.76	0.47
BAT mass(g)	0.15 ± 0.02	0.18 ± 0.06	0.17 ± 0.05	0.37 ± 0.07	0.26 ± 0.06	0.38 ± 0.06	55.27	< 0.01	0.62	0.55	6.02	0.01
Tg(μg/g)	300.06 ± 46.67	294.38 ± 21.41	302.63 ± 49.34	283.38 ± 33.52	275.44 ± 27.00	288.19 ± 41.66	0.01	0.94	0.14	0.87	0.05	0.95
SCFAs (μmol/L)	44.27 ± 5.10	40.78 ± 6.73	46.06 ± 7.44	41.10 ± 7.70	37.75 ± 2.83	41.50 ± 5.46	6.98	0.01	3.78	0.03	0.07	0.93
UCP1(pg/ml)	85.62 ± 9.69	78.05 ± 8.75	86.87 ± 15.66	99.70 ± 13.38	87.99 ± 12.63	94.05 ± 15.59	0.83	0.37	2.71	0.08	0.07	0.94
FIAF (ng/L)	61.79 ± 13.81	57.55 ± 8.46	62.29 ± 9.66	64.19 ± 5.76	66.95 ± 9.95	63.64 ± 12.50	0.32	0.58	0.14	0.87	0.63	0.54
Leptin(pg/ml)	860.30 ± 138.40	841.05 ± 109.58	857.07 ± 112.82	794.16 ± 129.81	772.67 ± 128.32	796.83 ± 73.35	0.56	0.46	0.02	0.98	0.01	0.99
LBP((ng/ml)	9.22 ± 1.22	8.39 ± 1.42	9.17 ± 1.53	8.85 ± 1.23	8.25 ± 1.62	8.90 ± 0.66	1.46	0.24	1.79	0.18	0.10	0.91
TNF-α(ng/L)	940.47 ± 135.45	812.62 ± 120.03	941.81 ± 93.49	888.52 ± 39.00	798.78 ± 91.46	878.04 ± 106.58	0.34	0.56	1.46	0.25	0.22	0.80
Glu(mg/ml)	0.51 ± 0.12	0.48 ± 0.05	0.57 ± 0.08	0.47 ± 0.05	0.45 ± 0.12	0.47 ± 0.08	0.45	0.51	0.19	0.83	0.62	0.54
Tc(μg/g)	124.69 ± 12.96	118.66 ± 14.78	124.73 ± 11.04	127.49 ± 8.90	107.89 ± 14.78	122.52 ± 13.56	0.64	0.43	2.17	0.13	0.82	0.45

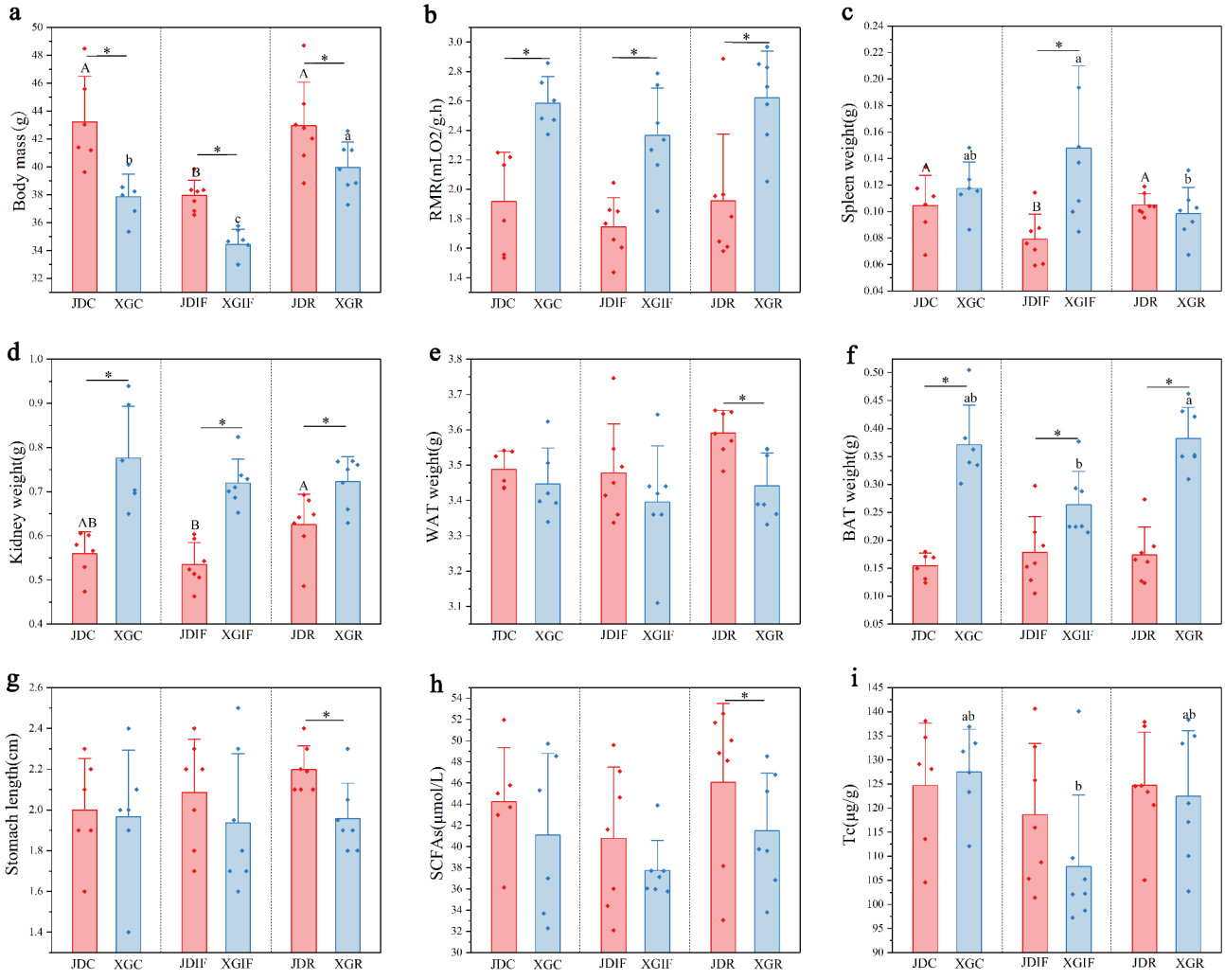

Fig. 1 Effects of IF on physiological and biochemical indices of *E. miletus*. (**a**: body mass; **b**: RMR; **c**: spleen mass; **d**: kidney mass; **e**: WAT mass; **f**: BAT mass; **g**: stomach length; **h**: serum SCFAs; **i**: serum Tc). (*: $P < 0.05$; uppercase letters indicate intergroup differences in JD regions, while lowercase letters indicate intergroup differences in XGLL regions)

the abundance of *Lactobacillus* ($P < 0.05$) (Fig. 4b). Additionally, *Odoribacter* abundance was significantly positively correlated with Tg, LBP, and Tc ($P < 0.05$) (Fig. 4b).

In the XGLL region, RMR and cecum length were significantly ($P < 0.05$) negatively correlated with the abundances of *Turicibacter* and *Lactobacillus*, respectively (Fig. 4c). Conversely, the abundance of *Ruminococcus* was significantly ($P < 0.05$) positively correlated with both body mass and cecum length (Fig. 4c). The mass of the spleen was significantly and positively correlated with the abundance of *Bacteroides* ($P < 0.05$) (Fig. 4c). *Treponema* abundance exhibited a significant positive correlation with Tg and TNF-α, while showing a significant negative correlation with FLAF ($P < 0.05$) (Fig. 4d). Additionally, UCP1 and TNF-α were significantly negatively correlated with *Turicibacter* and S24-7 (UG), respectively ($P < 0.05$). Furthermore, Glu demonstrated a significant positive correlation with *Bacteroides* abundance ($P < 0.05$) (Fig. 4d).

Redundant analyses of physiological indicators and gut-dominant OTUs revealed that the abundance of f_S24-7 was positively correlated with spleen mass and RMR, while it was negatively correlated with WAT mass and leptin (Fig. 4f). *g_Lactobacillus* was positively correlated with SCFAs and leptin, while it exhibited a negative correlation with BAT mass and kidney mass (Fig. 4f). *g_Lactobacillus* was positively correlated with UCP1 and lung mass. Additionally, Tg showed a positive correlation with o_Clostridiales and f_Rikenellaceae (Fig. 4f).

Co-occurrence network analysis of gut microbiota

The gut-dominant OTU correlation network encompasses the top 200 OTUs ranked by relative abundance, forming a network structure with 200 nodes and 994 edges. In this network, there are 910 positive edges and

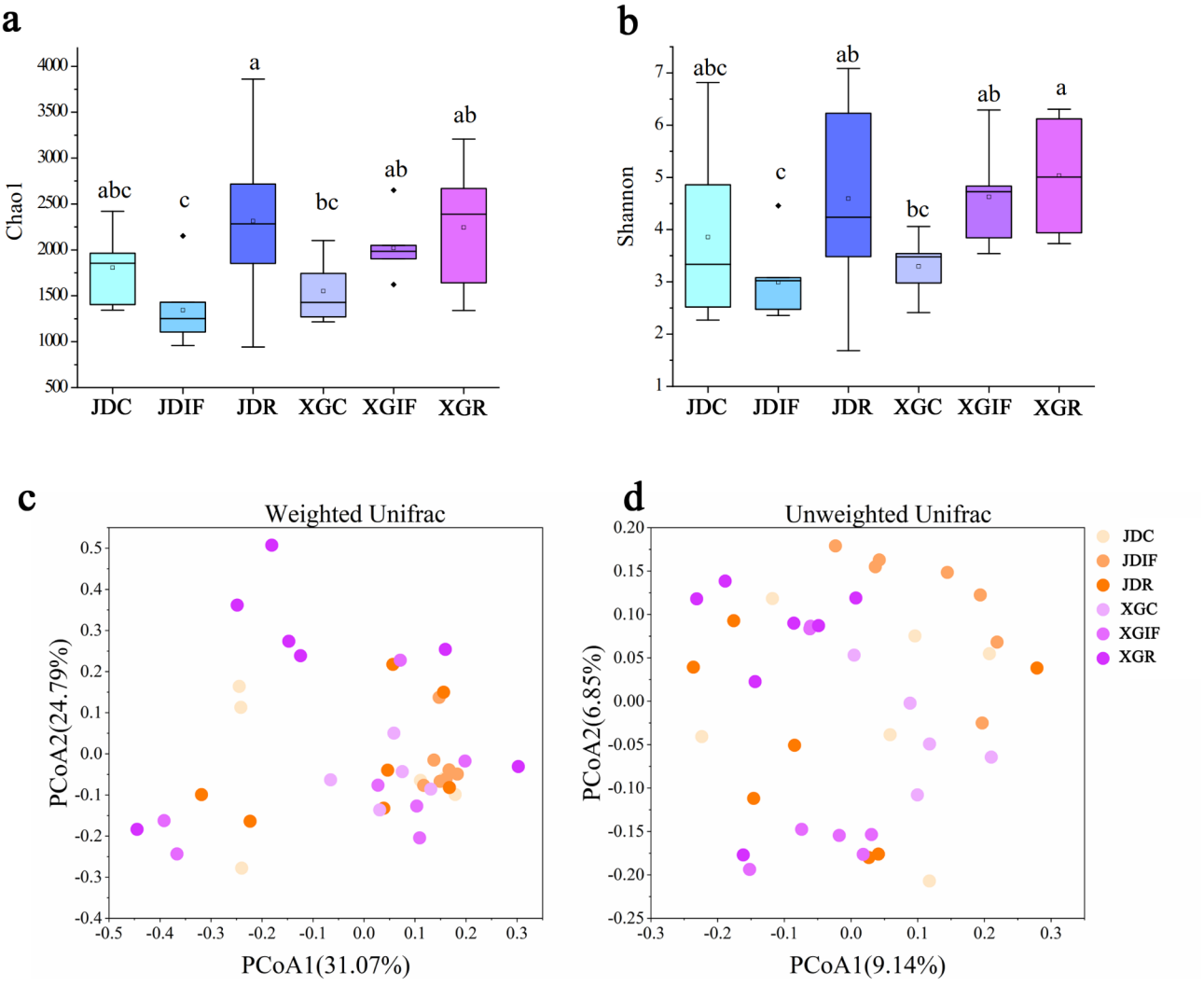

Fig. 2 Gut microbiota diversity of *E. miletus*. (**a**: α diversity Chao1 index; **b**: α diversity Shannon index; **c**: β diversity massed unifrac; **d**: β diversity unmassed unifrac). (Different letters indicate differences between groups)

Table 4 PERMANOVA test for *E. miletus* gut microbiota

PERMANOVA	Massed Unifrac			Unmassed Unifrac		
	F	R^2	*P*	F	R^2	*P*
JDC vs. JDIF	0.791	0.067	0.394	1.247	0.102	0.217
JDIF vs. JDR	0.848	0.066	0.402	1.019	0.078	0.418
XGC vs. XGLIF	0.935	0.086	0.377	1.031	0.093	0.364
XGIF vs. XGR	1.558	0.115	0.203	2.004	0.143	0.047*
DLC vs. XGC	0.637	0.066	0.472	0.774	0.079	0.563
DLIF vs. XGIF	0.889	0.069	0.402	0.774	0.061	0.648
DLR vs. XGR	1.437	0.107	0.242	1.286	0.097	0.209

84 negative edges. Different modules are clearly distinguished by distinct colors, as depicted in Fig. 5.

Discussion

Effect of IF on physiological and biochemical indices

E. miletus has been found to display high plasticity under short-term food restriction. It adapts by regulating body mass and energy metabolism, and is more sensitive to food restriction at higher altitudes [32]. In the present study, IF was found to significantly reduce the body mass of *E. miletus*. However, RMR and most body composition metrics showed only a slight downward trend. This indicates that the body mass loss in *E. miletus* may mainly stem from the depletion of short-term energy reserves.

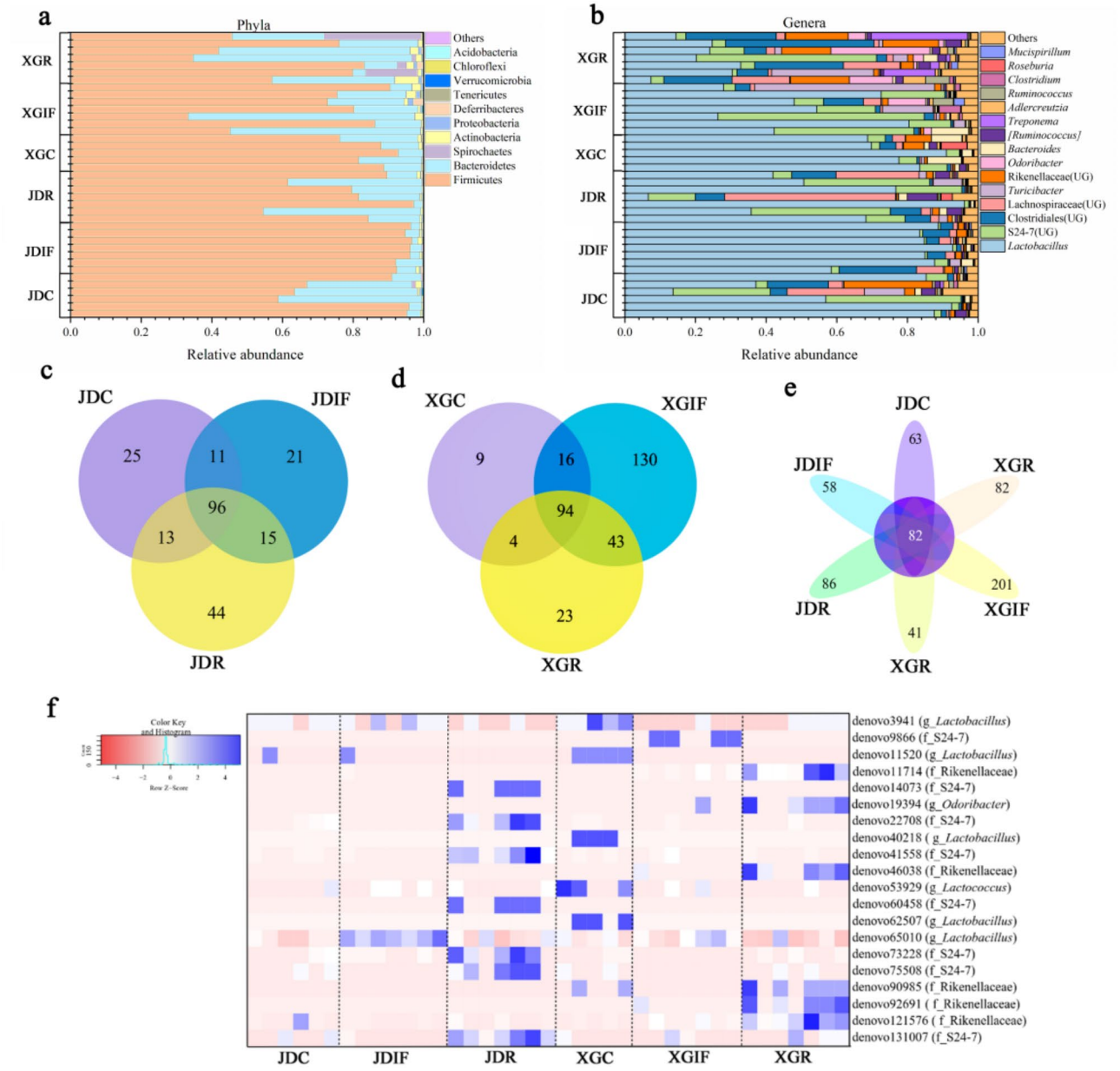

Fig. 3 Composition of gut microbiota in the *E. miletus*. (**a**: composition of gut microbiota levels; **b**: genus-level composition of gut microbiota; **c**: Venn diagram for JD region; **d**: Venn diagram for XGLL region; **e**: Venn diagram for different groups; **f**: enrichment analysis of gut microbiota between different groups)

This is a strategy that allows for rapid body mass adjustments under IF conditions while safeguarding tissues crucial for vital physiological functions. The combination of rapid body mass regulation and metabolic homeostasis demonstrates the high adaptability of *E. miletus* to IF.

Previous studies have shown that IF promotes glycolipid metabolism and enhances immunomodulation [15, 33]. In this study, Tc content only showed a decreasing tendency under IF conditions. Moreover, at high altitudes, IF increased the mass of the spleen, an immune organ. Conversely, at low altitudes, the opposite effect was observed. There were no significant changes in serum immune-related factors, which might be due to the long-term domestication under IF or the unique adaptive mechanisms of *E. miletus* to IF. Further research is required to explore these aspects.

Compared to higher altitudes, body mass loss in the lower body occurs more significantly at lower altitudes. Additionally, the lower RMR and BAT mass at lower altitudes can be ascribed to the scarcity of food resources in high - altitude habitats during winter. In contrast, under

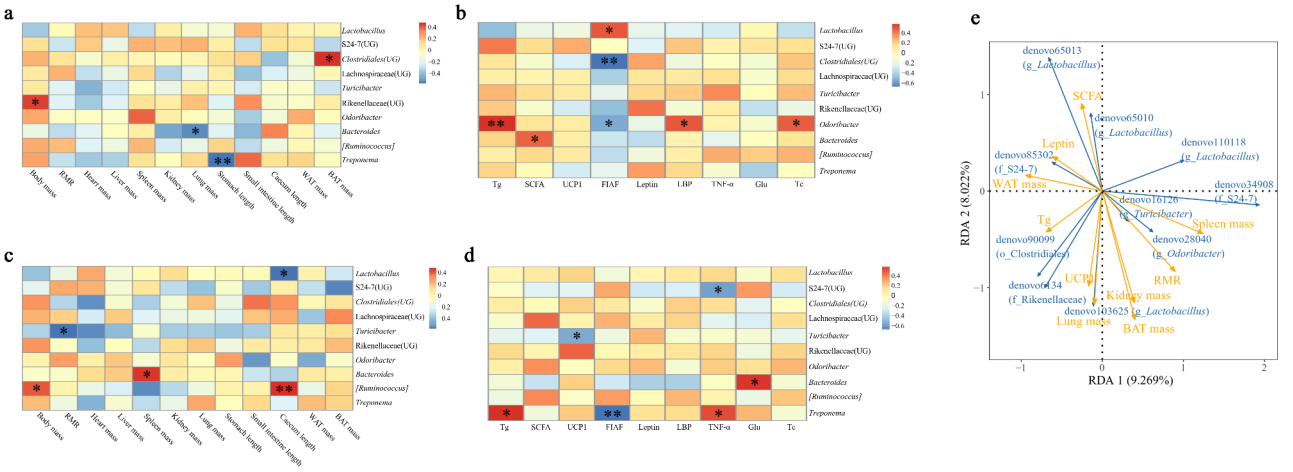

Fig. 4 Correlation analysis of gut microbiota and physiological and biochemical indices in the *E. miletus*. (**a**: analysis of physiological correlates in the JD region; **b**: analysis of biochemical correlation in the JD region; **c**: analysis of physiological correlates in the XG region; **d**: analysis of biochemical correlation in the XG region; **e**: redundancy analysis)

IF conditions, *E. miletus* exhibits a more stable pattern of body mass regulation at higher altitudes.

Effects of IF on the diversity and composition of gut microbiota

Dietary alterations can rapidly reshape gut microbial communities, and food restriction has the capacity to impact the gut microbiota of animals. In *E. miletus*, the dominant phyla are consistent with those observed in other rodents, mainly consisting of Firmicutes and Bacteroidetes. These phyla play pivotal roles in host nutrient regulation and absorption, energy metabolism, and safeguarding the intestine against invasion by foreign pathogens [34–35].

The predominant genera in *E. miletus* are Lactobacillus, S24–7(UG), and Clostridiales(UG), which are also among the major bacterial genera present. Lactobacillus mainly metabolizes simple sugars and generates lactic acid, which exerts both inhibitory effects on pathogens and immune-enhancing effects within the organism [36–37]. S24–7(UG) and Clostridiales(UG) help the host degrade complex polysaccharides and are the main flora responsible for the production of acetic and butyric acids [38–39]. Dominant bacteria are crucial in helping *E. miletus* extract energy from food under IF conditions.

At higher altitudes, the abundance of *Lactobacillus* decreased sequentially in both the intermittent fasting (IF) and re - feeding groups. Conversely, taxa such as S24–7(UG), Rikenellaceae(UG), and *Odoribacter* were significantly enriched. Rikenellaceae(UG) and *Odoribacter* are important constituents of the intestinal microbiota, playing a pivotal role in host health and maintaining the balance of the intestinal ecosystem [40–42].

At low altitudes, *Lactobacillus* was significantly enriched under IF conditions, while S24–7(UG) showed significant enrichment after heavy feeding. Gut microbes form diverse ecosystems. For instance, *E. miletus* can increase the abundance of energy - metabolism - associated flora under IF conditions, facilitating its energy acquisition. Unlike *Lactobacillus*, which depends on fermentation products, S24-7(UG) can directly and efficiently catabolize complex carbohydrates [36, 39].

The gut flora of *E. miletus* at different altitudes demonstrated distinct adaptation patterns under IF conditions. It is hypothesized that the flora at high altitudes tend to efficiently utilize food resources, while those at low altitudes prioritize the effective utilization of metabolites. Although gut microbes constitute a complex and dynamic system, the gut microbes of *E. miletus* mainly rely on cooperative relationships to assist the host in better coping with food - scarcity periods.

Most studies have shown that IF enhances the gut microbial diversity in hosts, and greater diversity enables more efficient extraction of energy from a limited quantity of food [14]. The impact of IF on the α-diversity of *E. miletus* gut microbiota varies with altitude. At high altitudes, IF increased the α-diversity of gut microorganisms in *E. miletus*, and this diversity continued to increase after heavy feeding. Under the same conditions, IF led to a 3.4-fold increase in the number of endemic bacteria in *E. miletus* at high altitudes compared to low altitudes. This suggests that *E. miletus* at high altitudes may adopt a strategy of enhancing gut bacterial flora diversity to improve substrate degradation capacity.

Conversely, at low altitudes, the α-diversity of *E. miletus* gut microorganisms decreased, along with a corresponding reduction in the number of endemic bacteria. It is hypothesized that gut microorganisms at low altitudes may specialize in decomposing specific substances to gain a competitive edge. Higher diversity often implies

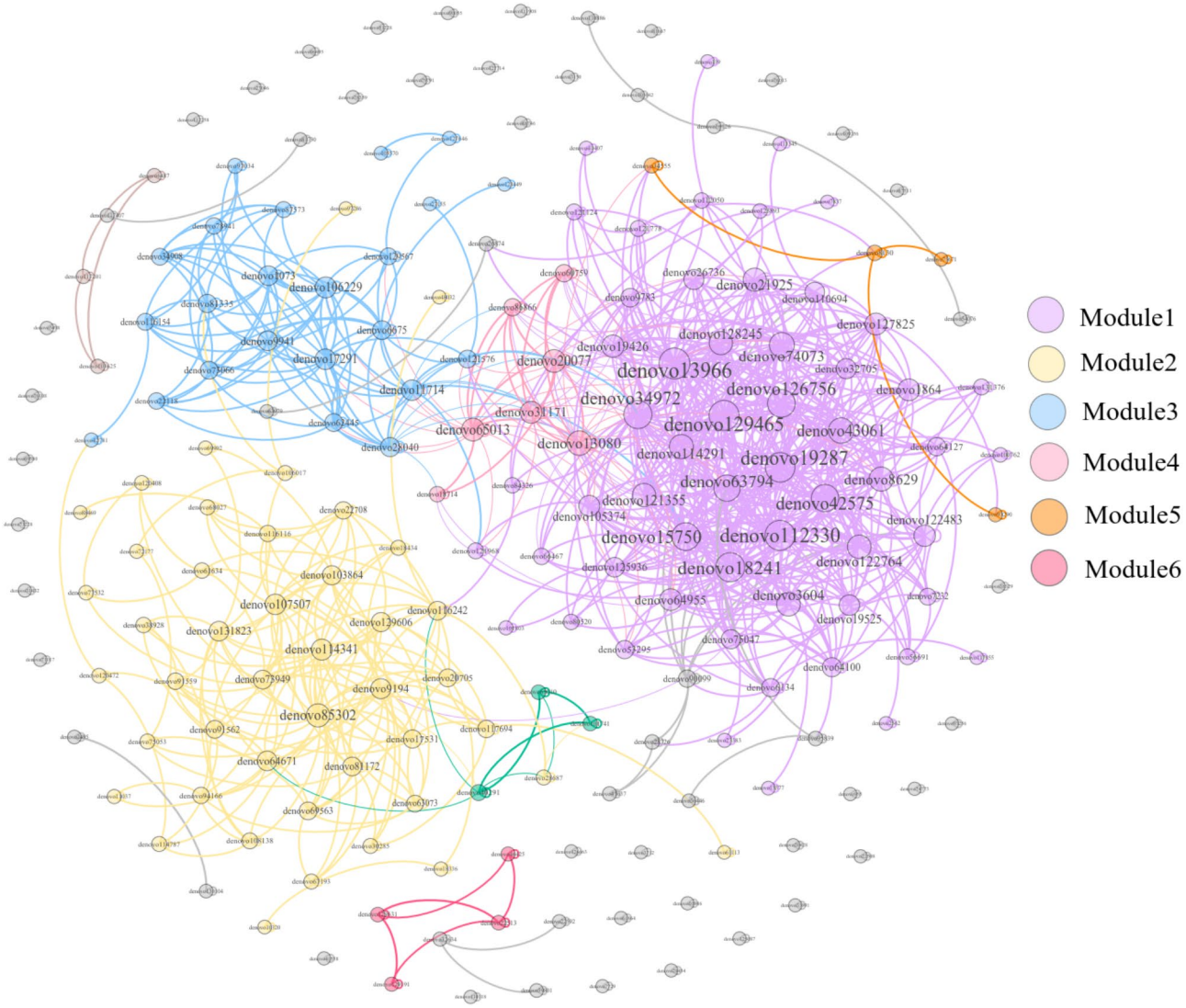

Fig. 5 Network of dominant OTU co-occurrence in *E. miletus* faces

functional redundancy, where redundant strains can substitute for each other's metabolic functions, thus maintaining the overall stability of the microbial community [43]. However, compared to high altitudes, the stability of the gut flora in *E. miletus* may be lower at low altitudes.

Interactions between gut microbiota and physiological and biochemical indicators under IF conditions

At low altitudes, body mass and BAT mass were significantly and positively correlated with Rikenellaceae (UG) and Clostridiales (UG), respectively. BAT mass is a critical indicator of heat production in animals, and both genera can promote the probiotic digestion of the host [38, 42]. Meanwhile, FLAF was negatively correlated with Clostridiales (UG) and *Odoribacter*. A decrease in *Odoribacter* has been associated with various microbiota-related diseases [41]. It has been shown that Rikenellaceae (UG) might be the key functional flora mediating mass loss and thermogenesis in *E. miletus* under IF conditions. In the same context, Clostridiales (UG) and *Odoribacter* may exert anti-inflammatory effects. Lung mass and stomach length were significantly and negatively correlated with *Bacteroides*, whereas spleen mass and Glu were significantly and positively correlated with *Bacteroides*. *Bacteroides* are important keystone genera of bacteria that aid in breaking down food and generating the nutrients and energy needed by the body [44–45]. In *E. miletus*, *Bacteroides* play a crucial role mainly in nutrient acquisition, and their metabolites may indirectly regulate glycolipid metabolism.

In high-altitude regions, RMR, cecum length, UCP1, and TNF-α showed significant negative correlations with *Turicibacter*. A relationship was identified between *Turicibacter* and host lipid metabolism traits, with

Turicibacter being found to reduce host bile acids and alter genes related to lipid metabolism [46]. [*Ruminococcus*] was significantly and positively correlated with body mass and cecum length. [*Ruminococcus*] plays essential roles in food digestion, maintaining intestinal barrier function, and regulating immunity and energy metabolism [47]. *Turicibacter* may have important impacts on energy metabolism and immune regulation in *E. miletus* under IF conditions. In contrast, [*Ruminococcus*] may serve as a crucial functional flora for maintaining energy acquisition and body mass during IF. UCP1 and TNF-α were significantly negatively correlated with S24-7(UG). Redundancy analysis indicated that S24-7(UG) was positively correlated with spleen mass and RMR, and negatively correlated with WAT mass and Leptin. Previous studies on S24-7(UG) have mainly focused on energy metabolism, and the results suggest that its secondary metabolites, generated during nutrient catabolism, may also influence the health of *E. miletus*.

At low altitudes, lung mass and stomach length showed a significant negative correlation with *Treponema* abundance. In contrast, at high altitudes, *Treponema* abundance had a significant positive correlation with Tg and TNF-α, and a significant negative correlation with FLAF. Research has shown that *Treponema* is involved in cellulose degradation. However, most studies indicate that *Treponema* is a diverse group of bacteria, including several well - known human pathogens [48]. These pathogens can penetrate the barrier function of endothelial cells in the host's blood vessels and then diffuse through the bloodstream to distant tissues and organs [49–50]. Although IF has multiple beneficial effects on the organism, prolonged or excessive fasting may compromise the organism's protective response to pathogens [16–18, 51]. Based on these findings, it is hypothesized that *Treponema* may support food digestion in *E. miletus*. However, it may also pose potential risks to the host's serum metabolism and immune system. Nevertheless, due to the significant interspecies phenotypic diversity within the genus and the inability of this study to clarify its species-specific classification, the underlying mechanism still needs to be elucidated through further in-depth research.

The results of the redundancy analysis indicated that *Lactobacillus* was positively associated with SCFA, Leptin, UCP1, and lung mass, but negatively associated with BAT mass and kidney mass. Most studies have demonstrated that intermittent fasting increases the abundance of gut probiotics. Moreover, alterations in the gut microbiota can generate beneficial metabolites, which may alleviate diseases related to metabolism and inflammation [12–14]. In the study of *E. miletus*, gut microorganisms like *Lactobacillus* may mediate host gut and organismal homeostasis through their metabolites, thereby enhancing adaptation to food-scarce environments.

In consideration of the synergistic effects of physiological and biochemical indicators and gut microbes, *E. miletus* can effectively adapt to IF conditions. Gut microbial metabolites play a crucial role in enhancing a species' adaptive ability to food shortage stress. They achieve this by participating in the regulation of energy metabolism and the maintenance of immune homeostasis within the host organism. Given the variation in the colonization of gut microbial communities at different altitudes, the interactions among these communities also vary.

Similarities and differences in the effects of IF and food restriction (FR) on energy metabolism and gut microbiology in *E. miletus*

Wild animals encounter food uncertainty in winter, with unpredictability in both the quantity and timing of available sustenance [52]. The amount of food exerts a significant impact on the gut microbiota of these animals. In response, the gut microbiota may deploy diverse strategies to assist the host in adapting to varying levels of food intake. It carried out an 80% food restriction (FR) experiment on *E. miletus*. In comparison with the present study, they identified differences in the composition and diversity of gut microorganisms, as well as in the microorganisms' interactions with physiological indicators [26].

Relative to the FR experiment, the gut microbe diversity in different regions of the IF group showed substantial changes. There was an increase in the number of both endemic and shared genera. It is likely that the IF conditions imposed more stress on *E. miletus*, necessitating a more diverse gut microflora to support the host in energy acquisition. In the FR experiment, the food - restricted group had the most enriched operational taxonomic units (OTUs). Conversely, the current study revealed that the majority of OTUs were enriched in the re - fed group. This implies that E. miletus may require more time to recover from stress under IF conditions compared to FR.

Most of the dominant microbial flora played similar roles under both experimental conditions, although the FR experiment had a greater enrichment of probiotics than the present study. Notably, in both experiments, Treponema showed significant associations with most physiological indices. However, as relatively little research has been conducted on this bacterium, it could be a focal point for future investigations. The cooperative relationships observed in the present study were more prominent than those in the FR experiment. It is possible that the microorganisms mitigated the impact of the unfavorable IF environment by strengthening their cooperative interactions with one another. In summary, the stress endured by *E. miletus* under IF might be greater.

The gut microbiota aids the host in coping with IF stress by rapidly modifying its composition, diversity, and interactions.

Conclusion

In conclusion, IF led to a significant reduction in the body mass of *E. miletus*. Although the RMR showed a decreasing tendency, this decrease did not reach statistical significance. *E. miletus* adapted to IF conditions mainly by modifying the composition and abundance of gut microbiota, rather than through substantial changes in community structure. IF had a significant impact on the relative abundance of *Lactobacillus* and S24–7(UG). The diverse correlations between different gut microorganisms and physiological indicators might be due to the distinct gut microbial populations in the two regions. Moreover, the effect of IF on *E. miletus* may differ at different altitudes. *E. miletus* at low altitudes experienced more substantial mass loss compared to those at high altitudes. Under IF conditions, the gut microbial diversity and endemic bacteria of low - altitude *E. miletus* were reduced. In contrast, *E. miletus* at high altitudes may have more stable regulatory mechanisms, enabling better adaptation to IF. However, the sequencing depth of gut microorganisms in this study was inadequate to identify specific strains. This research is the first to investigate the effects of IF on gut microbes and the physiological and biochemical indicators of *E. miletus* at different altitudes. It offers a new basis for understanding the adaptation mechanisms of small wild rodents under food-scarcity conditions.

Abbreviations

ANOVA	Analysis of variance
BAT	Brown adipose tissue
ELISA	Enzyme-linked immunosorbent assay
FIAF	Fasting-induced adipocyte factor
IF	Intermittent fasting
JD	Jindong
LBP	Lipopolysaccharide binding protein
mean ± SE	Mean ± standard error
RDA	Redundancy analysis
RMR	Resting metabolic rate
SCFAs	Short-chain fatty acids
TC	Cholesterol, UCP1:uncoupling protein 1
TG	Triglyceride
TNF-α	Tumor necrosis factor-α
WAT	White adipose tissue
XGLL	Xianggelila

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Author contributions

Zhu WL conceived the study, participated in design, and coordination and drafted the manuscript. Jia T and Zhang W carried out the studies of body mass, gut microbiome and physiological indexes. Fan LX carried out the morphological studies and revised the manuscript.

Data availability

The 16 S rRNA sequences used in this study can be found on the European Nucleotide Archive (http://www.ebi.ac.uk/ena) under accession number: PRJEB63217. The physiological data can be found on figshare (https://doi.org/10.6084/m9.figshare.28082315.v1).

Declarations

Ethics approval and consent to participate

The study was approved by the Animal Care and Use Committee of Yunnan Normal University (Accession number: 13-0901-011). In addition, the method of euthanasia on *Eothenomys miletus* was performed in accordance with the American Veterinary Medical Association (AVMA) guidelines for the euthanasia of animals (2020). Carbon dioxide have been used as euthanasia methods for *Eothenomys miletus* with low concentrations in AVMA guidelines for euthanasia. *Eothenomys miletus* is listed as Least Concern in the International Union for Conservation of Nature (IUCN) red list status (https://www.iucnredlist.org) and not endangered or protected in China. All sampling was permitted by the local level authority in scientific research.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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