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Probiotic *Lactobacillus kefiranofaciens* K6 attenuates physiological and behavioral alterations and modulates the gut microbiota in a mouse model of overtraining syndrome

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Exercise is widely recognized for its substantial health benefits in promoting well-being and preventing disease across diverse populations; however, inappropriate training intensity combined with insufficient recovery can lead to overtraining syndrome (OTS), characterized by sustained performance decline, physiological maladaptation, and psychological disturbances. Probiotics have emerged as a promising nutritional strategy for modulating host metabolism and the gut microbiota, yet effective interventions for OTS remain limited. In the present study, a well-established OTS-like mouse model induced by a progressive treadmill protocol was used to evaluate the effects of *Lactobacillus kefiranofaciens* K6 supplementation on performance decline, physiological maladaptation, psychological disturbances, and gut microbiota dysbiosis. Mice were randomly assigned to Sedentary, Exercise, OTS, and OTS-K6 groups and received the designated interventions for 10 weeks. K6 supplementation was associated with attenuation of OTS-induced reductions in body weight, appetite, body composition, endurance performance, glucose tolerance, and tissue glycogen content, as well as improvements in peripheral fatigue biomarkers, including muscle injury markers (CK and LDH) and energy metabolism indicators (glucose, lactate, and ammonia). In addition, K6 administration was associated with improved intestinal barrier-related gene expression (*Claudin-1*, *ZO-1*, and *ZO-2*), reduced tissue and systemic inflammatory responses (*TNF-α*, *IL-6*, neutrophil counts, PLR, and NLR), and support of immune function as indicated by lymphocyte levels. K6 supplementation also alleviated OTS-induced anxiety-like behaviors. Furthermore, gut microbiota composition was altered by exercise, OTS induction, and K6 supplementation, with K6 modulating OTS-related dysbiosis by reducing *Heminiphilus faecis* and increasing *Paramuribaculum intestinale*. Moreover, changes in *Duncaniella dubosii* and *P. intestinale* may also provide potential benefits in the Exercise group. Excessive training stress in this experimental OTS-like mouse model was associated with multiple metabolic, inflammatory, behavioral, and microbiota-related disturbances, whereas K6 supplementation was associated with attenuation of several OTS-related alterations. These findings suggest that microbiota-targeted nutritional strategies may support physiological resilience under excessive training stress; however, the results should be interpreted as associative and hypothesis-generating, as mechanistic relationships were not directly examined.

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1. Introduction

Athletes often engage in periodized training based on the principles of overload and supercompensation to enhance their competitive performance and physical capacity.[1] However, long-term imbalance between high-intensity training and insufficient recovery can lead to the development of overtraining syndrome (OTS), which is characterized by a decline in sport-specific performance accompanied by mood disturbances that may persist even after weeks or months of rest.[2] OTS is highly prevalent among athletes, although its incidence varies depending on the type of sport. Studies have shown that

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athletes who have previously experienced OTS are at higher risk of recurrence. For example, surveys on elite runners revealed that 60% of female and 64% of male athletes had experienced at least one episode of OTS, compared to 33% in non-elite adult runners.[3] Moreover, the prevalence was 37% in individual sports and 17% in team sports,[4] indicating that sport type and training modality influence the likelihood of OTS development. Clinically, OTS presents with a wide array of symptoms, including chronic fatigue, sleep disturbances, appetite loss, weight reduction, anxiety, increased stress, reduced motivation and concentration, and emotional instability. It may also cause dysregulation in multiple physiological systems, including the endocrine, immune, musculoskeletal, and nervous systems.[5] Multiple hypotheses have been proposed to elucidate the complex pathophysiology of overtraining syndrome (OTS), including the cytokine, glutamine, central fatigue, glycogen depletion, autonomic dysregulation, oxidative stress, and hypothalamic dysfunction hypotheses. Collectively, these theories underscore the multisystemic nature of OTS and its impact on immune, metabolic, neuroendocrine, and psychological function.[6]

The human gut harbors 10 to 100 trillion microorganisms, forming the body's largest and most complex internal ecosystem. These microorganisms span multiple bacterial phyla, including Bacteroidetes, Firmicutes, Actinobacteria, Proteobacteria, Verrucomicrobia, Fusobacteria, Cyanobacteria, and Tenericutes. Notably, Bacteroidetes and Firmicutes constitute approximately 90% of the gut microbiota.[7] Functionally, the gut microbiota acts as a metabolic organ, engaging in a symbiotic relationship with the host and playing a critical role in maintaining health.[8] While previous study has shown that both physical activity and nutritional factors can alter the gut microbial composition, their combined or individual influence may directly or indirectly affect host metabolism and physiology. Moreover, through the gut–brain axis, such changes may influence cognitive function, emotional regulation, and systemic physiological processes.[9] However, the role of the gut microbiota in the physiological alterations associated with overtraining—especially under complex interactions of training and non-training factors—remains poorly understood.

In recent years, probiotics have been increasingly studied for their role in health promotion. Beyond enhancing immune function and gastrointestinal health, a growing body of evidence supports their application in disease prevention and treatment. Gut dysbiosis has been implicated in numerous health conditions, including antibiotic-associated diarrhea, inflammatory bowel disease, chronic constipation, Crohn's disease, chronic systemic illnesses, neurodegenerative diseases, cardiovascular disease, and colorectal cancer. Probiotic supplementation has been shown to restore gut microbial balance and alleviate related symptoms.[10] In addition to dietary interventions, the therapeutic strategies such as functional probiotics and fecal microbiota transplantation are being developed from the perspective of gut microbiota regulation.[11] Clinical research has also provided evidence that psychobiotics—probiotics with mental health benefits—can alleviate symptoms of depression and anxiety. These probiotics produce neuroactive compounds such as γ-aminobutyric acid and serotonin, which may act *via* the gut–brain axis. Their potential mechanisms include anti-inflammatory effects and the suppression of hypothalamic–pituitary–adrenal (HPA) axis activation, thereby contributing to improved emotional regulation.[12] Beyond immune modulation, probiotics may influence multiple physiological axes through gut microbiota alteration, including the gut–brain axis, gut–muscle axis, HPA axis, and hypothalamic–pituitary–gonadal axis, thereby contributing to systemic physiological adaptation.[13]

Importantly, probiotic effects are highly strain-specific, and the selection of candidate strains should be supported by prior functional evidence. *L. kefiranofaciens* K6 is a probiotic strain originally isolated from kefir grains and has recently attracted attention for its functional properties under stress-related conditions. In a previous study, K6 supplementation was shown to attenuate chronic inflammation, reduce exercise-induced tissue injury, and improve stress adaptation in a sleep deprivation animal model (mouse).[14] Although sleep deprivation and overtraining syndrome represent distinct physiological stress paradigms, both conditions share several underlying biological features, including chronic low-grade inflammation, metabolic dysregulation, impaired recovery capacity, and neuropsychological disturbances mediated by stress-responsive pathways such as inflammatory signaling and hypothalamic–pituitary–adrenal axis activation. These overlapping physiological characteristics suggest that probiotic strains capable of modulating inflammatory and stress-related responses in one stress context may represent promising candidates for investigation in other stress-associated conditions. Based on this rationale, *L. kefiranofaciens* K6 was selected in the present study as a functional probiotic candidate to examine its potential associations with physiological adaptation under excessive training stress. Nevertheless, whether K6 exerts similar regulatory effects under the complex physiological stress induced by excessive training remains unknown and therefore warrants further investigation.

Given the known adverse health consequences of improper training programs, it is ethically unfeasible to induce OTS in human participants for experimental purposes. Therefore, the present study employed an established animal model of overtraining syndrome developed in a previous study.[15] From a preventive perspective, this study aimed to investigate the effects of functional probiotics *L. kefiranofaciens* K6 on OTS-related alterations, including exercise performance decline, peripheral fatigue, physiological maladaptation, psychological disturbances, and gut microbiota dysbiosis.

2. Methods and materials

2.1. Experimental design and procedures

Thirty-two six-week-old male C57BL/6 mice (BioLASCO, Taipei, Taiwan) were housed under controlled conditions (24 ± 2 °C, 50–60% humidity, 12 h light/dark cycle) with *ad libitum* access

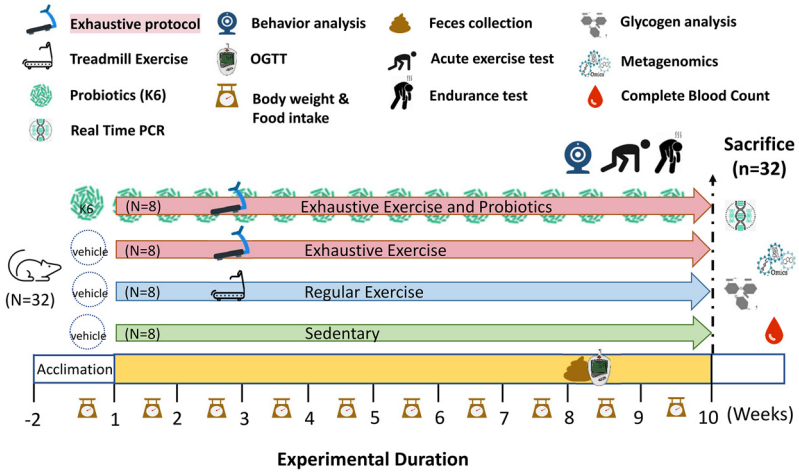

Fig. 1 Experimental design and procedures for investigating the effects of probiotic supplementation and OTS induction in an animal model. Animals were randomly assigned to one of four groups: Sedentary, Exercise, OTS, and OTS + K6. Overtraining syndrome (OTS) was induced using a treadmill running-to-exhaustion protocol for ten consecutive weeks without sufficient rest. Probiotic supplementation and the designated exercise protocols were administered to the relevant groups throughout the experimental period, including both pre-treatment and intervention phases. Assessments were conducted during the experimental period and after sacrifice, and included measurements of growth curves, exercise capacity, inflammatory markers, tight junction integrity, neuropsychological behaviors, glucose tolerance, hematological parameters, peripheral fatigue, glycogen content, and gut microbiota composition. K6: *Lactobacillus kefiranofaciens* K6.

to water and a standard chow diet (No. 5001; PMI Nutrition International, Brentwood, MO, USA). The chow diet provided 3.35 kcal g^{-1} of metabolizable energy and consisted of 28.5% protein, 13.4% fat, and 58.1% carbohydrates. After a two-week acclimatization period, mice were randomly assigned to one of four groups (n = 8): Sedentary, Exercise, OTS, and OTS supplemented with *L. kefiranofaciens* K6 (OTS-K6). The intervention lasted 10 weeks, during which animals underwent group-specific treadmill protocols and/or probiotic supplementation. Body weight, food intake, and time to exhaustion were monitored (Fig. 1). All experimental procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the National Research Institute of Chinese Medicine (approval number: 113-355-1). All animal experiments were conducted in accordance with the ARRIVE guidelines and relevant institutional and national regulations for the care and use of laboratory animals.

2.2. Implementation of regular exercise and overtraining syndrome induction

Based on a previous protocol,[15] regular exercise involved treadmill running at 15 meters per min, 20 min per session, 3 times per week. OTS was induced through progressive treadmill running 5 times per week, with incremental increases in speed (starting at 15 m min^{-1}, +3 m min^{-1} every 10 min) and a 5% incline, continued until exhaustion. The running time to exhaustion during each session was recorded to monitor changes in endurance capacity during the intervention period. Exhaustion was defined as the inability of the mouse to maintain running pace, characterized by remaining in the "fatigue zone" for 10 consecutive seconds despite gentle stimulation. The fatigue zone was defined as the area at the rear of the treadmill belt, within approximately one body length from the shock grid. When mice entered this zone, mild stimuli (*e.g.*, air puff or light touch) were applied to encourage continued running. If the mouse failed to resume running and remained in the fatigue zone for 10 seconds, the test was terminated and the time to exhaustion was recorded as an indicator of endurance performance.

2.3. Probiotics supplementation

L. kefiranofaciens K6 was kindly provided by Professor Yen-Po Chen. The strain was cultured in de Man, Rogosa and Sharpe (MRS) broth (Difco™, USA) at 37 °C for 24 hours, harvested by centrifugation, and washed twice with phosphate-buffered saline (PBS) to remove residual culture medium. The bacterial pellet was then resuspended in 20% (w/v, weight/volume) skim milk powder solution (NZMP, New Zealand), which served as a cryoprotectant, and stored at −80 °C for 24 h prior to freeze-drying. The freeze-drying procedure was conducted following standard probiotic preservation protocols, and the resulting lyophilized powder was stored at −20 °C until use. The viable bacterial counts were verified after lyophilization by standard plate counting on MRS agar following rehydration of the powder. This procedure ensured accurate quantification of colony-forming units (CFU) and confirmed the administered dose. For supplementation, the lyophilized powder was freshly suspended in sterile saline to achieve a final dose of 1×10^8 CFU per mouse per day and administered by oral gavage. Mice in the Sedentary, Exercise, and OTS groups received an equivalent volume of sterile saline containing the same concentration of skim milk powder (without bacteria) as the vehicle control. The probiotic suspension was administered at a volume of 200 μL per mouse per day *via* oral gavage. This

volume is within the recommended administration range for mice (approximately 10 μL g^{-1} body weight) and therefore represents a physiologically acceptable gavage volume that contributes minimally to total dietary intake.

To provide translational interpretation of the probiotic dose, a body surface area-based dose conversion was considered. In this commonly used interspecies scaling method, the human body surface area is standardized as 1, and species-specific coefficients are applied according to the relative body surface area. For mice, the conversion coefficient is 12.3. Based on the administered dose in this study (1×10^8 CFU day^{-1} per 25 g mouse), the human-equivalent intake corresponds to approximately 1.95×10^{10} CFU day^{-1} for a 60 kg adult. This estimated intake falls within the typical range used in human probiotic supplementation studies and remains well below commonly recommended upper intake levels for probiotic consumption.

2.4. Aerobic endurance performance and assessment of peripheral biochemical variables

Aerobic endurance was evaluated using a weight-loaded forced swimming test (FST) following a 12 h fast. In this test, mice were subjected to swimming with an additional weight attached to the tail until exhaustion. Exhaustion was defined as submersion for 5 seconds without resurfacing. The total swimming time until exhaustion was recorded as an indicator of aerobic endurance performance. Treadmill running to exhaustion was primarily used to monitor endurance performance changes during the OTS induction protocol. Because treadmill training was not performed in the Sedentary group and differed between training groups, a weight-loaded forced swimming test was additionally used as a standardized endurance assessment that could be applied to all groups, allowing cross-group comparison of aerobic endurance capacity.

Peripheral fatigue-related biochemical markers were assessed using a separate acute swimming challenge consisting of 20 minutes of continuous swimming without additional weight. Blood samples were collected immediately before and after this 20 min swimming test to determine biochemical indicators of peripheral fatigue, including blood glucose (GLU), ammonia (NH_3), lactate dehydrogenase (LDH), lactate, and creatine kinase (CK), using an automated biochemical analyzer (Hitachi 7060; Hitachi, Tokyo, Japan).

2.5. Oral glucose tolerance test

At the 8th week of the exercise intervention, the mice were fasted for 12 h before being orally administered glucose at a dose of 1 g kg^{-1} of body weight. Whole blood (0.6 μL) was sampled from the tail at the indicated time points: baseline, 15, 30, 60, and 120 min. This was performed using a glucometer (Accu-Chek®; Roche, Taipei, Taiwan) to measure plasma glucose fluctuations. During the fasting period, mice were maintained under their standard group-housing conditions rather than being individually housed. This approach was adopted to minimize stress associated with sudden social isolation, which may itself influence metabolic responses. All groups were maintained under identical housing conditions during the fasting period.

2.6. Complete blood count (CBC) analysis

Following CO_2 euthanasia, cardiac puncture was immediately performed to collect whole blood samples. Blood was drawn into microcentrifuge tubes containing K_2-EDTA as an anticoagulant for subsequent complete blood count (CBC) analysis using an automated hematology analyzer (ADVIA 2010, Bayer, NY, USA). The parameters measured included white blood cell count (WBC), neutrophils (Neu), lymphocytes (Lym), monocytes (Mono), eosinophils (Eos), basophils (Bas), red blood cell count (RBC), hemoglobin concentration (Hb), and platelet count (PLT). In addition, the neutrophil-to-lymphocyte ratio (NLR) and platelet-to-lymphocyte ratio (PLR) were calculated as indicators of systemic inflammatory status. The elevated NLR and PLR observed in the OTS group may reflect systemic inflammatory stress and immune imbalance induced by excessive training load, as these composite hematological indices are commonly used indicators of inflammation and physiological stress.

2.7. Quantitative real-time polymerase chain reaction

The proximal colon, liver, and muscle tissues were extracted using the RNAzol RT kit (Molecular Research Center, OH, USA). Next, 1 μg of RNA was reverse transcribed into cDNA using the Magic RT cDNA synthesis kit (DBU-RT-100, BioGenesis Technologies, Taipei, Taiwan). Quantitative polymerase chain reaction was conducted using 2× EvaGreen qPCR Master Mix (31045, Biotium, Fremont, CA, USA) on an ABI 7500 FAST Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). The cDNA templates were analyzed for the expression of inflammatory genes (*TNFA*, *IL1B*, and *IL6*), tight junction genes [occludin (*OCLN*), claudin-1 (*CLDN1*), zonula occludens-1 (*TJP1*), and zonula occludens-2 (*TJP2*)], and an internal control gene (*GAPDH*). The primers used to quantify gene expression were referenced from a previous study.[14] Gene expression was estimated using the threshold cycle values, and relative mRNA expression levels were calibrated using *GAPDH* expression.

2.8. Behavioral neuropsychology assessments

Behavioral tests, including the open field test (OFT) and elevated plus maze (EPM), were conducted to evaluate anxiety-like behaviors in mice following the intervention, based on a previously described protocol.[15] Behavioral activity was recorded and analyzed using AnyMaze software (Stoelting, Wood Dale, IL, USA). Parameters included travel distance, time in open arms (EPM), center zone time, and fecal boli. Apparatuses were sanitized between trials.

2.9. Gut microbiota analysis

Fecal samples collected during the open field test were immediately stored at −80 °C until analysis. Microbial genomic DNA was extracted using the QIAamp PowerFecal

DNA Kit (Qiagen, Germany) according to the manufacturer's instructions.

Full-length 16S rRNA genes (V1–V9 regions) were amplified and sequenced using the PacBio Sequel IIe platform. High-fidelity (HiFi) reads with a quality threshold of RQ > 30 were used to ensure high sequencing accuracy. Raw sequencing reads were subjected to quality filtering to remove low-quality sequences and chimeric reads before downstream microbiome bioinformatic analyses. High-quality sequences were processed using the DADA2 pipeline to generate amplicon sequence variants (ASVs). Samples with insufficient sequencing depth were excluded from downstream analyses. Taxonomic classification of ASVs was performed using reference databases integrated within the analysis workflow. Microbial alpha and beta diversity analyses were conducted to evaluate community richness and compositional differences among the groups. Differentially abundant taxa were identified using linear discriminant analysis effect size (LEfSe), with statistical significance determined by the non-parametric Kruskal–Wallis test and an LDA score threshold of >4.0. To statistically evaluate differences in overall microbial community composition among the groups, permutational multivariate analysis of variance (PERMANOVA) was conducted based on the distance matrices derived from beta diversity metrics. PERMANOVA is a nonparametric multivariate statistical method that partitions variation in the microbial community structure according to explanatory variables. The analysis was performed using the "adonis" function in the vegan package in R with 999 permutations. The pseudo-F statistic, coefficient of determination (R^2) and permutation-based *p*-values were reported to assess the significance and magnitude of group differences.

2.10. Statistical analysis

Data are presented as mean ± SD. Normality was assessed using the Kolmogorov–Smirnov test. One-way or two-way repeated-measures ANOVA was used to assess group differences. When significant main effects were detected in ANOVA analyses, Tukey's *post hoc* multiple comparison test was applied to control the family-wise error rate across pairwise group comparisons. For nonparametric data, the Mann–Whitney and Kruskal–Wallis tests were used. SPSS (v22.0, IBM, New York, NY, USA) was used for all analyses, with significance set at $p < 0.05$.

3. Results

3.1. Growth curves, dietary intake, and body composition

A significant interaction between time and group was observed in the growth curves ($F(30, 280) = 9.99$, $p < 0.0001$, $\eta^2 = 0.512$), along with significant main effects of both time ($F(10, 280) = 50.39$, $p < 0.0001$, $\eta^2 = 0.643$) and intervention ($F(3, 28) = 11.06$, $p < 0.0001$, $\eta^2 = 0.542$), indicating differential weight gain patterns among the groups over the 10-week intervention (Fig. 2A). At week 10, the OTS group exhibited significantly lower body weight than the Sedentary, Exercise, and OTS-K6 groups. The OTS-K6 group also showed significantly lower body weight than the Sedentary group but did not differ from the Exercise group. The significant time × group interaction indicates that body weight trajectories differed among the experimental groups over the intervention period. Specifically, the OTS group exhibited a progressive attenuation of body weight gain compared with the Sedentary and Exercise groups, whereas the OTS-K6 group showed partial recovery of body weight gain during the later stages of the intervention.

Significant differences in dietary and water intake were also observed (food: $F(3, 71) = 3.86$, $p = 0.013$; water: $F(3, 71) = 2.71$, $p = 0.048$) (Fig. 2B). The OTS group consumed significantly less food and water than the Sedentary and OTS-K6 groups, with no significant differences between the OTS-K6, Sedentary, and Exercise groups.

At the endpoint on body composition, significant group differences were found in the weights of the liver, muscle, testes, heart, and epididymal fat ($F(3, 28) = 3.48$–13.79, all $p < 0.05$) (Table 1). The OTS group showed significantly lower muscle, testes, and fat pad weights compared with both the Sedentary and Exercise groups, and these weights were also lower than the OTS-K6 group. The OTS-K6 group did not differ

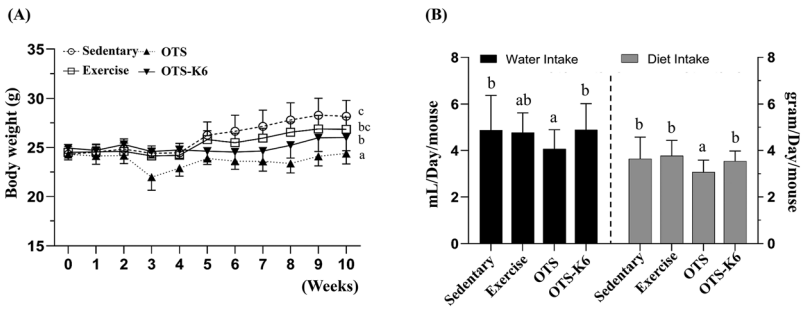

Fig. 2 Effects of probiotic supplementation and OTS induction on growth curve and dietary intake. Data are presented as mean ± SD ($n = 8$). (A) Body weight growth curve. Data were analyzed using two-way repeated-measures ANOVA with time and group as factors. Significant main effects of time and group, as well as a significant time × group interaction, were observed. (B) Diet and water intake during the experimental period. Data were analyzed using one-way ANOVA followed by Tukey's multiple comparison test. Columns subscripted with different letters (a, b, and c) indicate significant differences between the groups ($p < 0.05$).

Table 1 Effects of exercise, overtraining, and K6 supplementation on body compositions

Characteristics	Sedentary	Exercise	OTS	OTS-K6
Liver (g)	1.21 ± 0.08^{c}	1.13 ± 0.09^{bc}	1.03 ± 0.08^{a}	1.11 ± 0.11^{ab}
Muscle (g)	0.33 ± 0.02^{b}	0.32 ± 0.02^{b}	0.29 ± 0.02^{a}	0.33 ± 0.02^{b}
Testes (g)	0.20 ± 0.01^{b}	0.19 ± 0.01^{b}	0.17 ± 0.02^{a}	0.20 ± 0.01^{b}
Heart (g)	0.15 ± 0.01^{b}	0.14 ± 0.01^{b}	0.13 ± 0.01^{a}	0.14 ± 0.01^{ab}
EFP (g)	0.41 ± 0.04^{b}	0.40 ± 0.05^{b}	0.29 ± 0.04^{a}	0.37 ± 0.03^{b}

Data are presented as the mean ± SD (n = 8) and analyzed using one-way ANOVA followed by Tukey's multiple comparison test. Data in the same row with different superscript letters (a, b, and c) differ significantly at $p < 0.05$ as determined by one-way ANOVA. EFP: epididymal fat pad.

significantly from the Sedentary or Exercise groups. For liver weight, the OTS group showed a significantly lower value than that for the Sedentary and Exercise groups, while the OTS-K6 group showed a modest weight reduction compared with Sedentary mice only. Heart weight was reduced in the OTS group relative to the Sedentary and Exercise groups, with no significant differences involving the OTS-K6 group.

3.2. Exercise endurance performance

Endurance capacity was evaluated by monitoring treadmill running time to exhaustion during training sessions and by a forced swimming test. The treadmill running time to exhaustion recorded during the OTS training sessions in the OTS and OTS-K6 groups, with two representative time points per week, is shown in Fig. 3A. Repeated measurements of treadmill exhaustion time revealed significant main effects of time (F(15, 210) = 7.27, $p < 0.0001$, $\eta^2 = 0.342$) and treatment (F(1, 14) = 8.41, $p = 0.012$, $\eta^2 = 0.375$). The mice in the OTS-K6 group demonstrated significantly greater running endurance than the mice in the OTS group from the 7th to the 16th measurement point. In the FST (Fig. 3B), a significant group effect was observed (F(3, 28) = 6.89, $p = 0.001$). The OTS group exhibited significantly reduced swimming endurance compared with the Sedentary, Exercise, and OTS-K6 groups. The OTS-K6 group outperformed the OTS group, though its performance remained significantly below the Exercise group.

3.3. Glucose tolerance

Glucose tolerance was assessed using an oral glucose tolerance test (OGTT) to evaluate systemic glucose regulation. Two-way repeated-measures ANOVA revealed a significant interaction between time and treatment in the glucose response curves (F(12, 112) = 16.58, $p < 0.0001$), along with significant main effects of time (F(4, 112) = 374.8, $p < 0.0001$) and treatment (F(3, 28) = 6.49, $p < 0.0001$) (Fig. 4A). No significant differences among groups were observed at baseline (F(3, 28) = 0.76, p = 0.527). At 15 minutes, a significant group effect was detected (F(3, 28) = 21.10, $p < 0.0001$), with both the OTS and OTS-K6 groups exhibiting significantly higher glucose levels than the Sedentary and Exercise groups. However, glucose levels in the OTS-K6 group were significantly lower than those in the OTS group. From 30 to 120 minutes, significant group differences were observed at 30 min (F(3, 28) = 6.41, p = 0.002), 60 min (F(3, 28) = 6.44, p = 0.002), and 120 min (F(3, 28) = 6.34, p = 0.002). At these time points, the OTS group consistently showed elevated glucose levels compared with the other groups, whereas the OTS-K6 group did not differ significantly from the Sedentary or Exercise groups. Analysis of the area under the curve (AUC) revealed a significant group effect (F(3, 28) = 13.91, $p < 0.0001$) (Fig. 4B). The OTS group showed significantly higher AUC values than the Sedentary, Exercise, and OTS-K6 groups, while no significant differences were observed among the latter three groups.

3.4. Peripheral fatigue-related biochemical variables

Peripheral fatigue-associated biochemical parameters were evaluated *via* an acute swimming test following 8 weeks of intervention (Fig. 5A–E).

Blood glucose levels differed significantly among groups both pre- and post-exercise (F(3, 28) = 3.63–4.68, all $p < 0.05$). Before exercise, the OTS group exhibited significantly lower

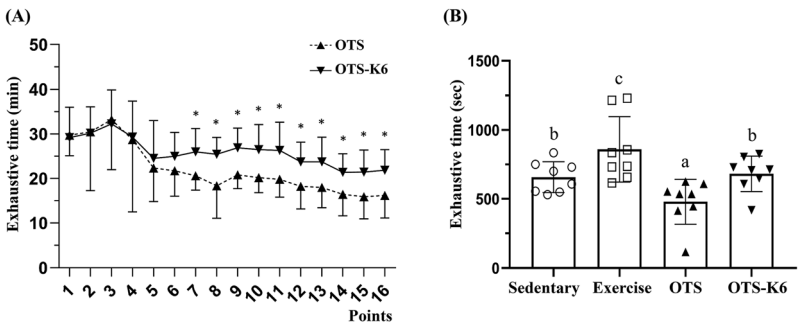

Fig. 3 Effects of probiotic supplementation and OTS induction on endurance performance. Data are presented as mean ± SD (n = 8). (A) Time to exhaustion on the treadmill test. Data were analyzed using two-way repeated-measures ANOVA with time and group as factors. Significant main effects of time and group, as well as a significant time × group interaction, were observed. (B) Time to exhaustion in the forced swimming test. Data were analyzed using one-way ANOVA followed by Tukey's multiple comparison test. Columns subscripted with different letters (a, b, and c) indicate significant differences between the groups ($p < 0.05$); * indicates a significant difference between the groups.

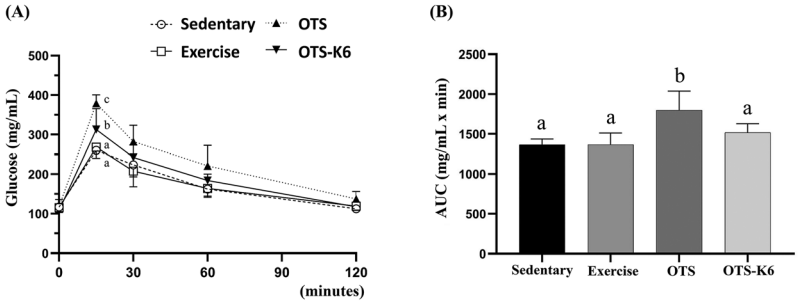

Fig. 4 Effects of probiotic supplementation and OTS induction on glucose tolerance. Data are presented as mean ± standard deviation (n = 8). (A) Oral glucose tolerance test (OGTT) curve. Data were analyzed using two-way repeated-measures ANOVA with time and group as factors. Significant main effects of time and group, as well as a significant time × group interaction, were observed. (B) Area under the OGTT curve. Data were analyzed using one-way ANOVA followed by Tukey's multiple comparison test. Columns subscripted with different letters (a, b, and c) indicate significant differences between the groups (p < 0.05).

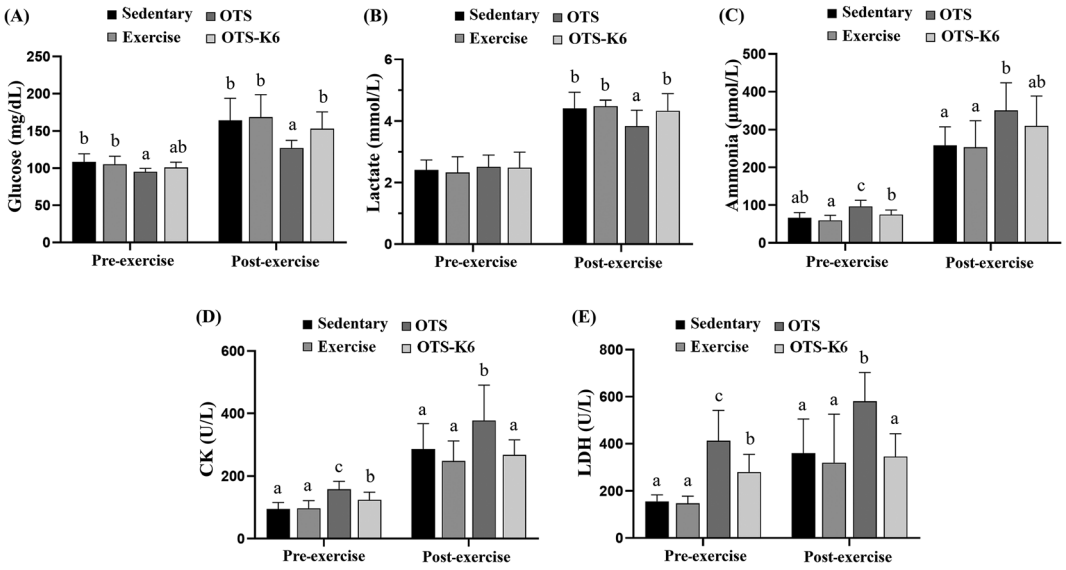

Fig. 5 The effects of probiotic supplementation and OTS induction on peripheral fatigue-related biochemical markers. OTS treadmill training and probiotic supplementation were implemented for 8 weeks, and biochemical markers related to peripheral fatigue were measured during an acute swimming exercise test. (A) Glucose, (B) lactate, (C) ammonia, (D) creatine kinase (CK), and (E) lactate dehydrogenase were assessed pre- and post-exercise. Data are presented as the mean ± SD (n = 8) and analyzed using one-way ANOVA followed by Tukey's multiple comparison test. Columns subscripted with different letters (a, b, c, and d) indicate significant differences between the groups (p < 0.05).

glucose levels than the Sedentary and Exercise groups, but not the OTS-K6 group. Post-exercise, glucose levels remained significantly reduced in the OTS group compared to all other groups, while no differences were observed among the OTS-K6, Sedentary, and Exercise groups. Blood lactate concentrations showed significant group differences only after exercise (F(3, 28) = 3.15, p = 0.041), with the OTS group displaying significantly lower lactate levels than the other three groups. Blood ammonia levels varied significantly both pre- and post-exercise (F(3, 28) = 3.70–10.53, all p < 0.05). Before exercise, the OTS group had higher ammonia levels than all other groups. The OTS-K6 group also showed elevated ammonia compared to the Exercise group but did not differ from the Sedentary group. Post-exercise, ammonia levels in the OTS group remained significantly higher than those in the Sedentary and Exercise groups, with no significant difference from the OTS-K6 group. Muscle injury-related biomarkers—CK and LDH—differed significantly across groups pre- and post-exercise (F(3, 28) = 4.05–20.34, all p < 0.05). Before exercise, both CK and LDH levels were significantly elevated in the OTS group relative to all others, with the OTS-K6 group also showing higher levels than the Sedentary and Exercise groups. After exercise, the OTS group maintained significantly higher CK and LDH concentrations compared to the other groups, while OTS-K6 values were comparable to those of the Sedentary and Exercise groups with no significance.

3.5. The glycogen content

Glycogen levels in the liver and gastrocnemius muscle were assessed using the glycogen–iodine colorimetric method. Significant group differences were found for both hepatic ($F(3, 28) = 7.45$, $p < 0.0001$) and muscle glycogen ($F(3, 28) = 8.08$, $p = 0.001$) (Fig. 6A and B).

Hepatic glycogen was significantly lower in the OTS group compared to the Sedentary and Exercise groups, but not significantly different from the OTS-K6 group. The OTS-K6 group had lower hepatic glycogen than the Exercise group, but did not differ from the Sedentary group. For muscle glycogen, the OTS group exhibited significantly lower levels than all other groups. In contrast, no significant differences were observed among the OTS-K6, Sedentary, and Exercise groups.

3.6. Hematological profiles

One-way ANOVA revealed significant group differences in neutrophils (Neu), lymphocytes (Lym), monocytes (Mono), platelets (PLT), neutrophil-to-lymphocyte ratio (NLR), and platelet-to-lymphocyte ratio (PLR) ($F(3, 28) = 3.29–22.87$, all $p < 0.05$) (Table 2).

Neutrophil counts were significantly elevated in both the OTS and OTS-K6 groups compared with the Sedentary and Exercise groups, with the OTS-K6 group showing significantly lower counts than the OTS group. Lymphocyte counts were significantly reduced in the OTS group relative to all other groups, while no differences were observed among the OTS-K6, Sedentary, and Exercise groups. Monocyte counts were significantly lower in both the OTS and OTS-K6 groups compared to the Sedentary and Exercise groups, with no difference between the two OTS groups. Platelet counts and PLR were significantly elevated in the OTS group *versus* all other groups, while the OTS-K6, Sedentary, and Exercise groups did not differ. For NLR, the OTS group showed significantly higher values than the other three groups. The OTS-K6 group also had a significantly higher NLR than the Sedentary group but did not differ from the Exercise group.

3.7. Anxiety-like behavioral assessments

Anxiety-like behaviors were evaluated using the open field test (OFT) and elevated plus maze (EPM), with group differences observed in several key parameters. In the OFT, significant group effects were found for both the percentage of time spent in the center area ($F(3, 28) = 14.83$, $p < 0.0001$) and the number of fecal boli ($F(3, 28) = 10.08$, $p < 0.0001$) (Fig. 7B and D). The

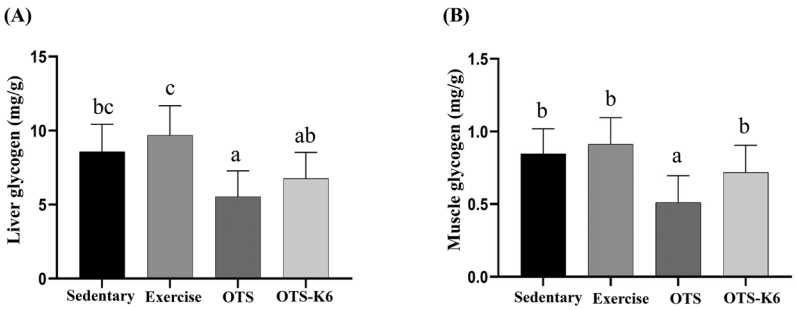

Fig. 6 Effects of probiotic supplementation and OTS implementation on glycogen content. (A) Liver glycogen and (B) muscle glycogen. Data are presented as the mean ± SD ($n = 8$) and analyzed using one-way ANOVA followed by Tukey's multiple comparison test. Columns subscripted with different letters (a, b, and c) indicate significant differences between the groups ($p < 0.05$).

Table 2 Effects of consecutive exhaustive exercise and probiotic interventions on complete blood count analysis

Parameter	Sedentary	Exercise	OTS	OTS-K6
WBC (10^3 μL^{-1})	4.2 ± 2.1	4.6 ± 1.8	3.9 ± 2.0	3.5 ± 1.2
Neu (%)	13.9 ± 2.5^{a}	15.1 ± 2.3^{a}	21.6 ± 1.7^{c}	17.4 ± 1.2^{b}
Lym (%)	84.9 ± 3.1^{c}	81.1 ± 5.3^{b}	77.5 ± 1.9^{a}	81.8 ± 1.1^{bc}
Mono (%)	1.1 ± 1.2^{a}	3.3 ± 5.5^{a}	0.6 ± 0.4^{b}	0.7 ± 0.4^{b}
Eosi (%)	0.04 ± 0.05	0.25 ± 0.52	0.24 ± 0.59	0.04 ± 0.07
Baso (%)	0.01 ± 0.03	0.22 ± 0.48	0.03 ± 0.07	0.01 ± 0.01
RBC (million per μL)	8.8 ± 1.1	8.2 ± 1.4	8.2 ± 1.2	8.6 ± 0.9
Hb (g dL^{-1})	12.9 ± 1.7	12.5 ± 1.3	12.4 ± 1.3	12.6 ± 1.3
Platelet (10^3 μL^{-1})	1187 ± 212^{a}	1146 ± 233^{a}	1461 ± 283^{b}	1119 ± 247^{a}
PLR	0.38 ± 0.11^{a}	0.32 ± 0.06^{a}	0.55 ± 0.16^{b}	0.41 ± 0.07^{a}
NLR	0.17 ± 0.04^{a}	0.18 ± 0.03^{ab}	0.28 ± 0.03^{c}	0.21 ± 0.02^{b}

Data are presented as the mean ± SD ($n = 8$) and analyzed using one-way ANOVA followed by Tukey's multiple comparison test. Values in the same row with different superscript letters (a, b, and c) differ significantly; $p < 0.05$, by using one-way ANOVA. WBC, white blood cell count; Neu, neutrophil count; Lym, lymphocyte count; Mono, monocyte count; Eosi, eosinophil count; Baso, basophil count; RBC, red blood count; Hb, hemoglobin level; PLR, platelet/lymphocyte ratio; NLR, neutrophil/lymphocyte ratio.

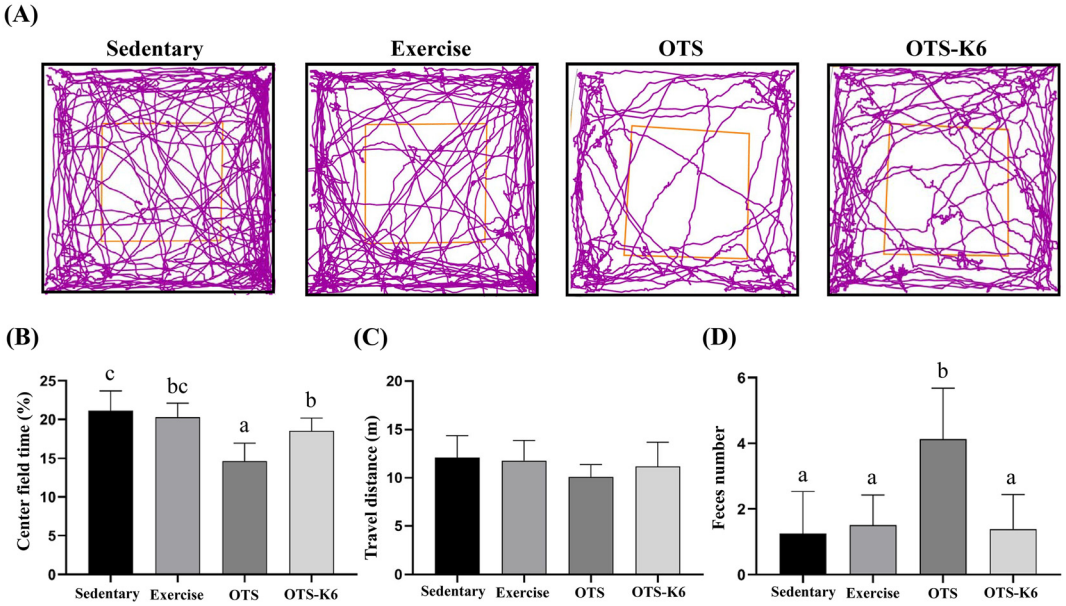

Fig. 7 Effects of probiotic supplementation and OTS implementation on behaviors from the open field test. (A) Representative trajectory diagrams. (B) Percentage of time spent in the center area. (C) Total travel distance. (D) Number of fecal boli produced during the test. These parameters were analyzed to assess the anxiety-like behavior. Data are presented as the mean ± SD (n = 8) and analyzed using one-way ANOVA followed by Tukey's multiple comparison test. Columns subscripted with different letters (a, b, and c) indicate significant differences between the groups ($p < 0.05$).

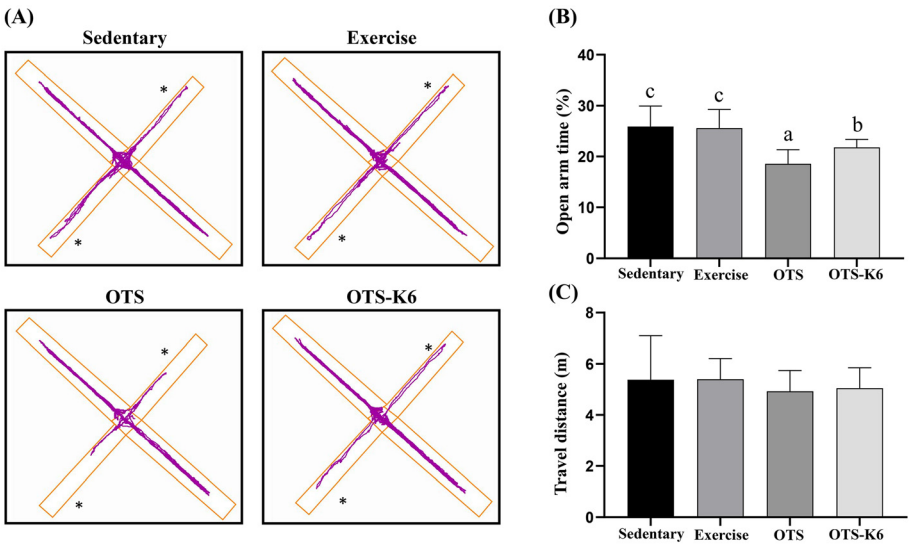

Fig. 8 Effects of probiotic supplementation and OTS implementation on behaviors from the elevated plus maze. (A) Representative trajectory diagrams. (B) Percentage of time spent in the open arms. (C) Total travel distance. These parameters were analyzed to assess the anxiety-like behavior. The arms marked with stars were designated as the open arms. Data are presented as the mean ± SD (n = 8) and analyzed using one-way ANOVA followed by Tukey's multiple comparison test. Columns subscripted with different letters (a, b, and c) indicate significant differences between the groups ($p < 0.05$).

OTS group spent significantly less time in the center area and produced more fecal boli compared to the Sedentary, Exercise, and OTS-K6 groups. The OTS-K6 group also spent less time in the center compared to the Sedentary group but did not differ from the Exercise group. In the EPM, a significant difference was observed in the percentage of time spent in the open arms ($F(3, 28) = 9.63$, $p < 0.0001$) (Fig. 8B). The OTS group spent significantly less time in the open arms than all other groups. The OTS-K6 group showed a significant increase compared to the OTS group, although still lower than the Sedentary and Exercise groups. No significant group differences were observed in total travel distance in either the OFT or EPM (Fig. 7C and 8C), indicating that overall locomotor activity was not affected by the treatment.

3.8. Intestinal tight junction gene expression

The expression of intestinal tight junction-related genes was assessed in cecal tissue to examine alterations in barrier-associated gene expression. Significant group differences were observed for *OCLN*, *TJP1*, and *TJP2* expressions ($F(3, 28)$ = 8.15–16.03, $p < 0.0001$), whereas no significant difference was detected for *CLDN1* ($F(3, 28) = 1.05$, $p = 0.384$) (Fig. 9). The OTS group exhibited significantly reduced expression of *OCLN*, *TJP1*, and *TJP2* compared with all other groups, suggesting alterations in tight junction-related gene expression under overtraining conditions. K6 supplementation (OTS-K6) was associated with significantly higher *TJP1* and *TJP2* expression relative to the OTS group, although these levels remained lower than those observed in the Sedentary group. *OCLN* expression in the OTS-K6 group was comparable to that of the Sedentary and Exercise groups. Taken together, these findings indicate that OTS is associated with alterations in intestinal tight junction-related gene expression at the transcriptional level, and that K6 supplementation may be associated with partial normalization of these gene expression patterns.

3.9. Inflammatory gene expression in the intestine, muscle, and liver

Quantitative PCR analysis revealed significant group differences in *TNFA*, *IL1B*, and *IL6* expression across intestinal, muscle, and liver tissues following OTS induction and probiotic intervention (Fig. 10). For *TNFA*, the expression was significantly elevated in the intestine, liver, and muscle of the OTS group compared to the Sedentary and Exercise groups ($F(4, 25)$ = 4.23–7.16, all $p < 0.05$). K6 supplementation (OTS-K6) significantly attenuated this OTS-induced increase in all three tissues. *IL1B* expression was significantly affected only in the intestine ($F(4, 25) = 5.96$, $p = 0.003$), where the OTS group showed a marked increase compared to the Sedentary and Exercise controls. This elevation was significantly reduced in the OTS-K6 group. No significant differences were observed in *IL1B* expression in the muscle or liver. For *IL6*, significant upregulation was detected in all three tissues under OTS conditions ($F(4, 25)$ = 5.13–9.36, all $p < 0.05$). Probiotic supplementation significantly reduced *IL6* expression in the muscle but not in the intestine or liver, where the expression remained elevated despite intervention.

3.10. Gut microbiota composition and diversity

Gut microbial community composition was characterized using full-length 16S rRNA gene sequencing. Alpha diversity analysis revealed no significant differences among the experimental groups in either the number of observed species or the Shannon diversity index (Fig. 11A and B), indicating that microbial richness and overall diversity were comparable across groups.

In contrast, beta diversity analysis based on unweighted UniFrac distances demonstrated significant differences in the microbial community structure among the experimental groups. Specifically, the Sedentary group exhibited significantly higher beta diversity compared with both the OTS and OTS-K6 groups ($p < 0.05$), while the OTS-K6 group displayed significantly higher beta diversity than the OTS group, suggesting partial restoration of the microbial community structure following probiotic supplementation.

Principal coordinate analysis (PCoA) further illustrated distinct clustering of microbial communities among the four experimental groups. The first two principal coordinates explained 41.52% and 31.96% of the total variance, respect-

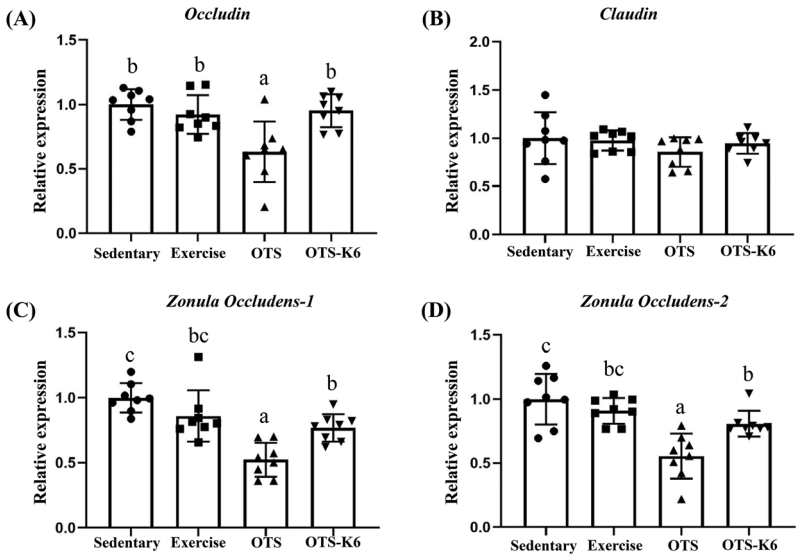

Fig. 9 Effects of probiotic supplementation and OTS implementation on intestinal tight junction. The expression levels of (A) occludin (*OCLN*), (B) claudin-1 (*CLDN1*), (C) zonula occludens-1 (*TJP1*), and (D) zonula occludens-2 (*TJP2*) genes were analyzed in the proximal large intestine (cecal) tissue. Data are presented as the mean ± SD (n = 8) and analyzed using one-way ANOVA followed by Tukey's multiple comparison test. Columns subscripted with different letters (a, b, and c) indicate significant differences between the groups ($p < 0.05$).

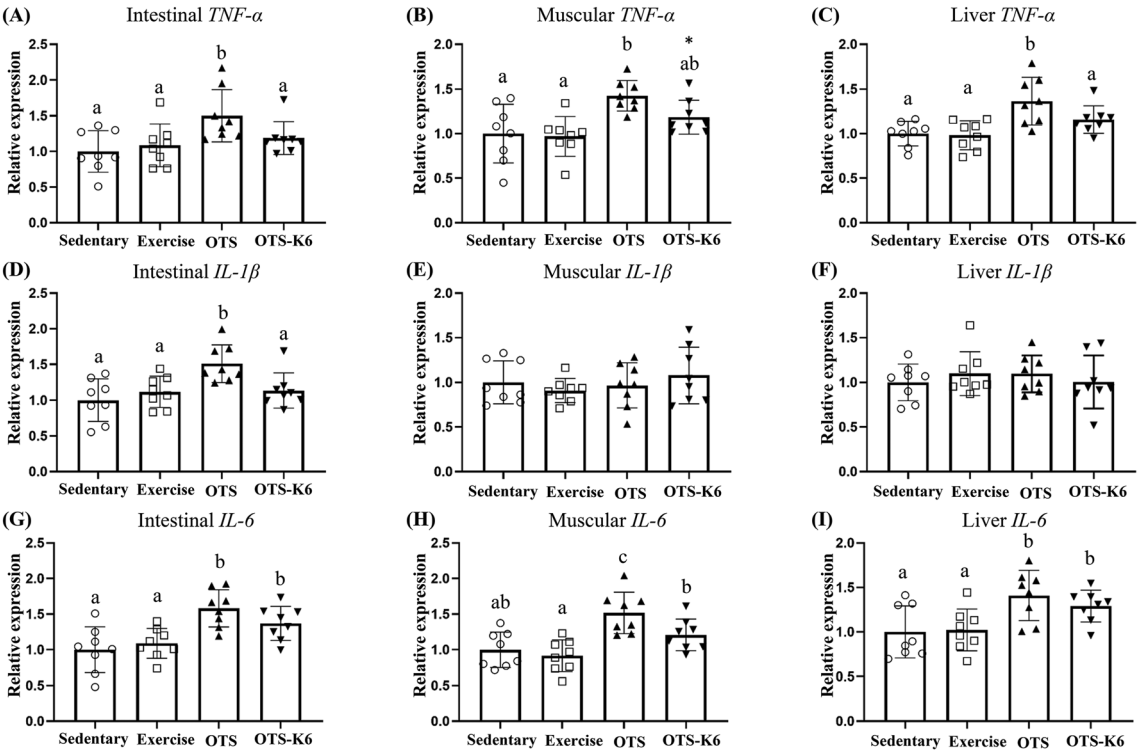

Fig. 10 Effects of probiotic supplementation and OTS implementation on inflammation levels. The expression levels of TNF-α, IL-1β, and IL-6 were measured on different tissues, including intestinal (A, D, and G), muscle (B, E, and H), and liver (C, F, and I). Data are presented as the mean ± SD (n = 8) and analyzed using one-way ANOVA followed by Tukey's multiple comparison test. Columns subscripted with different letters (a, b, and c) indicate significant differences between the groups ($p < 0.05$).

ively (p = 0.001), indicating clear separation of the microbial community composition among the treatment conditions. Similarly, partial least squares discriminant analysis (PLS-DA) revealed distinct clustering patterns across the groups, with the first two components explaining 7.34% and 8.9% of the total variance, respectively (Fig. 12A and B), further supporting the presence of group-specific microbial profiles.

To statistically evaluate differences in the overall microbial community composition between the groups, permutational multivariate analysis of variance (PERMANOVA) was performed based on the beta diversity distance matrix. Significant differences in the microbial community structure were observed between the Sedentary and OTS groups (F = 2.3323, R^2 = 0.2257, p = 0.028), Exercise and OTS groups (F = 2.2969, R^2 = 0.2231, p = 0.016), Exercise and OTS-K6 groups (F = 1.9863, R^2 = 0.1989, p = 0.026), and OTS and OTS-K6 groups (F = 1.8087, R^2 = 0.1844, p = 0.045). In contrast, comparisons between the Sedentary and Exercise groups (F = 1.6049, R^2 = 0.1671, p = 0.110) and between the Sedentary and OTS-K6 groups (F = 1.6011, R^2 = 0.1668, p = 0.111) did not reach statistical significance. These findings indicate that excessive training stress was associated with significant alterations in the microbial community composition, whereas probiotic supplementation was associated with partial shifts in the microbial structure relative to the OTS condition.

At the taxonomic level, heatmap and bar plot analyses identified several differentially abundant taxa across the experimental groups using LEfSe analysis (LDA score > 4.0) (Fig. 13A and C). The OTS group exhibited increased relative abundance of *Heminiphilus* and *Heminiphilus faecis* compared with the other groups, whereas these taxa were reduced following K6 supplementation in the OTS-K6 group. In contrast, *Paramuribaculum* and *P. intestinale* were enriched in the Exercise group but decreased in the OTS group; K6 supplementation was associated with partial restoration of *P. intestinale* abundance in the OTS-K6 group. Conversely, *Duncaniella dubosii* abundance was reduced in the Exercise group but increased in both the OTS and OTS-K6 groups.

At the species level, the dominant taxa across all groups included *Muribaculum gordoncarteri*, *Duncaniella freteri*, *Duncaniella muris*, and *Fusimonas intestini*, collectively accounting for more than 50% of the total microbial abundance (Fig. 13B). Overall, these results indicate that excessive training stress was associated with shifts in the gut microbial community composition, whereas probiotic supplementation was associated with partial modulation of the microbial community structure under OTS conditions.

4. Discussion

Previous studies on overtraining syndrome (OTS) have primarily relied on retrospective cohort studies and systematic reviews to propose hypotheses regarding its pathophysiology

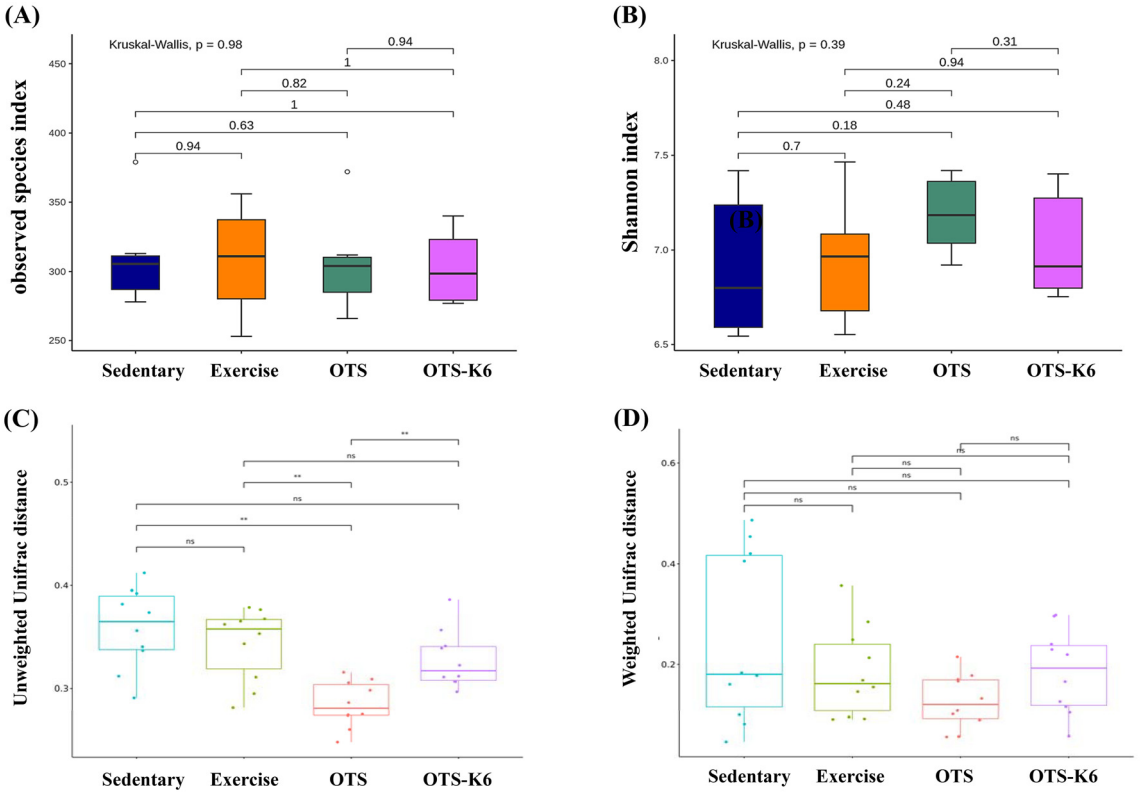

Fig. 11 Alpha and beta diversity plots illustrating differences in the gut microbiota structure following probiotic supplementation and OTS induction. The box plot corresponding to the alpha diversity including (A) observed species and (B) the Shannon index. (C) Unweighted UniFrac and (D) weighted UniFrac β-diversity plots of the four groups, analyzed using the ANOSIM algorithm to represent differences in community structure between the groups. The horizontal lines inside the boxes indicate the median; the lower and upper boundaries of each box represent the 25th and 75th percentiles, respectively. The whiskers indicate the minimum and maximum data points.

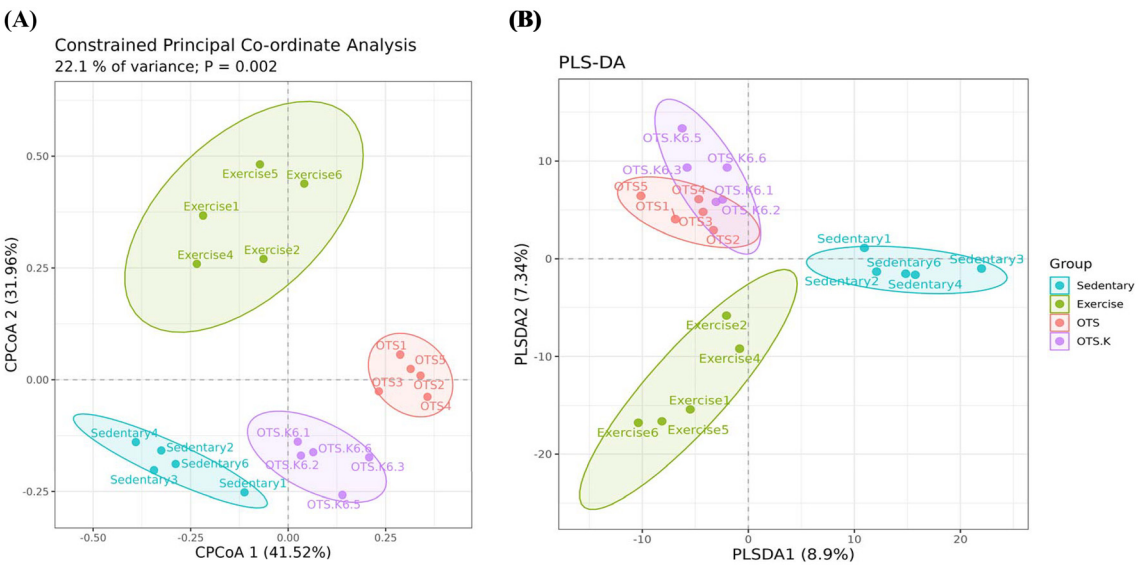

Fig. 12 Constrained PCoA and PLS-DA illustrating the structure of the microbiota composition. (A) Constrained principal coordinate analysis (PCoA) based on Bray–Curtis distances, constrained by the treatment groups. (B) Partial least squares discriminant analysis (PLS-DA) highlighting discriminative microbial features among the treatment groups. Each point represents an individual sample, and the colors indicate the different experimental groups. The percentage shown on each axis represents the proportion of total variance explained by the corresponding component. The center of each ellipse represents the group centroid with its corresponding confidence interval. Group differences in overall microbial community composition were further evaluated using permutational multivariate analysis of variance (PERMANOVA).

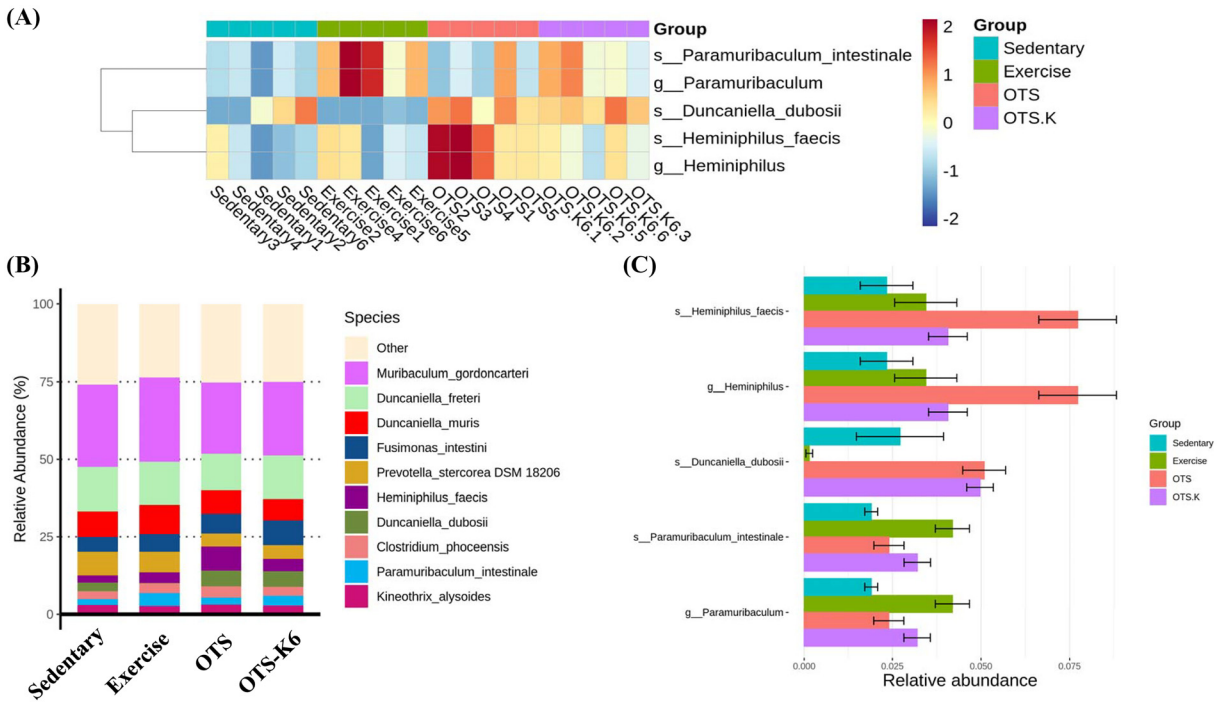

Fig. 13 Relative abundance of the gut microbiota based on LEfSe analysis. (A) Heatmap illustrating differentially abundant taxa identified by LEfSe analysis at the species and genus levels. (B) Stacked column plot showing the relative abundance of gut bacterial taxa at the species level across the different groups. (C) Bar plot presenting the relative abundance of selected bacterial taxa.

and prevention strategies. From a nutritional perspective, maintaining adequate energy availability and balanced nutrient intake has been suggested as an important strategy for reducing OTS risk.[16] However, evidence regarding targeted nutritional interventions capable of mitigating the multisystem physiological disturbances associated with OTS remains limited. In the present study, we investigated a microbiota-targeted nutritional intervention using the probiotic strain *L. kefiranofaciens* K6 in a treadmill-induced OTS-like animal model designed to reproduce key physiological features associated with excessive training stress. The experimental paradigm reproduced several features commonly associated with excessive training stress, including reductions in endurance performance despite continued training stimuli, metabolic dysregulation reflected by impaired glucose tolerance and altered glycogen metabolism, systemic inflammatory and immune alterations, and anxiety-like behavioral disturbances. Within this model, K6 supplementation was associated with attenuation of several OTS-related physiological alterations, including reduced performance decline, peripheral fatigue, inflammatory responses, intestinal barrier-related changes, and behavioral disturbances. These findings may indicate that microbiota-targeted nutritional strategies could represent a complementary approach associated with physiological resilience during excessive training loads, although causal relationships cannot be inferred from the present study. In clinical settings, OTS is typically distinguished from functional overreaching by persistent performance decline and neuroendocrine dysregulation. Because comprehensive endocrine and recovery assessments were not performed here, the present paradigm should be interpreted as an OTS-like model reproducing key physiological features of excessive training stress rather than a definitive clinical representation of OTS.

OTS has been widely associated with appetite loss and reductions in body weight under excessive training stress. Insufficient recovery during prolonged training may trigger physiological disturbances including hormonal imbalance and neuroinflammatory responses mediated by cytokines such as IL-1β and TNF-α, which may impair appetite regulation and energy balance.[6,17] Experimental OTS models have similarly reported reductions in food intake, body weight, and skeletal muscle mass accompanied by inflammatory signaling and catabolic pathways in muscle tissue.[18–20] Consistent with these observations, OTS induction in the present study was associated with reduced body weight, food intake, and body composition-related parameters, including skeletal muscle and epididymal fat pad weights. K6 supplementation was associated with partial attenuation of these alterations, suggesting that probiotic supplementation may be associated with partial preservation of energy balance and body composition under excessive training stress.

Excessive training loads without adequate recovery have also been shown to induce physiological disturbances characteristic of OTS. Previous studies using exhaustive swimming or progressive treadmill protocols reported reductions in endurance capacity accompanied by inflammatory and metabolic

disturbances.[18,21] At the cellular level, excessive training has been associated with activation of muscle stress pathways and impaired insulin signaling in skeletal muscle,[22,23] while human studies have reported impaired mitochondrial function and glucose tolerance following excessive training loads.[24] Consistent with these reports, OTS induction in the present study was associated with reduced endurance performance, increased peripheral fatigue markers, and impaired glucose tolerance. K6 supplementation was associated with partial attenuation of these physiological disturbances, suggesting that probiotic supplementation may be associated with improved physiological responses under excessive training stress.

Prolonged intensive exercise is also associated with inflammatory responses and immune perturbations.[25] Experimental OTS models have reported reductions in lymphocyte counts together with elevations in neutrophils and platelets, reflecting systemic inflammatory stress under excessive training conditions.[15,26] Similar immune alterations have been observed following high-intensity exercise in both animal and human studies, where glucocorticoid responses, reduced glutamine availability, and leukocyte redistribution contribute to transient immunosuppression.[27–30] In line with these findings, OTS induction in the present study was associated with increased neutrophils, platelets, NLR, and PLR alongside reduced lymphocyte counts. K6 supplementation was associated with partial normalization of these immune-related markers, which may reflect an association between probiotic supplementation and modulation of immune parameters during excessive training stress.

Emerging evidence suggests that excessive training may disrupt gut homeostasis and contribute to systemic inflammation. OTS has been associated with increased intestinal permeability and gastrointestinal disturbances resulting from epithelial disruption and microbiota dysbiosis.[6,15,31,32] These alterations may promote inflammatory signaling involving cytokines such as IL-1β, IL-6, and TNF-α, which can influence metabolic and endocrine regulation.[33] IL-6 also functions as an exercise-responsive cytokine involved in metabolic regulation during prolonged exercise.[34] Consistent with these observations, the present model demonstrated altered expression of intestinal tight junction-related genes (*OCLN*, *TJP1* and *TJP2*), impaired glucose tolerance, and elevated inflammatory cytokine expression. K6 supplementation was associated with partial normalization of these alterations, suggesting an association between probiotic supplementation and changes in inflammatory signaling and intestinal barrier-related gene expression under excessive training stress.

Excessive training without adequate recovery has also been linked to adverse psychological outcomes, including anxiety and mood disturbances.[35] Experimental studies have shown that overtraining protocols can induce anxiety-like behaviors in rodents.[15] Increasing attention has therefore been focused on the gut–brain axis, through which the gut microbiota may influence emotional regulation and stress-related behaviors. This concept has led to the development of psychobiotics—probiotics that may influence mental health outcomes.[36] These effects may involve the modulation of neuroactive compounds such as γ-aminobutyric acid and serotonin as well as inflammatory signaling and hypothalamic–pituitary–adrenal axis activity.[37] Behavioral paradigms such as the open field test and elevated plus maze are widely used to assess anxiety-like behaviors in rodents.[38,39] In the present study, OTS induction was associated with anxiety-like behavioral alterations, whereas K6 supplementation was associated with attenuation of these behavioral responses. However, the present data do not provide mechanistic evidence for psychobiotic effects. These behavioral outcomes may reflect complex physiological responses to excessive training stress that could involve interactions between gut microbiota composition, inflammatory signaling, and host regulatory pathways, although such mechanisms were not directly evaluated in the present study.

Alterations in the gut microbiota composition have increasingly been reported in association with excessive training and host physiological responses. In the present study, several microbial taxa differed among the experimental groups, including increased abundance of *Heminiphilus faecis* and *Duncaniella dubosii* and reduced abundance of *Paramuribaculum intestinale* in the OTS group. These observations suggest that excessive training stress may be accompanied by shifts in gut microbial communities. Previous studies have shown that gut microbial communities regulate intestinal epithelial integrity, immune signaling, and systemic metabolic homeostasis through microbial metabolites, particularly short-chain fatty acids (SCFAs).[40–42] These metabolites have been reported to regulate tight junction protein expression, modulate inflammatory pathways, and interact with host metabolic regulatory systems, which have been reported to interact with host metabolic and immune pathways.

The abundance of *H. faecis* was elevated in the OTS group and reduced following K6 supplementation. Although this taxon has been associated with inflammatory responses in some disease models, its functional role in intestinal barrier regulation remains unclear.[43–47] Another taxon altered under OTS conditions was *D. dubosii*, which has been proposed as a conditionally pathogenic bacterium under inflammatory or immune-dysregulated states and may respond to host tryptophan metabolism.[48–51] Conversely, *P. intestinale* abundance was higher in the Exercise and OTS-K6 groups than in the OTS group. Members of the Muribaculaceae family possess metabolic pathways involved in carbohydrate degradation and microbial fermentation and may produce SCFAs such as propionate.[52–54] However, because microbial metabolites were not measured in the present study, these interpretations should be considered hypothesis-generating rather than evidence of microbiota-driven mechanisms.

Overall, OTS induction in this model was associated with alterations in gut microbiota composition, intestinal barrier-related gene expression, systemic inflammatory markers, metabolic dysregulation, and anxiety-like behaviors. Probiotic supplementation with *L. kefiranofaciens* K6 was associated with

partial shifts in microbial community composition. Because microbial metabolites were not directly quantified, the microbiota findings presented here should be interpreted primarily as descriptive observations of microbial community changes rather than evidence of functional microbiota-mediated mechanisms. Future studies integrating microbiome sequencing, metabolomics, and functional analyses will be necessary to clarify the mechanisms underlying microbiota–host interactions in the context of excessive training stress.

The present study has several limitations that should be acknowledged, particularly given the exploratory nature of the integrated physiological and microbiome analyses. First, endocrine markers related to physiological stress responses, such as corticosterone and testosterone, were not measured due to limitations in the available blood sample volume. Assessment of these hormones would provide additional insight into neuroendocrine responses during excessive training and could further support validation of the OTS model. Moreover, although the experimental protocol reproduced several physiological features reported in OTS-like models, the absence of endocrine markers limits the ability to clearly distinguish between overtraining syndrome and severe functional overreaching. Second, probiotic-only control groups (*e.g.*, Sedentary + K6 or Exercise + K6) were not included in the experimental design, making it difficult to determine whether the observed effects of K6 supplementation are specific to excessive training stress or represent more general physiological effects of probiotic administration. Third, skeletal muscle damage was evaluated using circulating biomarkers such as CK and LDH rather than histopathological examination, and intestinal barrier integrity was inferred from tight junction-related gene expression rather than functional measurements such as circulating endotoxin levels or intestinal permeability assays. Finally, microbial metabolites that may mediate microbiota–host interactions, including short-chain fatty acids (SCFAs), were not quantified, and formal *a priori* power analysis was not conducted for sample size determination. The relatively small sample size (n = 8 per group), combined with multiple physiological endpoints, may increase the risk of type I error. In addition, although Tukey's *post hoc* test was applied to control pairwise comparisons following ANOVA, future studies integrating larger multi-omics datasets may benefit from additional multiple testing correction strategies such as false discovery rate (FDR) adjustment. Future studies incorporating larger sample sizes, endocrine assessments, functional gut permeability assays, metabolomic analyses, and microbiota–host correlation approaches will be necessary to clarify the mechanistic relationships between probiotic supplementation, microbial alterations, and host physiological responses under excessive training stress.

5. Conclusion

Overtraining syndrome represents a significant challenge for athletic performance and long-term health when excessive training is performed without adequate recovery and nutritional support. In the present study, a treadmill-induced animal model demonstrated that excessive training stress was associated with physiological maladaptation, anxiety-like behavioral changes, and alterations in gut microbiota composition. Supplementation with *L. kefiranofaciens* K6 was associated with attenuation of several OTS-related alterations, including improvements in physiological, inflammatory, and behavioral responses. These findings suggest that microbiota-targeted nutritional strategies may be associated with improved physiological resilience under excessive training stress in this experimental OTS-like model. However, the present findings remain associative and hypothesis-generating, and mechanistic relationships between probiotic supplementation, gut microbiota alterations, and host physiological responses require further investigation. Appropriate regulation of training load, adequate recovery, targeted nutritional strategies, and psychological interventions remain essential for maintaining physiological and mental health in both athletes and the general population.

Author contributions

Yen-Po Chen contributed to conceptualization, investigation, formal analysis, probiotics isolation, and drafting the original manuscript. Ming-Han Gao contributed to the investigation and data curation. Wen-Chi Wei contributed to project administration and resources. Wen-Ching Huang was responsible for conceptualization, methodology, supervision, validation, visualization, manuscript review and editing, as well as funding acquisition. All authors have read and approved the submitted version of the manuscript.

Conflicts of interest

Yen-Po Chen, Ming-Han Gao, Wen-Chi Wei, and Wen-Ching Huang declare that they have no competing interests.

Ethics approval

All experimental procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the National Research Institute of Chinese Medicine, and the experimental protocol guidelines 113-355-1 were approved by the ethics committee of the IACUC. The study was conducted in compliance with the ARRIVE guidelines.

Data availability

The datasets used and analyzed in this study are available from the corresponding author upon reasonable request.

Acknowledgements

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References

1 V. B. Issurin, New horizons for the methodology and physiology of training periodization, *Sports Med.*, 2010, **40**(3), 189–206, DOI: 10.2165/11319770-000000000-00000.
2 A. Urhausen and W. Kindermann, Diagnosis of overtraining: What tools do we have?, *Sports Med.*, 2002, **32**(2), 95–102.
3 R. Meeusen, M. Duclos, C. Foster, A. Fry, M. Gleeson, D. Nieman, J. Raglin, G. Rietjens, J. Steinacker and A. Urhausen, European College of Sport Science, and American College of Sports Medicine, Prevention, Diagnosis, and Treatment of the Overtraining Syndrome: Joint Consensus Statement of the European College of Sport Science and the American College of Sports Medicine, *Med. Sci. Sports Exercise*, 2013, **45**(1), 186–205, DOI: 10.1249/MSS.0b013e318279a10a.
4 N. F. Matos, R. J. Winsley and C. A. Williams, Prevalence of nonfunctional overreaching/overtraining in young English athletes, *Med. Sci. Sports Exercise*, 2011, **43**(7), 1287–1294, DOI: 10.1249/MSS.0b013e318207f87b.
5 K. M. Myrick, Overtraining and Overreaching Syndrome in Athletes, *Nurse Pract.*, 2015, **11**(10), 1018–1022, DOI: 10.1016/j.nurpra.2015.08.038.
6 J. B. Kreher and J. B. Schwartz, Overtraining syndrome: a practical guide, *Sports Health*, 2012, **4**(2), 128–138, DOI: 10.1177/1941738111434406.
7 E. Rinninella, P. Raoul, M. Cintoni, F. Franceschi, G. A. D. Miggiano, A. Gasbarrini and M. C. Mele, What is the Healthy Gut Microbiota Composition? A Changing Ecosystem across Age, Environment, Diet, and Diseases, *Microorganisms*, 2019, **7**(1), 14, DOI: 10.3390/microorganisms7010014.
8 S. Schippa and M. P. Conte, Dysbiotic events in gut microbiota: impact on human health, *Nutrients*, 2014, **26**(12), 5786–5805.
9 O. O'Sullivan, O. Cronin, S. F. Clarke, E. F. Murphy, M. G. Molloy, F. Shanahan and P. D. Cotter, Exercise and the microbiota, *Gut Microbes*, 2015, **6**(2), 131–136, DOI: 10.1080/19490976.2015.1011875.
10 S. K. Kim, R. B. Guevarra, Y. T. Kim, J. Kwon, H. Kim, J. H. Cho, H. B. Kim and J. H. Lee, Role of Probiotics in Human Gut Microbiome-Associated Diseases, *J. Microbiol. Biotechnol.*, 2019, **29**(9), 1335–1340, DOI: 10.4014/jmb.1906.06064.
11 X. Meng, G. Zhang, H. Cao, D. Yu, X. Fang, W. M. de Vos and H. Wu, Gut dysbacteriosis and intestinal disease: mechanism and treatment, *J. Appl. Microbiol.*, 2020, **129**(4), 787–805, DOI: 10.1111/jam.14661.
12 T. G. Dinan, C. Stanton and J. F. Cryan, Psychobiotics: a novel class of psychotropic, *Biol. Psychiatry*, 2013, **74**(10), 720–726.
13 G. U. Varela-Trinidad, C. Domínguez-Díaz, K. Solórzano-Castanedo, L. Íñiguez-Gutiérrez, T. J. Hernández-Flores and M. Fafutis-Morris, Probiotics: Protecting Our Health from the Gut, *Microorganisms*, 2022, **10**(7), 1428, DOI: 10.3390/microorganisms10071428.
14 C. W. Chen, L. T. Wu, P. H. Chiu, Y. P. Chen and W. C. Huang, The probiotic *Lactobacillus kefiranofaciens*, K6 alleviates exercise and sleep deprivation-induced physiological dysregulation and neuropsychiatric disorders via modulation of inflammation, circadian rhythm, and stress response, *Int. J. Med. Sci.*, 2025, **22**(14), 3722–3736, DOI: 10.7150/ijms.115387.
15 Y. Chung, Y. T. Hsiao and W. C. Huang, Physiological and Psychological Effects of Treadmill Overtraining Implementation, *Biology*, 2021, **10**(6), 515.
16 M. E. la Torre, A. Monda, A. Messina, M. I. de Stefano, V. Monda, F. Moscatelli, F. Tafuri, E. Saraiello, F. Latino, M. Monda, G. Messina, R. Polito and D. Tafuri, The Potential Role of Nutrition in Overtraining Syndrome: A Narrative Review, *Nutrients*, 2023, **15**(23), 4916, DOI: 10.3390/nu15234916.
17 L. L. Smith, Cytokine hypothesis of overtraining: a physiological adaptation to excessive stress?, *Med. Sci. Sports Exercise*, 2000, **32**(2), 317–331, DOI: 10.1097/00005768-200002000-00011.
18 B. C. Pereira, A. L. da Rocha, J. R. Pauli, E. R. Ropelle, C. T. de Souza, D. E. Cintra, M. R. Sant'Ana and A. S. da Silva, Excessive eccentric exercise leads to transitory hypothalamic inflammation, which may contribute to the low body weight gain and food intake in overtrained mice, *Neuroscience*, 2015, **311**, 231–242, DOI: 10.1016/j.neuroscience.2015.10.027.
19 C. V. Coutinho de Oliveira, C. V. Barbosa, N. M. Massa, A. Pereira Rde, S. Félix Gda, S. Aquino Jde, E. M. de Oliveira and A. S. Silva, Carbohydrate supplementation attenuates decrement in performance in overtrained rats, *Appl. Physiol., Nutr., Metab.*, 2016, **41**(1), 76–82, DOI: 10.1139/apnm-2015-0393.
20 R. W. Alves Souza, A. F. Aguiar, I. J. Vechetti-Júnior, W. P. Piedade, G. E. Rocha Campos and M. Dal-Pai-Silva, Resistance training with excessive training load and insufficient recovery alters skeletal muscle mass-related protein expression, *J. Strength Cond. Res.*, 2014, **28**(8), 2338–2345, DOI: 10.1519/jsc.0000000000000421.
21 X. Yuan, S. Xu, H. Huang, J. Liang, Y. Wu, C. Li, H. Yuan, X. Zhao, X. Lai and S. Hou, Influence of excessive exercise on immunity, metabolism, and gut microbial diversity in an overtraining mice model, *Scand. J. Med. Sci. Sports*, 2018, **28**(5), 1541–1551, DOI: 10.1111/sms.13060.
22 B. C. Pereira, A. L. da Rocha, A. P. Pinto, J. R. Pauli, C. T. de Souza, D. E. Cintra, E. R. Ropelle, E. C. de Freitas,

A. M. Zagatto and A. S. da Silva, Excessive eccentric exercise-induced overtraining model leads to endoplasmic reticulum stress in mice skeletal muscles, *Life Sci.*, 2016, **145**, 144–151.

23 B. C. Pereira, A. L. da Rocha, A. P. Pinto, J. R. Pauli, L. P. de Moura, R. A. Mekary, E. C. de Freitas and A. S. da Silva, Excessive training impairs the insulin signal transduction in mice skeletal muscles, *J. Endocrinol.*, 2016, **230**(1), 93–104.

24 M. Flockhart, L. C. Nilsson, S. Tais, B. Ekblom, W. Apró and F. J. Larsen, Excessive exercise training causes mitochondrial functional impairment and decreases glucose tolerance in healthy volunteers, *Cell Metab.*, 2021, **33**(5), 957–970.

25 É. Cerqueira, D. A. Marinho, H. P. Neiva and O. Lourenço, Inflammatory Effects of High and Moderate Intensity Exercise-A Systematic Review, *Front. Physiol.*, 2020, **10**, 1550, DOI: 10.3389/fphys.2019.01550.

26 Z. Gholamnezhad, M. H. Boskabady, M. Hosseini and A. Aghaei, Effect of different loads of exercise and Nigella sativa L. seed extract on serologic and hematologic parameters in rat, *Indian J. Exp. Biol.*, 2019, **57**, 21–29.

27 M. Gleeson, Biochemical and immunological markers of over-training, *J. Sports Sci. Med.*, 2002, **1**(2), 31–41.

28 U. Sri and P. Majumdar, Effect of a weight lifting training session on leukocyte count and myokine (Interleukin-6) levels, *J. Phys. Educ. Sport.*, 2019, **19**, 2435–2440, DOI: 10.7752/jpes.2019.04369.

29 M. S. El-Sayed, C. Sale, P. G. Jones and M. Chester, Blood hemostasis in exercise and training, *Med. Sci. Sports Exercise*, 2000, **32**(5), 918–925, DOI: 10.1097/00005768-200005000-00007.

30 P. Wahl, S. Mathes, W. Bloch and P. Zimmer, Acute Impact of Recovery on the Restoration of Cellular Immunological Homeostasis, *Int. J. Sports Med.*, 2020, **41**(1), 12–20, DOI: 10.1055/a-1015-0453.

31 O. Fiala, M. Hanzlova, L. Borska, Z. Fiala and D. Holmannova, Beyond physical exhaustion: Understanding overtraining syndrome through the lens of molecular mechanisms and clinical manifestation, *Sports Med. Health Sci.*, 2025, **7**(4), 237–248, DOI: 10.1016/j.smhs.2025.01.006.

32 E. Ribichini, G. Scalese, A. Cesarini, C. Mocci, N. Pallotta, C. Severi and E. S. Corazziari, Exercise-Induced Gastrointestinal Symptoms in Endurance Sports: A Review of Pathophysiology, Symptoms, and Nutritional Management, *Dietetics*, 2023, **2**, 289–307, DOI: 10.3390/dietetics2030021.

33 A. L. da Rocha, A. P. Pinto, E. B. Kohama, J. R. Pauli, L. P. de Moura, D. E. Cintra, E. R. Ropelle and A. S. R. da Silva, The proinflammatory effects of chronic excessive exercise, *Cytokine*, 2019, **119**, 57–61, DOI: 10.1016/j.cyto.2019.02.016.

34 B. K. Pedersen and C. P. Fischer, Physiological roles of muscle-derived interleukin-6 in response to exercise, *Curr. Opin. Clin. Nutr. Metab. Care*, 2007, **10**, 265–271.

35 M. A. Peluso and L. H. Guerra de Andrade, Physical activity and mental health: the association between exercise and mood, *Clinics*, 2005, **60**(1), 61–70, DOI: 10.1590/s1807-59322005000100012.

36 L. Ķimse, A. Reinis, L. Miķelsone-Jansone, S. Gintere and A. Krūmiņa, A Narrative Review of Psychobiotics: Probiotics That Influence the Gut-Brain Axis, *Medicina*, 2024, **60**(4), 601, DOI: 10.3390/medicina60040601.

37 K. Ross, Psychobiotics: Are they the future intervention for managing depression and anxiety? A literature review, *Explore*, 2023, **19**(5), 669–680, DOI: 10.1016/j.explore.2023.02.007.

38 E. Zimcikova, J. Simko, I. Karesova, J. Kremlacek and J. Malakova, Behavioral effects of antiepileptic drugs in rats: Are the effects on mood and behavior detectable in open-field test?, *Seizure*, 2017, **52**, 35–40, DOI: 10.1016/j.seizure.2017.09.015.

39 D. Sawada, Y. Kuwano, H. Tanaka, S. Hara, Y. Uchiyama, T. Sugawara, S. Fujiwara, K. Rokutan and K. Nishida, Daily intake of *Lactobacillus gasseri* CP2305 relieves fatigue and stress-related symptoms in male university Ekiden runners: A double-blind, randomized, and placebo-controlled clinical trial, *J. Funct. Foods*, 2019, **57**, 465–476.

40 A. Koh, F. De Vadder, P. Kovatcheva-Datchary and F. Bäckhed, From Dietary Fiber to Host Physiology: Short-Chain Fatty Acids as Key Bacterial Metabolites, *Cell*, 2016, **165**(6), 1332–1345, DOI: 10.1016/j.cell.2016.05.041.

41 M. G. Rooks and W. S. Garrett, Gut microbiota, metabolites and host immunity, *Nat. Rev. Immunol.*, 2016, **16**(6), 341–352, DOI: 10.1038/nri.2016.42.

42 B. Dalile, L. Van Oudenhove, B. Vervliet and K. Verbeke, The role of short-chain fatty acids in microbiota-gut-brain communication, *Nat. Rev. Gastroenterol. Hepatol.*, 2019, **16**(8), 461–478, DOI: 10.1038/s41575-019-0157-3.

43 J. K. Park, D. H. Chang, M. S. Rhee, H. Jeong, J. Song, B. J. Ku, S. B. Kim, M. Lee and B. C. Kim, Heminiphilus faecis gen. nov., sp. nov., a member of the family Muribaculaceae, isolated from mouse faeces and emended description of the genus Muribaculum, *Antonie van Leeuwenhoek*, 2021, **114**(3), 275–286, DOI: 10.1007/s10482-021-01521-x.

44 H. Wang, Y. Xi, F. Gu, L. Peng and J. Li, Protective Effects of a Polyherbal Mixture on Intestinal Injury via the NF-κB Signaling Pathway and Gut Microbiota Modulation in Hyperuricemic Mice, *Foods*, 2025, **14**(7), 1118, DOI: 10.3390/foods14071118.

45 Y. Zhu, B. Chen, X. Zhang, M. T. Akbar, T. Wu, Y. Zhang, L. Zhi and Q. Shen, Exploration of the Muribaculaceae Family in the Gut Microbiota: Diversity, Metabolism, and Function, *Nutrients*, 2024, **16**(16), 2660, DOI: 10.3390/nu16162660.

46 X. Yang, Z. Wang, J. Niu, R. Zhai, X. Xue, G. Wu, Y. Fang, G. Meng, H. Yuan, L. Zhao and C. Zhang, Pathobionts from chemically disrupted gut microbiota induce insulin-dependent diabetes in mice, *Microbiome*, 2023, **11**(1), 62, DOI: 10.1186/s40168-023-01507-z.

47 J. Zhang, D. Zhang, Y. Xu, J. Zhang, R. Liu, Y. Gao, Y. Shi, P. Cai, Z. Zhong, B. He, X. Li, H. Zhou, M. Chen and Y. X. Li, Large-scale biosynthetic analysis of human microbiomes reveals diverse protective ribosomal peptides, *Nat. Commun.*, 2025, **16**(1), 3054, DOI: 10.1038/s41467-025-58280-w.

48 J. Chen, Q. Pan, L. Lu, X. Huang, S. Wang, X. Liu, J. Lun, X. Xu, H. Su, F. Guo, L. Yang, L. You, H. Xiao, W. Luo, H. F. Liu and Q. Pan, Atg5 deficiency in basophils improves metabolism in lupus mice by regulating gut microbiota dysbiosis, *Cell Commun. Signaling*, 2025, **23**(1), 40, DOI: 10.1186/s12964-025-02041-1.

49 S. C. Forster, S. Clare, B. S. Beresford-Jones, K. Harcourt, G. Notley, M. D. Stares, N. Kumar, A. T. Soderholm, A. Adoum, H. Wong, B. Morón, C. Brandt, G. Dougan, D. J. Adams, K. J. Maloy, V. A. Pedicord and T. D. Lawley, Identification of gut microbial species linked with disease variability in a widely used mouse model of colitis, *Nat. Microbiol.*, 2022, **7**(4), 590–599, DOI: 10.1038/s41564-022-01094-z.

50 H. C. Kim, H. J. Kim, J. La, W. H. Park, S. H. Park, B. H. Kang, Y. Kim and H. K. Lee, Gut microbiota dysbiosis induced by brain tumors modulates the efficacy of immunotherapy, *Cell Rep.*, 2025, **44**(7), 115825, DOI: 10.1016/j.celrep.2025.115825.

51 M. Yamashita, Potential Role of Neuroactive Tryptophan Metabolites in Central Fatigue: Establishment of the Fatigue Circuit, *Int. J. Tryptophan Res.*, 2020, **13**, 1178646920936279, DOI: 10.1177/1178646920936279.

52 I. Lagkouvardos, T. R. Lesker, T. C. A. Hitch, E. J. C. Gálvez, N. Smit, K. Neuhaus, J. Wang, J. F. Baines, B. Abt, B. Stecher, J. Overmann, T. Strowig and T. Clavel, Sequence and cultivation study of Muribaculaceae reveals novel species, host preference, and functional potential of this yet undescribed family, *Microbiome*, 2019, **7**(1), 28, DOI: 10.1186/s40168-019-0637-2.

53 C. Fang, J. Cheng, W. Jia and Y. Xu, *Akkermansia muciniphila* Ameliorates Alcoholic Liver Disease in Experimental Mice by Regulating Serum Metabolism and Improving Gut Dysbiosis, *Metabolites*, 2023, **13**, 1057.

54 Z. Wang, S. Kang, Z. Wu, X. Liu, X. Zhang, Y. Wu, Y. Wen, X. Zhou, G. Zhang, J. Wang and D. Han, *Muribaculum intestinale* restricts Salmonella Typhimurium colonization by converting succinate to propionate, *ISME J.*, 2025, **19**(1), wraf069, DOI: 10.1093/ismejo/wraf069.