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Lactobacillus kefiranofaciens and *Lactobacillus kefiri* are the key species in kefir for reversing oxytetracycline-induced intestinal damage in zebrafish

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ABSTRACT

Increased accumulation of oxytetracycline (OTC) in environmental water bodies could potentially lead to its accumulation in human body, thereby damaging human intestinal tract. Dietary interventions could be helpful for recovery of intestinal morphology and function. Therefore, this study set zebrafish as model to explore the potential of kefir supplementation in the recovery of intestinal damage caused by exposure to OTC. In experiments by zebrafish, the rearing units used were glass tanks, each with volume of 5 L. The tanks were stocked with 12 zebrafish each. For each treatment, there were 8 replicate tanks. The zebrafish were treated with OTC followed by the addition of kefir to the food. The results showed positive improvements with kefir supplementation. Kefir treatment mitigated intestinal inflammation by reducing the levels of TNF-α, IL-6, and IL-1β; enhancing the activity of the antioxidant enzymes catalase, superoxide dismutase, glutathione peroxidase and increasing the gene expression of intestinal tight junction proteins (ZO-1a and ZO-1b). These effects were beneficial for reversing reduced integrity of intestinal barrier caused by OTC. Moreover, kefir helped to reverse the disruption of gut microbiota caused by OTC and further impacted host metabolism. Specifically, *Lactobacillus kefiranofaciens* and *Lactobacillus kefiri*, which were derived from the kefir microbiota, were found to be enriched in the zebrafish intestine. This helped to inhibit the increased abundance of some *Proteobacteria* species induced by OTC treatment. Liver metabolomics analysis revealed that kefir improved OTC-induced disruptions in the tricarboxylic acid cycle, glycerophospholipid and amino acid metabolism. The differentially abundant metabolites identified included a total of 80 types after OTC exposure, with the abundance of 74 kinds significantly reversed following kefir treatment. Correlation analysis revealed that certain *Proteobacteria* species and above *Lactobacillus* species were closely linked with metabolic inhibition in zebrafish caused by OTC and metabolic restoration caused by kefir treatment, respectively.

1. Introduction

Oxytetracycline (OTC) is a widely used broad-spectrum antibiotic in the veterinary and aquaculture industries[1]. Its mechanism of action involves inhibiting bacterial protein synthesis,

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hindering the growth and spread of pathogenic bacteria within organisms. However, extensive use of OTC in animal production has led to its significant accumulation in the environment due to its excretion in animal feces and urine. This could result in OTC residues contaminating water sources and soil, which posed a potential public health threat[2-3]. The excessive accumulation of OTC in the bodies of individuals who consume contaminated food or water could ultimately have detrimental effects on their health. One notable impact of OTC on the human body is its effect on the gut microbiota. In previous studies, OTC substances were proved to have diverse effects on the composition, diversity, and microbial-mediated functions of gut bacteria[4-5]. The OTC could lead to reduction in gut microbiota diversity and affect microbial metabolic activities and functions, potentially resulting in altered digestion, impaired immune responses[6]. OTC might also promote the spread and dissemination of resistance genes, leading to antibiotic resistance in certain pathogenic bacteria and making treatment more challenging[7-8]. Additionally, OTC affect intestinal morphology and causes severe damage by altering the thickness of the muscularis, height of the plica, and number of mucous cells[9]. Importantly, these effects might persist long after exposure to OTC has ceased. For example, an antibiotic mixture containing OTC caused significant disruptions in microbial-mediated nitridation and potentially long-lasting disturbances in nitridation for more than 5 months[10]. Furthermore, OTC induced significant alterations in the intestinal morphology of grouper, which did not fully recover even after 14 days of OTC exposure[9]. These findings suggest that additional interventions, such as dietary changes, might still be necessary to fully recover intestinal morphology and function.

Probiotics provide beneficial effects to the host when consumed in sufficient quantities and are, therefore, ingested through yogurt, fermented milk beverages, or in the form of capsules as supplements. Once they reach the intestine, specific probiotic strains could modify the composition of the gut microbiota, subsequently impacting the metabolic processes of the host. Various potential mechanisms might contribute to the beneficial effects of these probiotic strains. For example, probiotics can reduce intestinal pathogenic bacteria by producing antimicrobial compounds such as bacteriocins and hydrogen peroxide[11]. Probiotics can also promote the synthesis of anti-inflammatory factors and suppress the generation of inflammatory mediators, thereby orchestrating immune responses. Notably, the health benefits of certain probiotic species, like *Bifidobacterium infantis* 35624 and *Lacticaseibacillus casei* DN-114001, have been reported severally[12-13]. These include alleviating symptoms associated with irritable bowel syndrome, preventing antibiotic-associated diarrhea, and offering protection against infections. Kefir, a traditional fermented milk product, is well known for its health benefits, including its ability to reduce cholesterol, combat inflammation, and pathogens[14]. Based on previous studies, it was hypothesized that the probiotic strains in kefir could also regulate the gut microbiota, enhance host metabolism, and reverse intestinal damage caused by OTC. It is important to note that kefir is generally consumed as a whole by the human body. However, different species of probiotic bacteria derived from kefir might vary in their ability to colonize the intestine and their effects on the gut. Therefore, it is crucial to explore the key bacterial strains present in kefir that are responsible for reversing intestinal damage and altering host metabolism caused by OTC exposure.

In this study, zebrafish was utilized as a model organism to investigate the potential mechanism underlying the impact of kefir on OTC-induced intestinal damage, focusing on antioxidant activity, gut histopathological analysis, and immunohistochemistry. Additionally, this study explored the effect of the supplementation with kefir on gut microbiome and liver metabolites using 16S rRNA gene sequencing and liquid chromatography-mass spectrometry (LC-MS) analysis. Notably, the presence of *Lactobacillus kefiranofaciens* and *Lactobacillus kefiri* in the intestine of zebrafish after consumption of kefir, albeit temporarily, was identified for the first time. Despite their limited abundance, these species were found to play a crucial role in reversing the altered intestinal microbiota and host metabolism induced by OTC, as indicated by correlation analysis.

2. Materials and methods

2.1 Chemicals reagents

Kefir grains were purchased from Huacheng Biological Corporation (Changchun, China). *Lactobacillus rhamnosus* GG (LGG) was obtained from the China General Microbiological Culture Collection Center. Catalase (CAT) assay kit (BC0205), superoxide dismutase (SOD) assay kit (BC0175), glutathione peroxidase (GPx) assay kit (BC1190) and malondialdehyde (MDA) assay kit (BC0025) were purchased from Beijing Solarbio Science & Technology Co., Ltd. A fast DNA SPIN extraction kit was obtained from MP Biomedicals (Santa Ana, CA, USA). The rest of the chemical reagents used in this study were obtained from a nearby commercial vendor and were of high purity, making them suitable for analytical applications.

2.2 Method

2.2.1 Kefir production

The cultivation of kefir was conducted following the methodology described previously, with some adjustments[15]. Typically, kefir is prepared by introducing kefir grains, which constitute approximately 5%–10% of the weight/volume of milk, and fermenting it for 12–48 h. In our study, we added 10 g of kefir grains to 100 mL of sterilized skim goat milk. The mixture was then stored in a container partially open to air. Notably, extended fermentation periods could lead to toxin buildup or decreased efficacy of beneficial compounds. Therefore, the mixture was fermented for 24 h at 37 ℃. At intervals of 6 h, 5 mL of the fermented kefir sample (with 5 replicates for each time point) was collected for the purpose of analyzing the structural progression of bacterial communities using 16S rRNA amplicon sequencing and bioinformation. A detailed explanation of the methodology was given in section 2.2.7. After the fermentation process, the grains were sieved and discarded. The average pH of kefir was found to be 4.5. The apparent viscosity values of kefir samples reached 102 mPa·s. Following dilution, the kefir was passed through filter paper with a 12 μm pore size to eliminate large insoluble fat or protein particles. The resulting kefir was subsequently lyophilized and mixed with food for the zebrafish experiments.

2.2.2 Experimental design for adult zebrafish

The research was adhered strictly to our institution's ethical guidelines for animal experimentation. The care of zebrafish was

performed based on the previous study[16]. The experimental design of adult zebrafish was according to the study with a few modifications[17]. The procedure was illustrated in Fig. S1. The adult zebrafish used in the experiments were divided into 4 groups. The control groups consisted of zebrafish that received no treatment. The model control groups comprised zebrafish treated only with OTC. The concentration of OTC in the culture water was 100 ng/mL according to the concentration described previously[18]. The positive control groups were under the treatment with OTC followed by addition of LGG to the food. The experimental groups were treated with OTC followed by the addition of kefir to the food. Groups for each treatment consisted of 8 replicate glass tanks, with 6 males and 6 females randomly distributed into each tank with a volume of 5 L. Zebrafish were fed brine shrimp (Artemia) 3 times a day, either with or without the addition of LGG or kefir (with the concentration of 10^6 CFU/mL in the food). Notably, no fish deaths were recorded throughout the entire duration of the experiment. At the end of each treatment, tissues from the liver or intestine of the zebrafish were dissected for analysis of antioxidant enzyme activities, histopathology, immunohistochemistry, gene expression and hepatic metabolomics. The intestinal contents were collected for microbiome analysis.

2.2.3 Antioxidant activity analysis of the zebrafish

The assessment of antioxidant activity in zebrafish was carried out following the methodology outlined in previous study[19]. Liver tissues were obtained from 10 zebrafish per group at the end of the treatments. Subsequently, the fish livers were homogenized in ice-cold potassium phosphate buffer (50 mmol/L, pH 7.0) at a ratio of 1:8 (*m*/*V*). The resulting homogenate solution was then centrifuged at 10 000 r/min and 4 ℃ for 10 min. After that, the supernatant was retained for the determination of antioxidant enzyme activities and MDA levels. Specifically, the levels of MDA, SOD, GPx and CAT were quantified using specific kits and following the respective protocols for each measurement.

2.2.4 Histopathological analysis of the zebrafish intestine

The intestines of adult fish were procured for histopathological examination. The collected samples were subjected to fixation using 10% formalin, followed by embedding in paraffin wax. Sections of 5 μm thickness were prepared and stained with haematoxylin and eosin (H&E). The samples were then dried and mounted using neutral mounting medium. Each treatment was replicated with 6 samples. Histopathological analysis of the middle gut segments was performed using an optical microscope (IMT 2, Olympus Corp., Tokyo, Japan).

2.2.5 Quantitative real-time PCR (qRT-PCR)

The gene expression levels of tight junction proteins in the intestine of zebrafish were assessed by qRT-PCR assay. After anesthesia, 6 intestinal tissues were collected from zebrafish in each experimental group for RNA extraction, followed by cDNA synthesis *via* reverse transcription. The resulting cDNA was analyzed through qRT-PCR. The primers utilized in our study are listed in Table S1. The expression levels of the target genes were normalized to those of *β*-actin, and data analysis was performed using the $2^{-\Delta\Delta Ct}$ method to calculate relative gene expression, adjusted for internal reference.

2.2.6 Zebrafish gut immunohistochemistry

Immunohistochemical experiments, which included immunofluorescence staining and TUNEL staining, were also performed. Each treatment was replicated with 6 samples. For detailed information regarding the methods, please refer to our supporting information.

2.2.7 Microbiome analysis

16S rRNA sequencing analysis was subsequently performed to investigate the structure of the bacterial communities in the intestinal contents of adult zebrafish. For zebrafish gut microbiota analysis, the guts of 60 zebrafish from each group were collected, and every 10 guts were set as one replicate, with 6 replicates in total. For detailed information regarding the methods, please refer to our supporting information.

2.2.8 Metabolomic analysis

Metabolomic analysis was performed to investigate the metabolic changes in the liver induced by OTC and kefir treatment. Liver tissues were collected from 60 adult zebrafish in each group, and every 10 livers were combined into one sample, resulting in 6 replicates for each group. For detailed information regarding the methods, please refer to our supporting information.

2.2.9 Statistical analysis

This study employed independent samples t tests for comparing 2 distinct groups, using a significance level set at $P < 0.05$ to indicate statistical significance. The relationships between the species-level gut microbiota and various liver metabolites were assessed using Spearman's rank correlation tests. These correlations were visualized through a heatmap created in R software. Additionally, a correlation heatmap illustrating the species-level gut microbiota was produced and displayed using Origin 2022. The data was shown as the mean ± standard deviation.

3. Results

3.1 Changes in the bacterial community structure of kefir across various fermentation time

The analysis of the bacterial community structure was conducted across various fermentation time points of kefir. A total of 723 700 bacterial sequences were obtained from the fermented samples and classified into operational taxonomic units (OTUs) at a 97% sequence similarity threshold, resulting in 1 531 bacterial OTUs. These genes were further assigned to 410 specific genera. Analysis of the microbial community revealed that *Lactobacillus* was the predominant genus in samples from different fermentation time. Changes in microbial communities were primarily observed at the species level, as depicted in Fig. 1. The *α*-diversity results, including the Ace, Chao1, Shannon, and Simpson indices, demonstrated

variations among the samples (Fig. 1a). Notably, there was a significant increase in community richness with increasing fermentation time, as indicated by the Ace and Chao1 indices. Additionally, an increase in bacterial species diversity was observed in the later stages of fermentation, as reflected by the Shannon and Simpson indices. The principal component analysis (PCA) results for the samples at different fermentation time points are illustrated in Fig. 1b. Generally, PCA yields multiple principal components, and using two-dimensional analysis helps to more intuitively display the projection of data points onto these components. In our study, the variances of PC1 and PC2 were 76.97% and 17.45%, respectively. The total variance explained by these 2 principal components was 94.42%, indicating that PC1 and PC2 could explain the majority of the data variability. The distribution of data points on PC1 and PC2 revealed significantly distinct clusters between the groups that underwent fermentation for 12 to 24 h, indicating a substantial change in the community structure of kefir. Fig. 1c offers insights into the distribution of bacterial community abundance at the species level following a 24-h fermentation period. After 24 h of fermentation, the predominant bacterial populations in kefir consisted of *L. kefiranofaciens*, *L. kefiri*, unclassified_g_*Pseudomonas*, *Pseudomonas_fragi*, unclassified_f_Alcaligenaceae, *Lactobacillus_parakefiri* and *Bacillus_cereus*_03BB102. Overall, the findings suggested that the bacterial community structure in kefir significantly changed across different fermentation durations.

3.2 Results for the activities of antioxidant enzymes in zebrafish

The results depicting the activities of antioxidant enzymes in zebrafish are presented in Fig. 2. OTC administration led to significant decreases in antioxidant enzyme activities, with inhibition rates of 31.04% for CAT, 43.21% for SOD, and 37.38% for GPx, respectively. Moreover, the MDA level in the OTC group was 2.84 times higher than that in control group. Importantly, kefir substantially increased the CAT, SOD, and GPx activities but also reduced the MDA level compared to those in the OTC group. Overall, the results suggested that kefir effectively reversed the decrease in antioxidant activity induced by OTC in zebrafish.

3.3 Histopathological and immunohistochemical analysis of the intestine in zebrafish

Fig. 3a presents histopathological and immunohistochemical analyses of the zebrafish intestine under various treatments. As shown in Fig. 3a, normal intestinal villi in zebrafish exhibited distinct

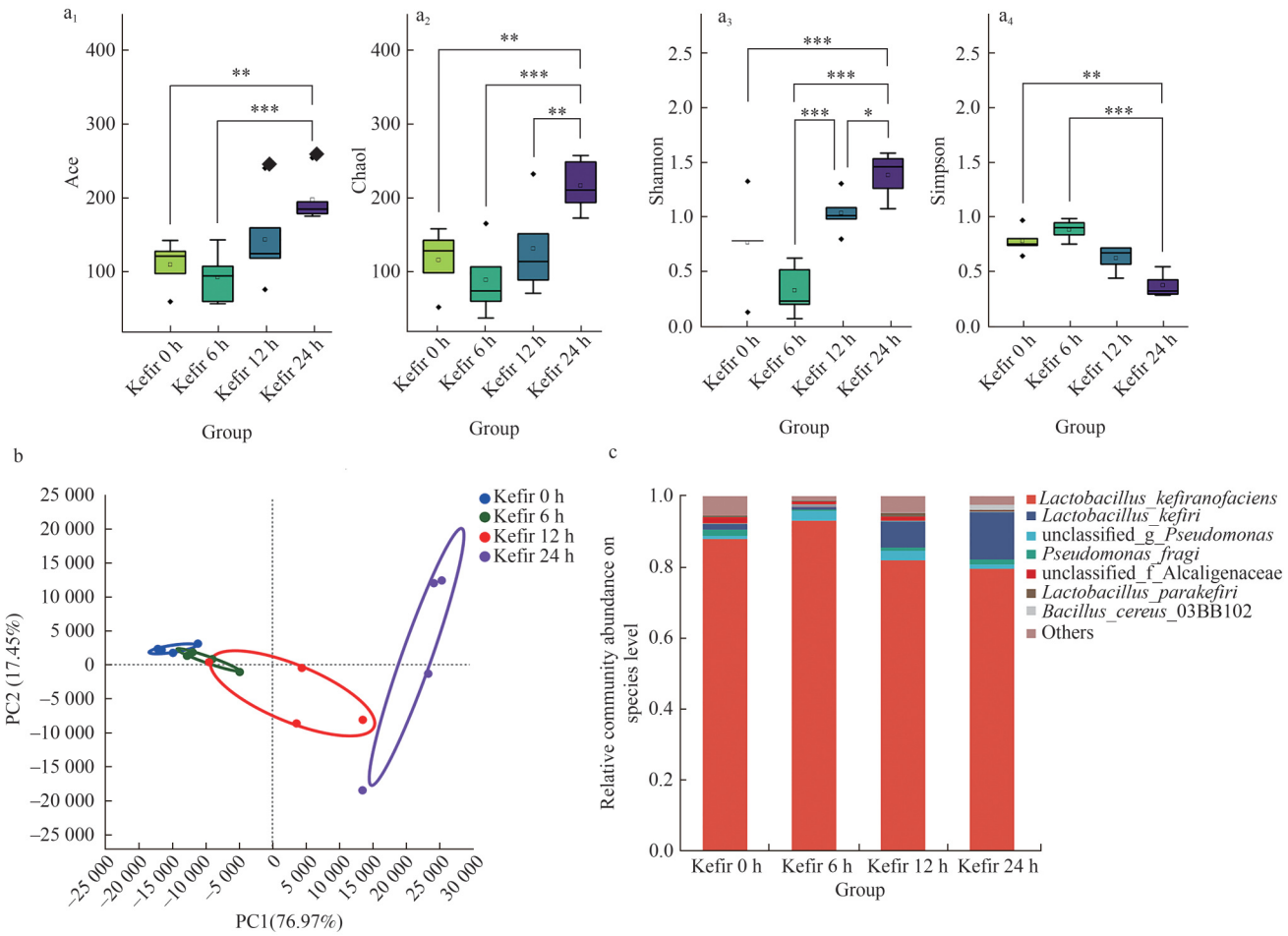

Fig. 1 Changes in the bacterial community structure in kefir across various fermentation times. (a) The results of α-diversity analysis. (b) PCA analysis for the bacterial community at the species level. (c) Distribution of bacterial community abundance at the species level. Statistical significance is denoted by $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

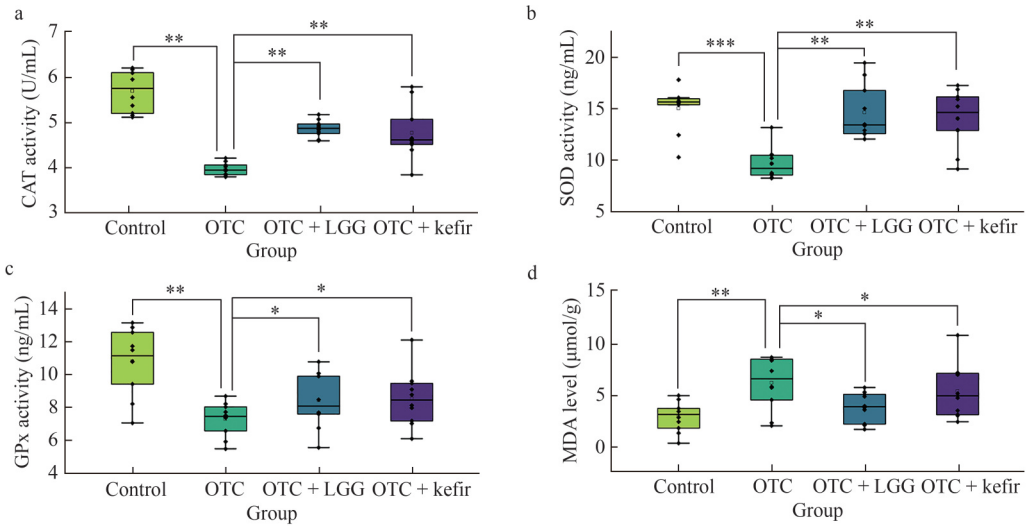

Fig. 2 Effects of OTC and kefir supplement on (a-c) antioxidant enzyme activities (CAT, SOD, GPx) and (d) MDA level of zebrafish. Statistical significance is denoted by $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

outlines and were covered by an intact epithelial layer containing goblet cells within the gut lumen. However, in the group treated with OTC, evident cilia defects (indicated by the green circle) were observed, along with partial shedding of cilia tissue into the mucus of the intestinal villi (indicated by the red circle). The arrangement of goblet cells appeared disordered (indicated by the yellow circle). Additionally, mild edema and loose connective tissue were observed at the basal region of the lamina propria (indicated by the blue circle). Comparison of the OTC-treated groups with the OTC + kefir-treated groups showed that the latter largely maintained an intact intestinal structure and exhibited normal characteristics. However, minor cilia defects (indicated by the green circle) and loose connective tissue (indicated by the blue circle) could still be observed. Fig. 3b shows the expression of genes related to tight junction proteins in the intestine of zebrafish. Kefir effectively reversed the downregulated expression of genes related to tight junction proteins (ZO-1a and ZO-1b) in the zebrafish intestine. Importantly, neither OTC treatment nor subsequent kefir supplementation significantly affected the expression of the *Occludin1* gene. The levels of tight junction proteins are largely linked with the integrity of the intestinal barrier. These findings suggested that OTC might reduce intestinal barrier function, but this effect could be reversed by kefir supplementation. Figs. 3c and d illustrates the immunohistochemical characteristics of the zebrafish intestine using immunofluorescence staining and TUNEL staining. A notable increase in TNF-α, IL-6, and IL-1β levels, along with an increase in the proportion of TUNEL-positive cells induced by OTC was clearly observed. These findings indicated that OTC triggered an inflammatory response and induced apoptosis in zebrafish intestinal tissues. Interestingly, kefir treatment mitigated the increased levels of inflammatory cytokines and intestinal tissue apoptosis induced by OTC. This was evidenced by reductions in IL-1β expression by 38.4%, IL-6 expression by 63.7%, TNF- α expression by 46.5%, and the number of TUNEL-positive cells by 50.23% compared to those in the OTC groups. TNF-α, IL-6, and IL-1β are crucial in intestinal immune responses and serve as valuable indicators for assessing intestinal inflammation[20]. These molecules have the potential to affect epithelial cell survival and thereby impact intestinal barrier integrity.

3.4 Analysis of the gut microbiota composition of zebrafish

We further employed 16S rRNA gene sequencing to examine the gut microbiota. The results were shown in Fig. 4. Overall, 939 114 bacterial sequences were identified. These sequences were subsequently grouped into 3 476 bacterial operational taxonomic units (OTUs), determined using a 97% sequence similarity threshold. The results of α-diversity metrics, including Ace, Chao1, Shannon, and Simpson indices, are given in Fig. 4a. According to the results, OTC treatment substantially decreased the Chao1 and Ace indices of the gut microbiota. Conversely, the consumption of kefir effectively counteracted the decreases in Chao1 and Ace, implying that kefir has the potential to increase species richness. Interestingly, no significant differences were observed in the Shannon and Simpson indices among the different groups. Subsequently, the similarities and differences within the gut microbiota were assessed through PCA, as illustrated in Fig. 4b. The total variance of the 2 principal components (PC1 and PC2) reached 87.65%, indicating that PC1 and PC2 could explain the majority of the data variability. The distribution of data points on PC1 and PC2 revealed significantly distinct clusters between the groups, indicating a variation in the community structure among the control, OTC and OTC + kefir groups. To identify changes in the gut microbiota composition, we analyzed the proportions of the predominant taxa within each treatment group. The predominant bacteria included Proteobacteria, Actinobacteria, Firmicutes, Fusobacteriota and Bacteroidota at the phylum level (Fig. S3). Compared with those in the control group, the percentages of Actinobacteria (5.88% *vs*. 41.81%), Bacteroidota (2.31% *vs*. 9.53%), and Firmicutes (0.34% *vs*. 1.71%) decreased in the OTC group. In the meanwhile, the greater percentage of Proteobacteria (84.75% *vs*. 39.44%) and Fusobacteria (7.22% *vs*. 5.18%) could be detected in the OTC group. Notably, compared with those in the OTC group, the proportions of Actinobacteria, Firmicutes, and Bacteroidota increased by 6.92%, 4.78%, and 7.3%, respectively, in the OTC + kefir groups, respectively. Proteobacteria decreased by 19.05% (Fig. S3). Analysis at the species level revealed that the dominant species mainly included s_unclassified_g_*Methylobacterium-Methylorubrum*, s_unclassified_f_Alcaligenaceae and s_*Gordonia_polyisoprenivorans* (Fig. 4c).

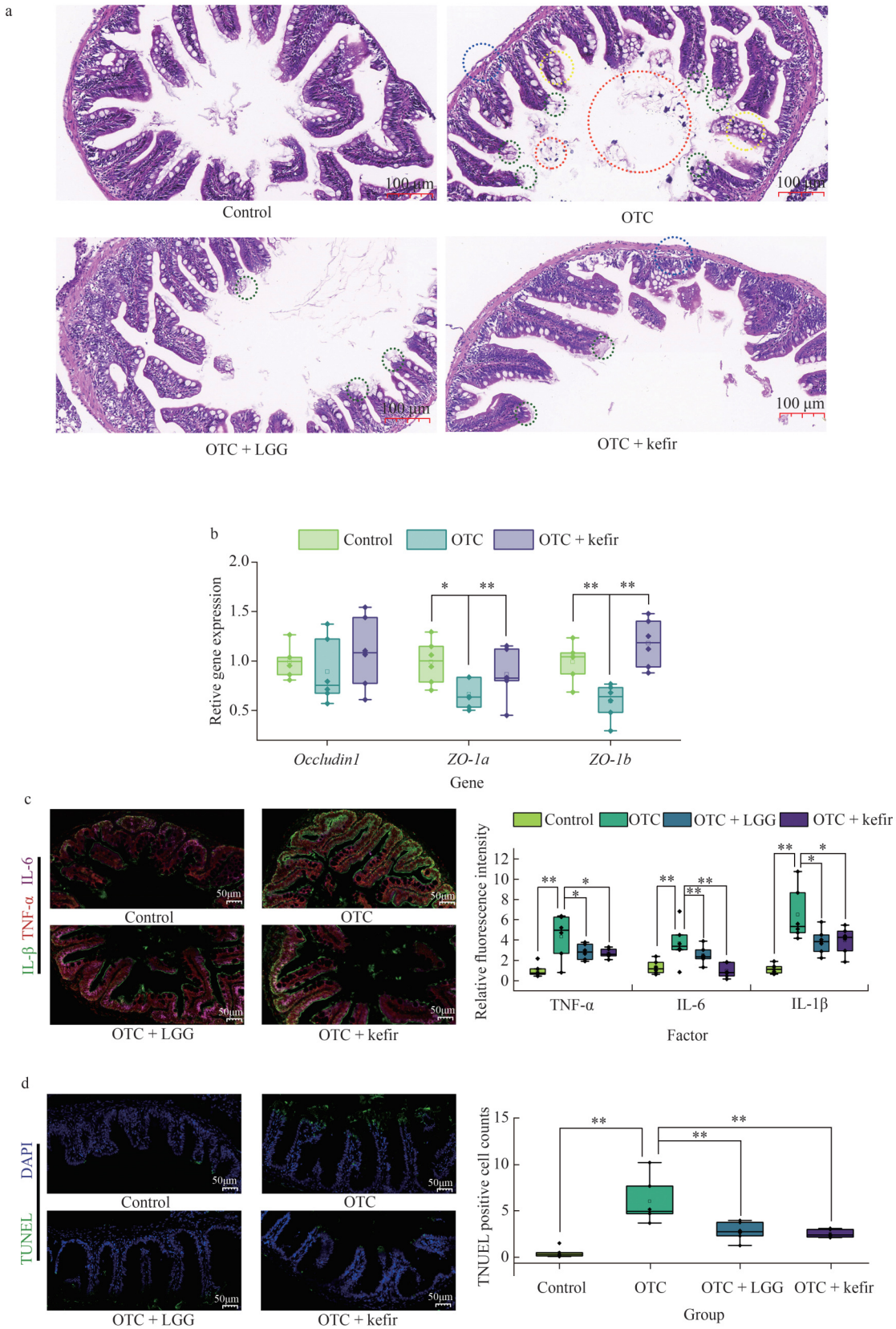

Fig. 3 Histopathological and immunohistochemical analysis of zebrafish intestines subjected to various treatments. (a) H&E staining of zebrafish intestine. The red, blue, yellow and green circles represented shredded cilia tissue, loose connective tissue, the goblet cells and cilia defects of the intestine. (b) Gene expression related to tight junction proteins in the intestine of zebrafish. (c) Triple immunofluorescence staining of zebrafish intestine included TNF-α (in red), IL-6 (in pink) and IL-1β (in green). (d) Intestinal DAPI and TUNEL staining images with quantitation of TUNEL positive cells. Statistical significance is denoted by $*P < 0.05$, $**P < 0.01$.

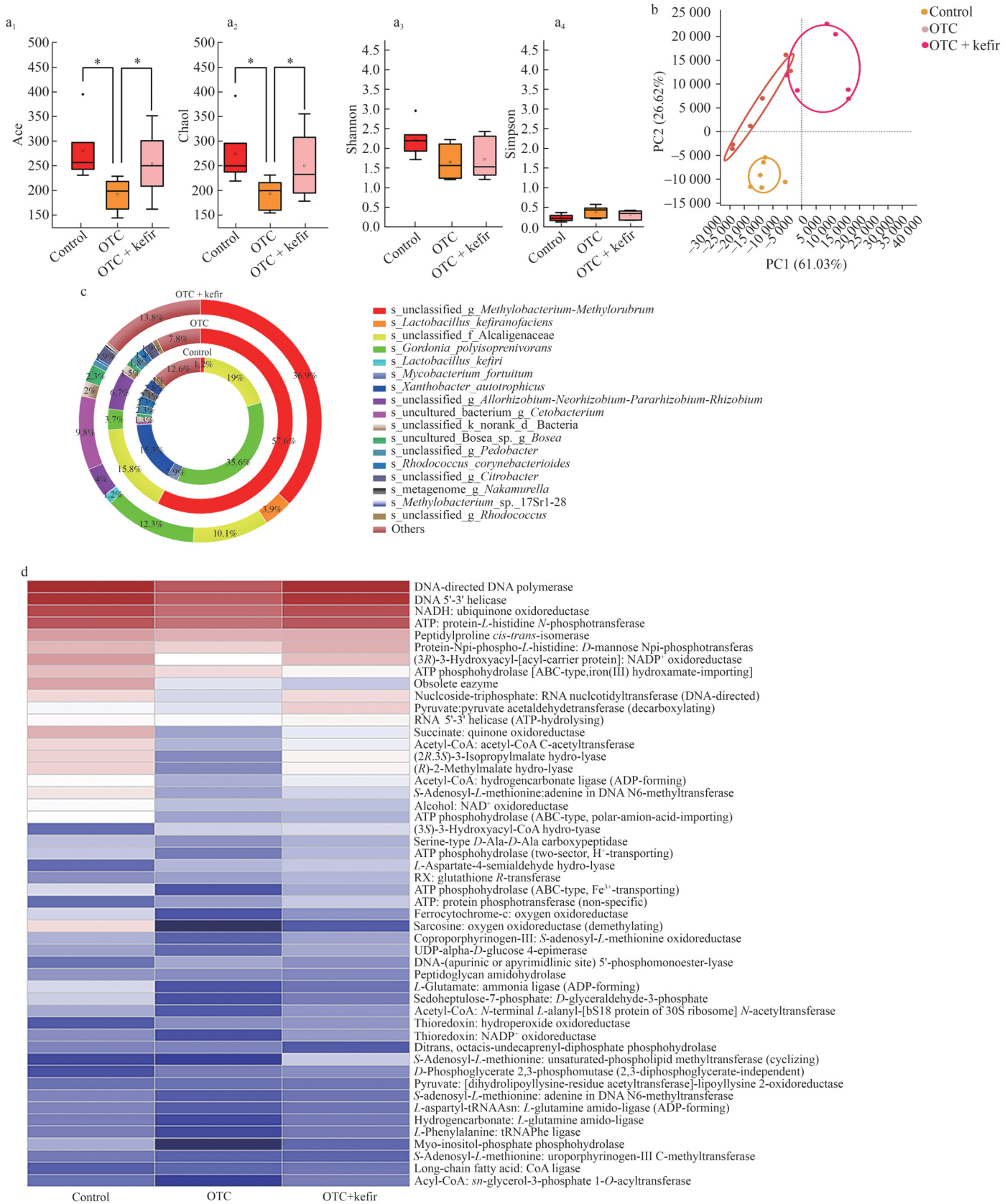

Fig. 4 Effects of kefir treatment on the composition of intestinal microbiota in zebrafish. (a) α-Diversity analysis of bacterial community. (b) PCA results for the bacterial community at the species level. (c) Relative abundances of the microbiota at the species level. (d) Enzymes encoded by gut microbiota involved in various metabolic and biochemical reactions as predicted by PICRUSt2. (e) Results for LEfSe analysis. Statistical significance is denoted by $^*P < 0.05$.

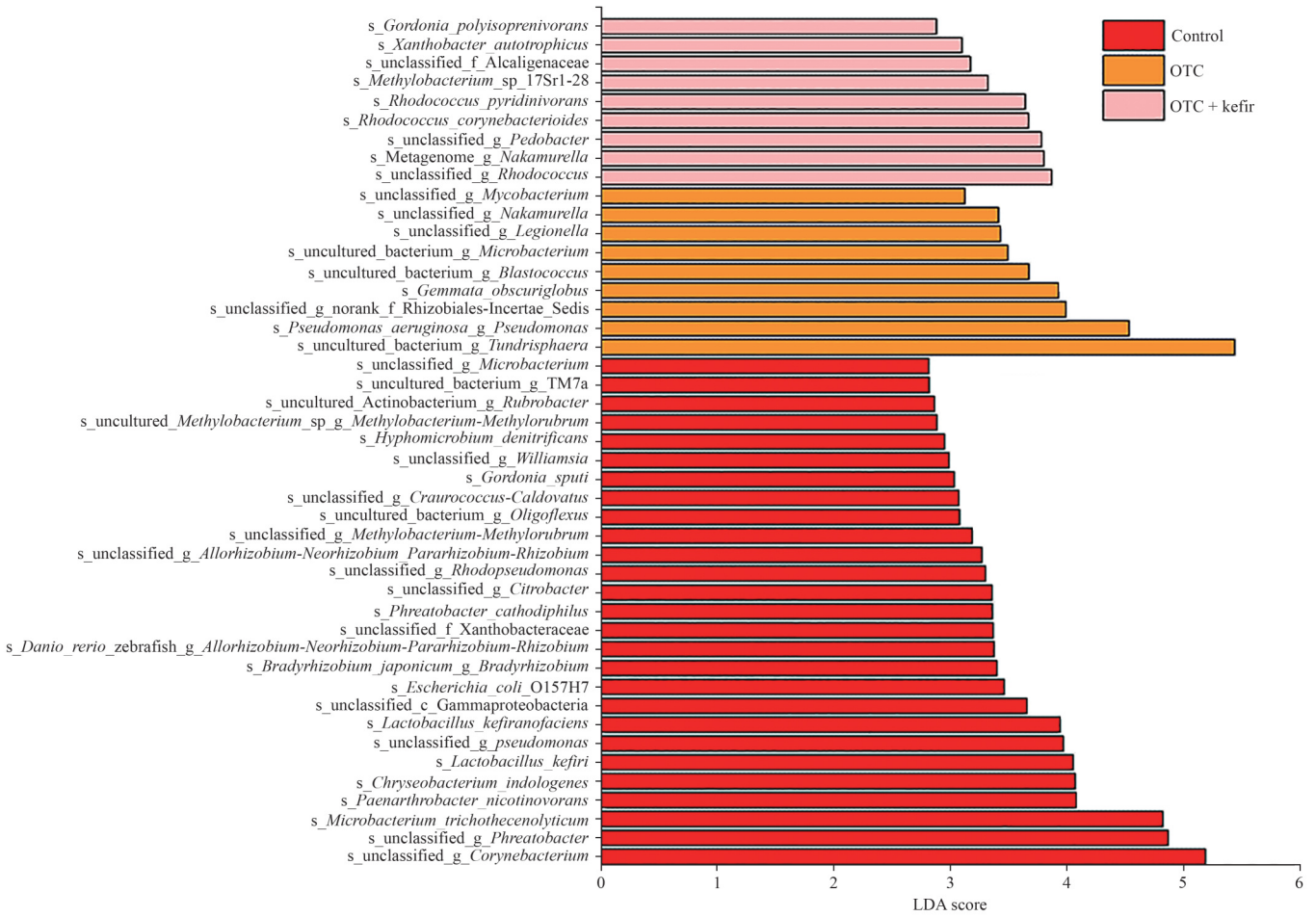

Fig. 4 (*Continued*)

Fig. 4d shows the enzymes encoded by the gut microbiota involved in various metabolic and biochemical reactions, as predicted by PICRUSt2. Overall, 50 kinds of enzymes were selected from the KEGG database, and they could be categorized into functions such as DNA replication and repair, protein modification and metabolism, energy production and lipid metabolism. Compared to those in the OTC groups, the intestinal microbiota of zebrafish in the OTC + kefir groups exhibited a greater ability to encode enzymes related to the aforementioned functions. To evaluate differences in gut microbiota composition among groups at the species level, we employed LDA scores to assess the extent of consistent changes in relative abundance among various treatments. Setting an LDA threshold score of 2.0, we identified a total of 27 distinctive features in the microbiota of the control groups, alongside 9 features each in the OTC and OTC + kefir groups (Fig. 4e).

3.5 Analysis of liver metabolomics in zebrafish

To explore the potential positive impacts of kefir on the amelioration of OTC-induced intestinal damage in adult zebrafish, untargeted metabolomics analysis was conducted. A total of 1 287 metabolites were identified in the liver tissue of adult zebrafish. The results of the PLS-DA analysis (Figs. 5a and b) revealed clear differentiation between groups in both the positive and negative ionization modes, as demonstrated by the distinct separation of all the samples within the 95% confidence intervals. The results demonstrated R^2_Y and Q^2 values of 0.982 and 0.993 in positive mode, 0.994 and 0.996 in negative mode, respectively. This indicated their suitability for subsequent analysis. Fig. 5c presents the top 50 differentially abundant metabolites categorized by groups. Among these, 39 metabolite types in the OTC groups demonstrated a decrease in abundance compared to those in the control groups, while 37 of these types exhibited a reversed impact on abundance when supplemented with kefir. This suggested that OTC and kefir might significantly impact the abundance of metabolites in zebrafish. The modified metabolites were subsequently classified and categorized into 14 distinct super classes within the HMDB database, as depicted in Fig. 5d. The 3 most prominent differentially abundant metabolite species, in terms of proportion, were identified as organic acids and derivatives (27.45%), lipids and lipid compounds (26.34%) as well as the organoheterocyclic compounds (12.59%). Subsequently, the differentially abundant metabolites between the experimental groups were subjected to pathway enrichment analysis for further investigation. A total of 23 differential pathways were identified, encompassing lipid metabolism, amino acid metabolism and carbohydrate metabolism (Fig. 5e). Notably, metabolic pathways exhibiting P values less than 0.002 included the TCA cycle (P = 0.000 009 073), lysine degradation (P = 0.000 027 82), ABC transporters (P = 0.000 042 38),

Fig. 5 Effects of OTC and kefir treatment on liver metabolomics in zebrafish. (a) PLS-DA score plot in the positive model. (b) PLS-DA score plot in negative model. (c) Pie-chart of HMDB superclass. (d) A heat map showing the top 50 differential metabolites. (e) Classification of the pathways related to the differential metabolites. Colors represent different pathways in the first level, and the abscissa represents the number of metabolites annotated to the pathway on the ordinate. (f) Analysis of KEGG pathway enrichment, where the abscissa represents the significance *P*-value and the ordinate represents the pathways.

arginine biosynthesis (P = 0.000 258 9), D-amino acid metabolism (P = 0.000 321 3), alanine, aspartate and glutamate metabolism (P = 0.000 813 1), cofactor biosynthesis (P = 0.000 825 4), and glycerophospholipid metabolism (P = 0.004 227) (Fig. 5f). Overall, our results suggested that OTC and subsequent kefir treatments could primarily influence the host's energy metabolism, glycerophospholipid metabolism, and amino acid metabolism.

3.6 Analysis of differential metabolites related to energy metabolism, glycerophospholipid metabolism and amino acid metabolism

The analysis focused on the relative abundance of differential metabolites involved in energy metabolism, glycerophospholipid metabolism, and amino acid metabolism within zebrafish groups. The results are depicted in Fig. 6a. These metabolic pathways primarily encompassed a total of 83 types ofdifferential metabolites. Among

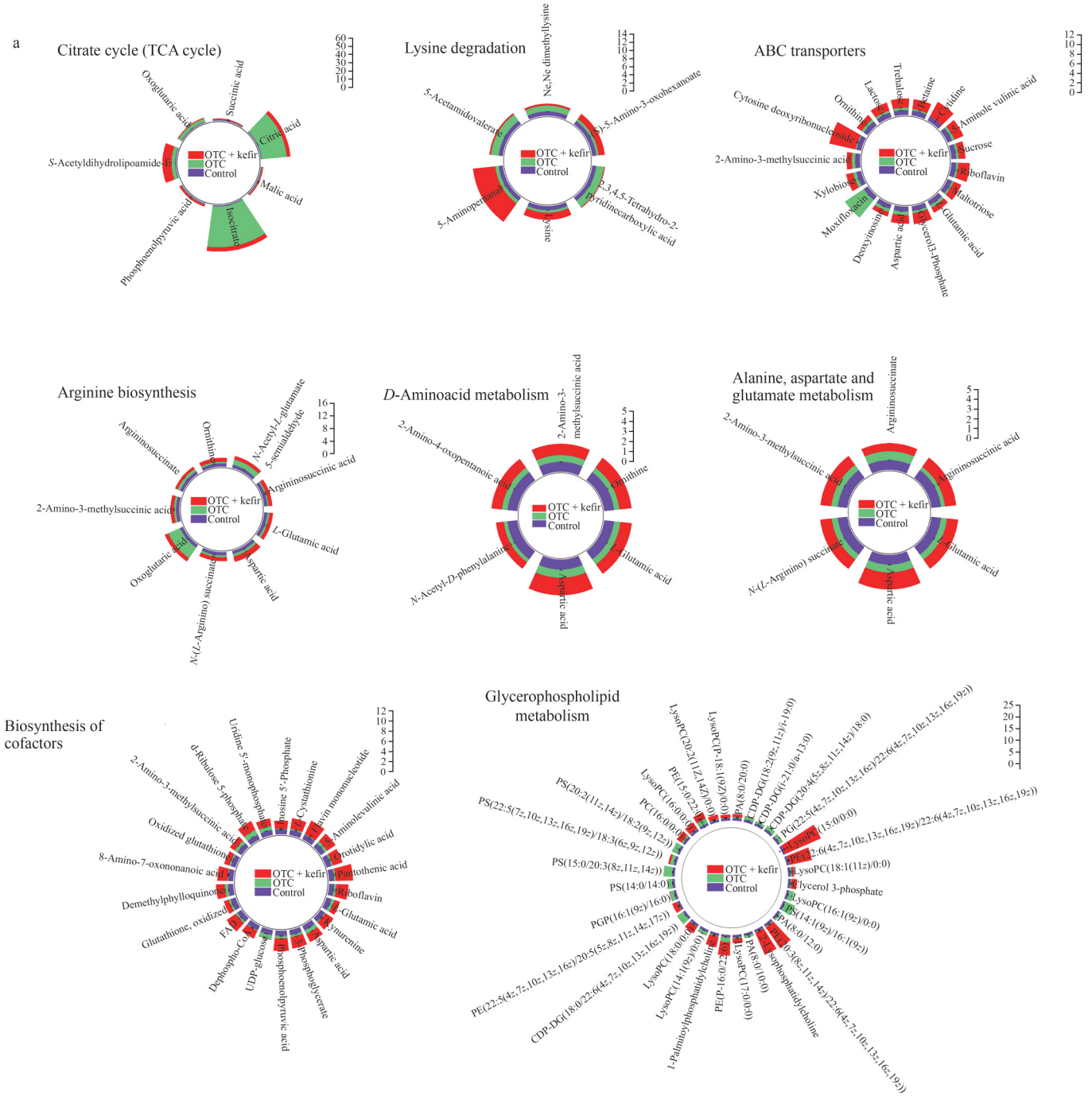

Fig. 6 Effect of OTC and kefir supplement on glycerophospholipid metabolism, energy metabolism, and amino acid metabolism in zebrafish. (a) Relative abundance of differential metabolites primarily involved in glycerophospholipid metabolism, energy metabolism, and amino acid metabolism among groups. (b) Schematic diagram illustrating significant alterations observed in major metabolic pathways associated with glycerophospholipid metabolism, the TCA cycle, and amino acid metabolism. The red minus sign indicates potential locations where pathways might be obstructed. Arrows corresponding to OTC denote reduced (red) or increased (blue) metabolite abundance compared to control groups. Arrows corresponding to kefir represent reduced (red) or increased (blue) metabolite abundance compared to OTC groups.

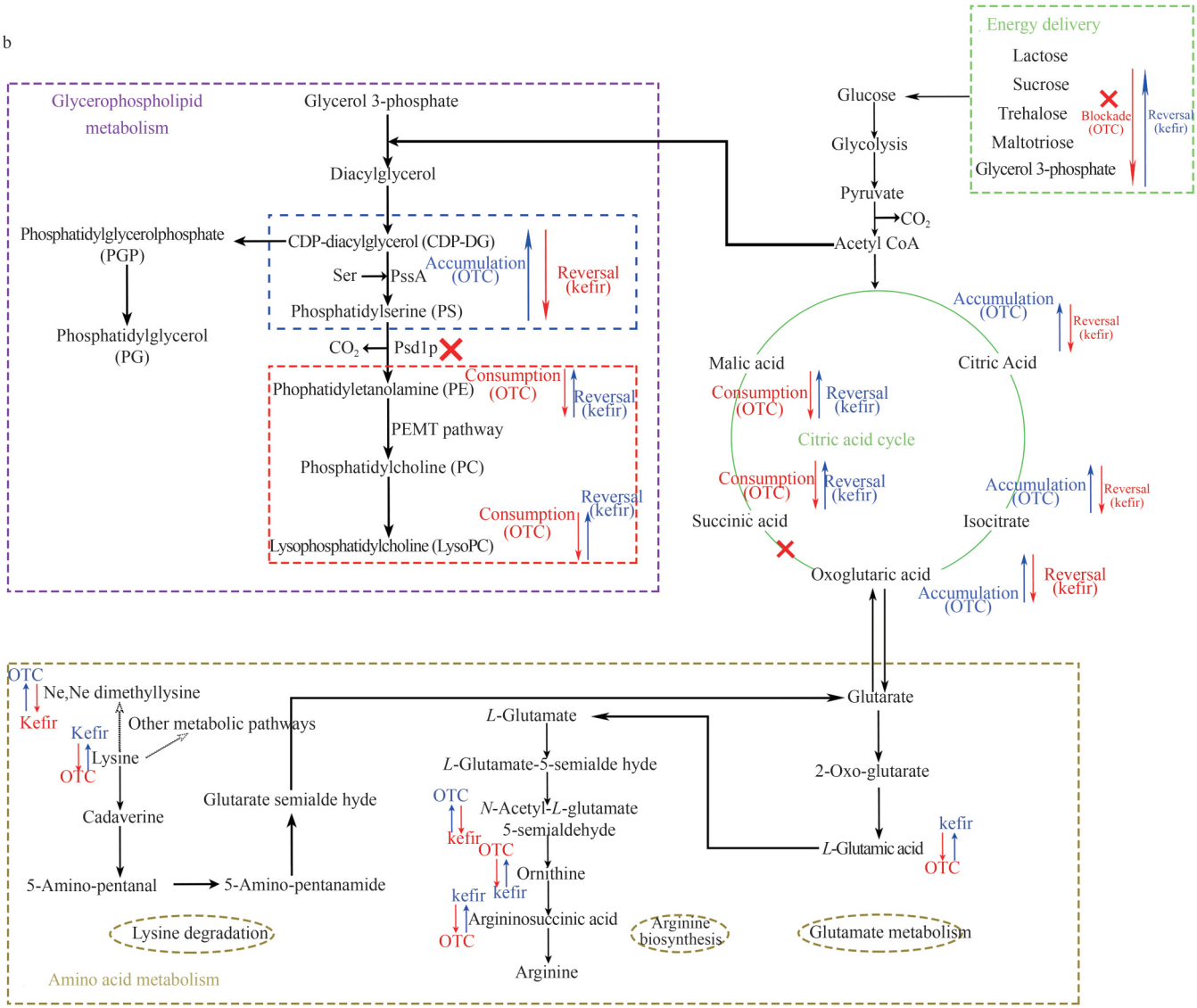

Fig. 6 (*Continued*)

these metabolites, 56 significantly decreased in abundance in the OTC group, and the decrease in the abundance of 54 of these metabolites could be reversed by kefir treatment. Additionally, the levels of 23 metabolites were upregulated in the OTC groups compared to the control groups, and 20 of them were downregulated in the OTC + kefir groups (Table S2). Among all differential metabolites, 24 were associated with energy metabolism or transport pathways (TCA cycle and ABC transporters), 32 were linked to glycerophospholipid metabolism, and 27 were associated with amino acid metabolism (lysine degradation, arginine biosynthesis, *D*-amino acid metabolism, alanine, aspartate, and glutamate metabolism). According to the results, OTC has the potential to strongly interfere with significant pathways related to glycerophospholipid metabolism, energy metabolism, and amino acid metabolism. The disruption of these pathways could be largely reversed by the addition of kefir. The main synthetic pathways of the corresponding metabolites were examined and are visually represented in Fig. 6b.

3.7 Correlation analysis

Correlation analysis was performed to examine the relationships between predominant microorganisms at the species level and differential metabolites associated with energy metabolism, glycerophospholipid metabolism, and amino acid metabolism among the different groups. Analysis between control and OTC groups revealed significant correlations between the relative abundance of specific species and the differential metabolite level. Specifically, species of s_*Gordonia_polyisoprenivorans*, s_*Mycobacterium_fortuitum*, s_*Xanthobacter_autotrophicus*, s_*metagenome*_g_*Nakamurella*, and s_*Methylobacterium*_sp. _17Sr1-28 were significantly positively correlated with 55, 43, 53, 55, and 54 metabolites, respectively. Conversely, species of s_unclassified_g_*Methylobacterium-Methylorubrum*, s_unclassified_g_*Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* and s_unclassified_g_*Citrobacter* exhibited negative correlations with 55, 56, and 55 metabolites, respectively (Fig. 7a).

In comparisons between the OTC and OTC + kefir groups, species including s_*L. kefiranofaciens*, s_*L. kefiri*, and s_*Mycobacterium_fortuitum*,

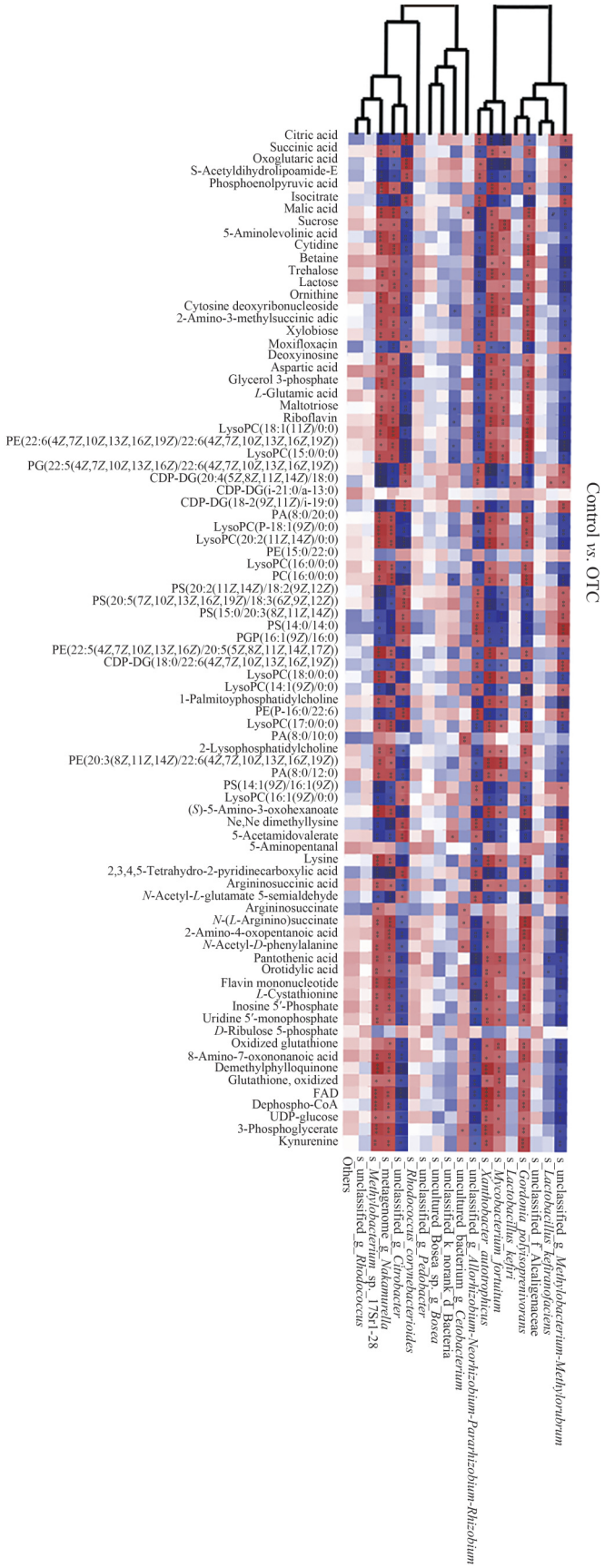

Fig. 7 Correlation analysis of main gut microbiota (species level) and different metabolites related to the glycerophospholipid metabolism, energy metabolism and amino acid metabolism of zebrafish. (a) Analysis between control groups and OTC groups. (b) Analysis between OTC groups and OTC + kefir groups. (c) Correlation heatmap between main gut microbiota at the species level. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

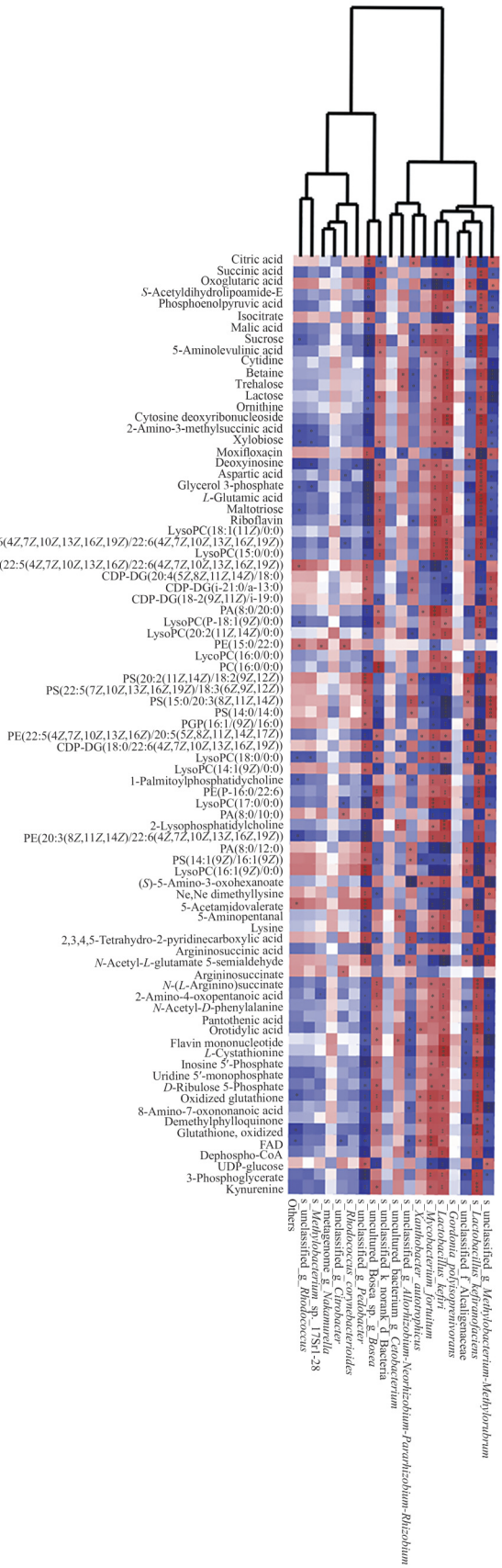

Fig. 7 (*Continued*)

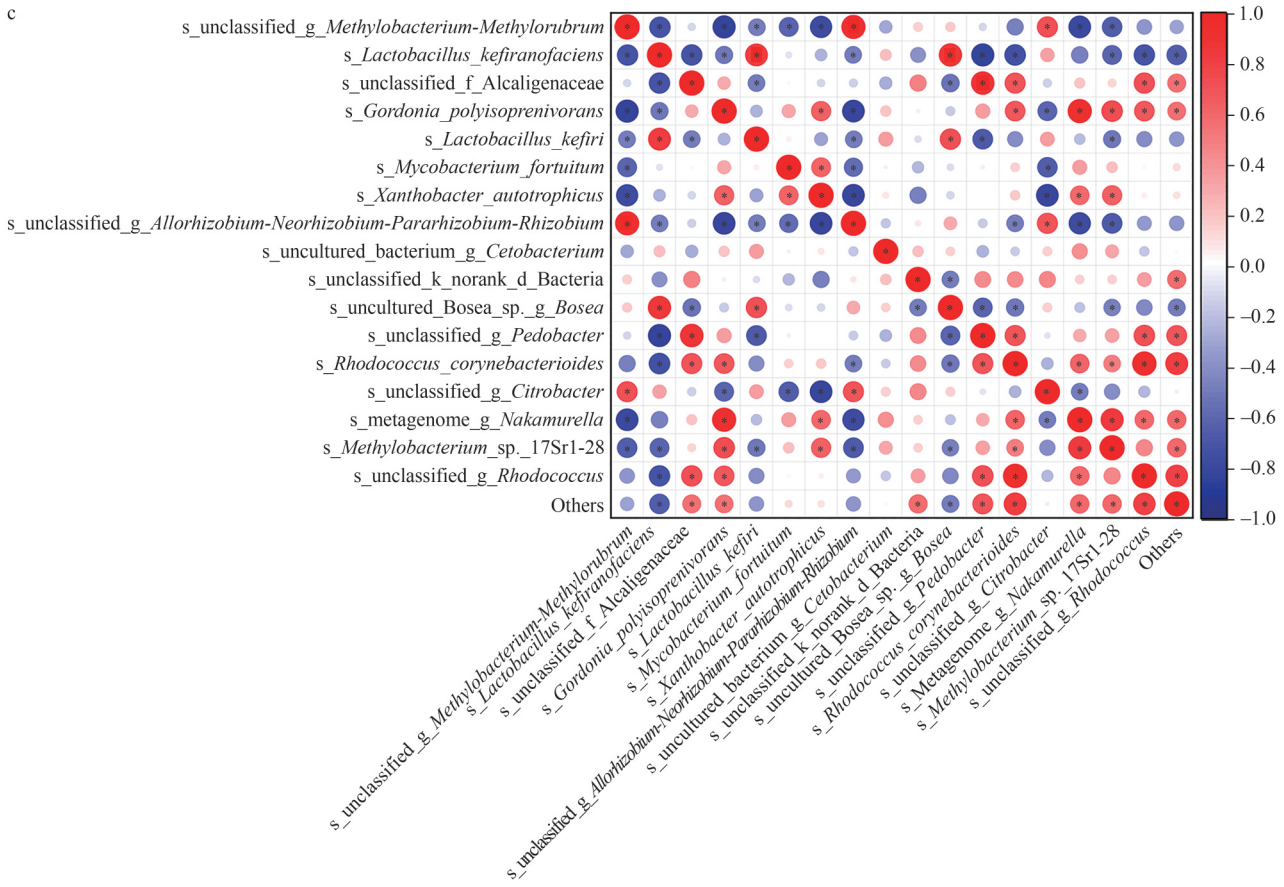

Fig. 7 (*Continued*)

demonstrated significant positive correlations with 57, 57, and 55 metabolites, respectively. A correlation analysis heatmap depicting the main gut microbiota at the species level was presented in Fig. 7c, revealing a notable negative correlation between s_*L. kefiranofaciens*, s_*L. kefiri*, and s_unclassified_g_*Methylobacterium-Methylorubrum*, s_unclassified_g_*Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*. These findings indicated a significant influence of the probiotics *L. kefiranofaciens* and *L. kefiri* on host metabolite alterations, particularly in relation to kefir supplementation.

4. Discussion

In this study, we found that kefir supplementation effectively mitigated OTC-induced gut damage in zebrafish, suggesting its potential as a beneficial dietary intervention strategy. However, further investigation into the mechanisms by which kefir reversed OTC-induced gut damage would be valuable. Analysis of the microbial community composition of kefir following a 24-h fermentation revealed various probiotics, predominantly *L. kefiranofaciens*, *L. kefiri*, unclassified_g_*Pseudomonas*, *P. fragi*, unclassified_f_Alcaligenaceae, *L. parakefiri* and *B. cereus*_03BB102. These findings align with some previous studies[21-23] on bacterial community structures in kefir, although some discrepancies might stem from variations in kefir grain origins.

The findings from the intestinal histopathology and immunohistochemistry analyses demonstrated that OTC exposure led to severe damage to intestinal tissue and caused intestinal inflammation. Specifically, OTC exposure resulted in evident cilia shedding or defects, disordered goblet cells, and epithelial cell apoptosis. This shedding further compromises the integrity of the intestinal barrier, exacerbating the detrimental effects of OTC on the intestines. The integrity of the intestinal barrier could also be related to tight junction proteins, which regulate the passage of molecules, ions, and exogenous substances between adjacent cell membranes[24]. Kefir supplementation reversed the decreased expression of tight junction genes (*ZO-1a* and *ZO-1b*) induced by OTC, thereby contributing to the restoration of intestinal barrier function. At the molecular level, another mechanism by which OTC induces the weakening of intestinal barrier function might be largely linked to oxidative stress, as supported by our experiments on antioxidant activities and some previous reports[25-26]. Research has shown that kefir protects the intestines through its antioxidant properties[27-28], particularly due to the primary types of *Lactobacillus* present in kefir. Previous studies have reported the antioxidant activity of specific *Lactobacillus* strains, such as *Lactobacillus kimchicus* DCY51, *Lactobacillus sake*, and *Lactobacillus plantarum* MC5[29-31]. Additionally, *Lactobacillus* could enhance the integrity of the intestinal barrier by promoting immune organ development and increasing the proportions of T cells and B cells[32]. Therefore, one of the mechanisms by which kefir reverses intestinal damage caused by OTC is by the protection of intestinal cilia cells from oxidative stress within the host, enhancing the gene expression of tight junction proteins and thereby preserving the integrity of intestinal barrier function.

Another mechanism by which kefir reverses OTC-induced gut damage might be its ability to restore the disrupted gut microbiota caused by OTC and further impact host metabolism. Our findings indicate that OTC exposure significantly decreased the richness of the microbiota, resulting in an altered structure of the intestinal flora. However, no significant difference in microbial diversity was found among the groups. Interestingly, our results differ from those of previous reports[5], which indicated that OTC exposure could lead to the decrease in both species richness and diversity. We speculate that these discrepancies might be attributed to variations in the duration and concentration of OTC treatment. In control groups, the intestinal flora was mainly composed of Proteobacteria, Actinobacteria, Bacteroidota, and Fusobacteria, which is similar with some previous reports[33]. Interestingly, the gut microbiota composition of zebrafish is quite different from that of mammals. While the mammalian gut is primarily dominated by Firmicutes, Bacteroidetes, and Actinobacteria, Proteobacteria are the predominant phylum in zebrafish[34-35]. This difference could be attributed to some factors like living environment, diet, and evolutionary divergence between fish and mammals. Compared with that of mammals, the microbiota of zebrafish reflects their aquatic environment, which exposes them to different microbial influences and pressures. The dominance of Proteobacteria in zebrafish might be associated with their ability to adapt to aquatic conditions rich in these bacteria. In the OTC treatment group, the proportion of Proteobacteria greatly increased compared to that in the control group, thereby leading to a reduction in the proportions of Actinobacteria, Bacteroidota, and Firmicutes. This observation implies that Proteobacteria might possess a heightened capacity for antibiotic resistance in comparison to other phyla within the gut of zebrafish, which was similar to the findings by previous studies[36]. Proteobacteria tends to accumulate antibiotic resistance genes during exposure to OTC[37]. However, the addition of kefir as a supplement led to a notable reversal impact on the changes induced by OTC. In the OTC + kefir group, a significant increase in the proportion of Firmicutes, especially *Lactobacillus,* was observed. *Lactobacillus* is considered to be key importance to the gut health of zebrafish[38]. Interestingly, the *Lactobacillus* species of the zebrafish in the OTC + kefir group mainly included *L. kefiranofaciens* and *L. kefiri*, which are considered to be derived from the kefir microbiota. In previous studies, both *L. kefiranofaciens* and *L. kefiri* demonstrated specific probiotic activities *in vitro*. *In vitro* experiments proved that *Lactobacillus kefiranofaciens* enhanced epithelial barrier function by increasing transepithelial electrical resistance[39]. Additionally, *L. kefiranofaciens* XL10 displayed favorable acid tolerance, cell surface hydrophobicity, and aggregation, which were advantageous for intestinal colonization[40]. Moreover, *L. kefiri* exhibited *in vitro* antioxidant and anti-tumor activities[41-42]. Notably, the colonization of microorganisms in the intestine poses significant challenges, demanding ability against harsh conditions including gastric acid, bile salts, and digestive enzymes[43]. Although our study did not conclusively demonstrate the long-term colonization of zebrafish intestines by *L. kefiranofaciens* and *L. kefiri*, we believe that their short-term enrichment in the zebrafish gut could be beneficial for the restoration of damaged gut microbiota.

The results of the metabolomic analysis indicate that exposure to OTC potentially alters hepatic metabolite abundance. In particular, the metabolic pathways of glycerophospholipid metabolism, the TCA cycle and amino acid metabolism were significantly inhibited among all pathways. In glycerophospholipid metabolism, phosphatidylserine (PS) synthesis involves the reaction between CDP-DG and serine. Subsequently, PS undergoes decarboxylation mediated by phosphatidylserine decarboxylase (PSD), which converts to phosphatidylethanolamine (PE) and further transforms into phosphatidylcholine (PC) and LysoPC[44]. Our findings indicated a notable decrease in LysoPC and PE levels following OTC treatment, suggesting inhibition of PE synthesis pathways, particularly *via* PSD, leading to the accumulation of CDP-DG and PS. PSD is not the only pathway for PE synthesis, but it is the primary pathway[45]. PS and PE are essential aminophospholipids that are critical for cell membrane structure and pivotal in various biological processes, including cell signaling. Reduced PE levels can compromise cell membrane fluidity, particularly affecting barrier function in intestinal epithelial cells. Moreover, increased PS synthesis rates might promote cellular apoptosis and contribute to inflammation[46]. In addition to glycerophospholipid metabolism, heterogeneous metabolic profiles were evident for TCA cycle intermediates. Specifically, the levels of malic acid, succinic acid, and phosphoenolpyruvic acid were lower in the OTC-treated groups than in the control groups, while the levels of oxoglutaric acid, *S*-acetyldihydrolipoamide-E, isocitrate, and citric acid were elevated. This suggests a blockade in the TCA cycle at the succinic acid stage in samples treated with OTC. Importantly, these heterogeneous metabolic profiles in the TCA cycle could further disrupt the synthesis or degradation of amino acids such as lysine, arginine, and glutamate. Notably, most of the changes in metabolite abundance induced by OTC could be reversed by kefir supplementation. This reversal impact could be partially attributed to alterations in the zebrafish gut microbiota caused by kefir supplementation. As predicted by PICRUSt2, the gut microbiota in the OTC + kefir group encoded enzymes with enhanced abilities to regulate energy production, lipid metabolism, protein modification, and metabolism compared with those in the OTC group. From this perspective, the finding indicated a possible therapeutic effect of probiotics in kefir on metabolic pathway impairment, as mentioned above.

Finally, we investigated the relationship between the main bacterial species in the intestinal microflora and differential metabolites. Studies have shown that changes in microbiota composition significantly impact host homeostasis, physiology, metabolic profiles, and disease susceptibility[47]. Our correlation analysis revealed that several key bacterial species—specifically, s_unclassified_g_*Methylobacterium-Methylorubrum*, s_unclassified_g_*Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* and s_unclassified_g_*Citrobacter* exhibited a negative correlation with the abundance of differentially abundant metabolites when comparing the control and OTC-treated groups. Notably, all three species belong to the Proteobacteria phylum. The inhibition of Proteobacteria has been largely linked with improvements in gut functions and inflammatory conditions, such as experimental colitis and cholangitis, through mechanisms involving *D*-amino acids[48]. Additionally, Proteobacteria have been shown to influence host metabolism, particularly amino acid levels, which could modulate responses to neurological stimuli such as cocaine[49]. These findings underscore the potential of Proteobacteria in regulating intestinal ecosystem health, despite its classification as a major phylum in the adult zebrafish gut microbiota.

Comparing the OTC and OTC + kefir groups, we observed a positive correlation between *L. kefiranofaciens* as well as the *L. kefiri*

and the abundance of most differentially abundant metabolites involved in glycerophospholipid metabolism, the TCA cycle, and amino acid metabolism. These *Lactobacillus* species might exert inhibitory effects on Proteobacteria, as suggested by correlation analysis heatmaps of the predominant gut microbiota. The mechanisms contributing to this inhibition could include nutrient competition, the production of antimicrobial compounds, or alterations in intestinal pH due to organic acid accumulation from kefir[50-52]. Furthermore, changes in the microbial balance between Proteobacteria and Firmicutes could affect host nutrient absorption[53]. Interestingly, other *Lactobacillus* species from kefir, such as *Lactobacillus parakefiri*, were not detected in the intestinal microflora in the OTC + kefir group, indicating that *L. kefiranofaciens* and *L. kefiri* are more likely to be the key species responsible for the intestinal restoration observed in OTC + kefir group.

5. Conclusion

This study demonstrated the potential of kefir supplementation in reversing intestinal damage induced by OTC in zebrafish. The beneficial effects of kefir on OTC-induced intestinal damage could be mainly attributed to 2 main factors. First, kefir contributes to the restoration of intestinal barrier integrity by improving the expression levels of tight junction proteins and increasing the activity of antioxidant enzymes. Second, the enrichment of *L. kefiranofaciens* and *L. kefiri* in the zebrafish intestine from kefir consumption was beneficial for inhibiting certain species within the Proteobacteria phylum, such as s_unclassified_g_*Methylobacterium-Methylorubrum* and s_unclassified_g_*Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* in the OTC groups. This inhibition helped to reverse the disordered metabolic profile (TCA cycle, glycerophospholipid metabolism, and amino acid metabolism) induced by OTC. Overall, our study identified the key species that reversed intestinal damage and altered host metabolism induced by OTC through kefir consumption.

Conflict of interest

All the authors declare no conflicts of interest.

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Appendix A. Supplementary information

Supplementary data associated with this article can be found, in the online version, at http://doi.org/10.26599/FSHW.2024.9250380.

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