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One-Health meets microbiota: Interactions between digestive tract microbiota, host, and environment

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One-Health meets microbiota: Interactions between digestive tract microbiota, host, and environment

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Differences in peripheral and central metabolites and gut microbiome of laying hens with different feather-pecking phenotypes

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Background: Feather pecking (FP) is a maladaptive behavior in laying hens that is associated with numerous physiological traits, including those involving the central neurotransmitter system and the immune system, which have been identified in many species as being regulated by the gut microbiota *via* the "microbiota-gut-brain" (MGB) axis. Yet, it is unknown whether and how gut microbiota influences FP by regulating multiple central neurotransmission systems and immune system.

Methods: This study was measured the prevalence of severe FP (SFP) in the commercial layer farm. The chicken flock with the highest frequency of SFP were selected for FP phenotype identification. Nontargeted metabolomics was performed to investigated the differences in the peripheral and central metabolites and 16S rDNA sequencing was performed to investigated the differences in gut microbiome of laying hens with different FP phenotypes. Correlation analysis was performed to determine the potential mechanism by which the disturbed gut microbiota may modulate host physiology and behavior.

Results: The results showed that pullets (12weeks of age) showed significantly higher SFP frequencies than chicks (6weeks of age) and adults (22weeks of age; $p<0.05$). Compared to neutrals (N), peckers (P) exhibited the stress-induced immunosuppression with the increased plasma levels of corticosterone and norepinephrine, and the decreased plasma levels of IgA, IL-1, IL-6 and tumor necrosis factor α ($p<0.05$). In the cecum, the relative abundances of *Bacteroides* and *Gemmiger* were higher in the P group, while *Roseburia*, *Ruminococcus2*, *Anaerostipes*, *Lachnospiracea_incertae_sedis* and *Methanobrevibacter* were more enriched in the N group. Moreover, increased plasma levels of L-tryptophan, beta-tyrosine and L-histidine were found in the P group ($p<0.05$). Notably, in the P group, hippocampal levels of L-tryptophan, xanthurenic acid, L-histidine and histamine were improved and showed a positive association with L-glutamic acid levels. Plasma levels of L-tryptophan, beta-tyrosine and L-histidine were both positively correlated with *Bacteroides* abundance but negatively correlated with *Methanobrevibacter* abundance.

Conclusion: Overall, these findings suggest that the development of FP may be affected by the gut microbiota, which regulates the central glutamatergic nerve system by altering the metabolism of tryptophan, histidine and tyrosine.

KEYWORDS

microbiota-gut-brain axis, laying hens, feather pecking, glutamatergic nerve system, metabolism, the commercial layer farm

1. Introduction

Feather pecking (FP) is a maladaptive behavior with an identified prevalence of 80% in all laying hens housing systems (Gunnarsson, 1999). FP was divided into gentle feather peck (GFP) and severe feather pecking (SFP). SFP, a detrimental type of FP, can cause feather loss and skin damage, and in some cases, this can escalate to severe injuries and cannibalism, while GFP is suggested to be similar to social exploration without damage (Kops et al., 2013). What's more, SFP can spread rapidly through learning among chickens, severely damaging animal welfare and causing economic losses (Rodenburg et al., 2013). Therefore, FP, especially SFP in laying hens is one of the most important unsolved behavioral issues in modern agriculture.

FP is multifactorial and has been linked to numerous behavioral characteristics, such as fearfulness, stress sensitivity and depression, but also to the central and peripheral physiological characteristics (Rodenburg et al., 2013). Deficiency or redundancy in the central serotonergic system can predispose birds to develop FP, while birds with high FP tendency generally have low rates of central serotonin (5-HT) and dopamine (DA) turnover at a young age but high turnover in the brain at an adult age (de Haas and van der Eijk, 2018). The brain transcriptomes of laying hens divergently selected for FP reveal the potential role of GABAergic and glutamatergic neurotransmitter systems in the development of FP (Falker-Gieske et al., 2020). Birds selected for high FP (HFP) and low FP (LFP) differ in innate and adapted immune characteristics (van der Eijk et al., 2019a,b). These studies above suggest that the occurrence of FP may be related to alterations in multiple central neurotransmission systems and immune system, however, there is a lack of clear evidence for the cause of these alterations.

The gastrointestinal tract is a complex ecosystem containing a large number of resident microorganisms that have been found to play an important role in the maintenance of host behavior *via* the "microbiota-gut-brain" (MGB) axis (Zheng et al., 2019). Recent evidence suggests that alterations in gut microbiota composition, *via*, for example, anti-, pre-or probiotic treatment, affect anxiety, stress sensitivity and fearfulness (Desbonnet et al., 2010; Ait-Belgnaoui et al., 2014). Moreover, the regulatory effects of the gut microbiota on serotonergic, dopaminergic, GABAergic and glutamatergic neurotransmitter systems in the central nervous system (CNS) and immune system have also been identified by many studies (Miller et al., 2011; Zheng et al., 2019; Hasebe et al., 2021). Notably, a growing number of studies are focusing on the potential role of the regulation of the gut microbiota in FP development. Laying hens that are divergently selected for FP (HFP and LFP) show significant differences in gut microbiota composition (Birkl et al., 2018; van der Eijk et al., 2019c). Early-life transplantation of microbiota from HFP birds influences the behavioral and physiological characteristics that are related to FP (van der Eijk et al., 2020). Collectively, these findings highlight the novel possibility that disturbances of gut microbiota or the MGB axis may contribute to the onset of FP in laying hens. Yet, it is unknown whether and how gut microbiota influences FP by multiple central neurotransmission systems and immune system. Metabolism is an important pathway of two-way communication between gut microorganisms and the brain (Zheng et al., 2019), however, there is a lack of systematic studies on the metabolism in laying hens with different FP phenotypes (including pecker, victim and neutral).

Outcomes from numerous research methods, including improving the house climate (light intensity, temperature, humidity and sound) and foraging condition (rearing intensity, feed shape and nonstarch polysaccharide concentration), indicate that the appropriate rearing environment plays a key role in alleviating the development of FP in laying hens (Lambton et al., 2010; Gilani et al., 2013). The physically, nutritionally, sensorially and socially restricted environment in which the majority of commercial laying hens hatch and live can be a powerful social and environmental chronic stressor that could induce a high occurrence rate of SFP (Maes et al., 2009). Therefore, the aim of this research was to investigate the prevalence of SFP in the commercial layer farm, as well as the metabolic characteristics and the gut microbial characteristics of laying hens with different FP phenotypes, and to reveal the potential correlation between gut microbiota and FP.

To address this issue, the frequency of SFP in the three stages of laying hens, which include chick, pellet and adult was investigated in a commercial layer farm. The chicken flock with the highest frequency of SFP were selected for FP phenotype identification and laboratory analysis. 16S ribosomal RNA (16S rRNA) gene sequencing analysis was performed to reveal the difference in the gut microbial communities of laying hens with different FP phenotypes. We conducted nontarget metabolomic analysis of the plasma and hippocampus to investigate metabolic changes in the peripheral and central systems. Finally, correlation analysis was performed to determine the potential mechanism by which the disturbed gut microbiota may modulate host physiology and behavior.

2. Materials and methods

2.1. Animals and housing conditions

This research was conducted in a commercial layer farm (Guangdong Lvyang Agricultural Co., LTD, Guangdong Province, China). First, preliminary FP observation was conducted for three consecutive days to select the chicken flock with the highest frequency of SFP among three different stages of chicken flocks (chick, pellet and adult stages of chicken) of beak-trimming Hyline gray laying hens. Specifically, 6 cages were randomly selected from laying hens at 6 weeks of age (40 chicks per cage), 12 weeks of age (25 pullets per cage) and 22 weeks of age (10 adults per cage), and pullet flocks at 12 weeks of age showed more SFP (Figure 1). Therefore, a total of 200 12-weeks-old beak-trimmed birds (8 cages, 25 birds per

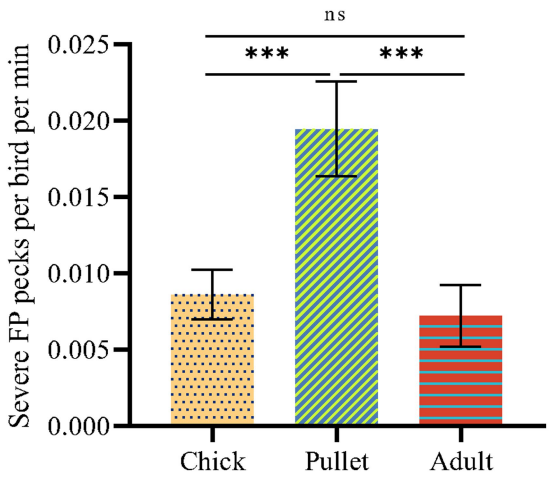

FIGURE 1
The SFP frequencies of birds at different stage of laying hens. The statistically significant differences between different groups are indicated as asterisks (*p<0.05, **p<0.01, and ***p<0.001).

cage) were then selected, individually identified using numbered silicone backpacks (6 × 6 × 0.5 cm; Birkl et al., 2017) and transferred to the top cage without disturbing the flock. Birds were kept in battery layer cages (120 l*60 W*45 H cm for chicks, 100 l*50*45 H cm for pullets and 120 l*60 W*45 H cm for adults) and reared under conventional management conditions on a commercial farm. One camera (HIKVISION DS-2CD2T55(D)-I3, Hangzhou, China) was installed 1 meter above each cage to enable a full view of the cage. The cages were arranged at intervals, so it was certain that there was no visual contact between the different cages. At 6 weeks of age, the light was on for 9 h, from 8:00 until 17:00. At 12 weeks of age, the light was provided from 6:00 to 22:00, and this stayed the same throughout the laying period. Birds had *ad libitum* access to well water and commercial layer feed. The animals used in this study were treated in accordance with the approval of the Scientific Ethics Committee of South China Agricultural University under approved permit number SYXK2014-0136.

2.2. Behavioral observations

For the preliminary FP observation, each cage was video recorded for 10 min (1 × in the morning between 8 and 11 am, 1 × in the after between 3 and 5 pm) on 3 consecutive days. FP was divided into GFP (subdivided into exploratory FP and stereotyped FP) and SFP as adapted from (Birkl et al., 2017). SFP was defined as follows: "A bird grips and pulls or tears vigorously at a feather of another bird with her beak, causing the feather to lift up, break or be pulled out. The recipient reacts to the peck by vocalizing, moving away or turning toward the pecking bird." The number of SFP events was observed using the video and recoded at the cage level. After individual identification and transfer, birds were provided 2 weeks before the experiment started. Backpacks were fastened around the wings *via* two elastic straps secured to the backpacks with metal eyelets (Mindus et al., 2021). At 14 weeks of age, FP behavior was observed at an individual level. SFP was observed from video recordings, and each observation lasted 20 min and was performed once in the morning (8:30–11:30) and once in the afternoon (14:00–17:00). The number of SFP events, either given or received, was summed over 4 consecutive days, thus including one morning and one afternoon observation with a total observation period of 40 min, and was used to identify FP phenotypes (Daigle et al., 2015). When a bird gave more than one and received zero or one severe FP, it was defined as a pecker (P). When a bird gave and received zero or one severe feather peck, it was defined as neutral (N). We did not include victims or feather pecker-victims in this study. All-occurrence sampling was used to record initiators and recipients of SFP interactions. An occurrence was defined as a sequence of uninterrupted behavior lasting more than 4 s aimed at the same bird (Birkl et al., 2017). All behavioral observations were performed by a trained, blinded observer.

2.3. Sample collection

After FP observation, one pecker and one natural were chosen from each cage, for a total of 16 hens. Blood samples were collected from the wing vein using EDTA-coated vacutainer tubes. Blood samples were stored on ice (maximum of 4 h) until centrifugation (4°C, 2,500 rpm, 15 min) for plasma separation. Plasma was aliquoted into 1.5 ml microtubes and stored at-80°C until further determination of plasma stress and immune indices. After blood sampling, the birds were euthanized by cervical dislocation to obtain the contents of the duodenum, ileum and caeca. Gut contents were stored in 2 ml cryovials at-80°C until further analysis. The hippocampus, considered the memory and learning area in both mammals and birds, was quickly sampled on ice and stored in liquid nitrogen (Colombo and Broadbent, 2000).

2.4. Measurement of plasma stress and immune indices

The plasma levels of interleukin-1 (IL-1), interleukin-6 (IL-6), immunoglobulin A (IgA), immunoglobulin G (IgG), tumor necrosis factor α (TNF-α), and corticosterone (CORT) (Item No#: YJ059829, YJ042757, YJ002792, YJ042771, YJ002790, YJ059881, Shanghai Enzyme Linked Biotechnology Co., Ltd., Shanghai, China) were measured using a commercially available ELISA kit following the manufacturer's protocol. The optical density of each sample was read at 450 nm using Nessler's reagent spectrophotometry (Shanghai Ao Yi Technology Co., Ltd., Shanghai).

2.5. DNA extraction and 16S rRNA gene sequencing

The total bacterial DNA of each sample of gut content was extracted using the QIAamp PowerFecal Pro DNA Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. DNA quality was examined by electrophoresis on a 1% agarose gel, and the final DNA concentration of each sample was determined using an ultrafine, ultraviolet spectrophotometer (Shanghai Ao Yi Technology Co. Ltd., Shanghai). DNA samples were stored at-80°C until further analysis.

The 16S rRNA gene amplicons were used to determine diversity and compare the structures of bacterial communities among samples to reveal taxonomic composition. Next-generation sequencing library preparations and Illumina NoveSeq sequencing were conducted at Novogene Co., Ltd., Tianjin, China. The V3-V4 hypervariable regions of the 16S rRNA genes were amplified with forward primers containing the sequence 5'-CCTAYGGGRBGCASCAG-3′ and reverse primers containing the sequence 5'-GGACTACNNGGGTATCT AAT-3′. Furthermore, indexed adapters were added to both ends of the 16S rDNA amplicons to generate indexed libraries ready for downstream NGS sequencing on the Illumina NovaSeq platform. The quality of each DNA library was validated with an Agilent 2,100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, United States), and the concentration was measured by a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Carlsbad, CA, United States). DNA libraries were multiplexed and loaded on an Illumina NovaSeq6000 instrument according to the manufacturer's instructions (Illumina, San Diego, CA, United States). Sequencing was performed using the 2 × 300 bp paired-end configuration. Image analysis and base calling were performed by NovaSeq Control Software embedded in the NovaSeq instrument.

2.6. Nontargeted metabolomics

Thawed plasma samples at 4°C were vortexed for 1 min after thawing and mixed evenly as described in previous research (Zelena et al., 2009) with some modifications. For metabolite extraction, cold methanol (stored at-20°C) was added at a ratio of 400 µl per 100 µl of plasma. The samples were then vortexed for 1 min and centrifuged for 10 min at 4°C and 13,000 rpm. The samples were kept on ice between the steps. The supernatant was transferred to a new 2 ml centrifuge tube and dissolved in 150 µl of 2-chloro-l-phenylalanine (4 ppm) solution prepared with 80% methanol water (stored at 4°C). The supernatant was filtered with a 0.22 µm membrane and inserted into HPLC vials for analysis. To extract hippocampal metabolites, samples weighing 60 mg were ground at 50 Hz for 1 min in 1 ml of cold tissue extract (75% 9:1 methanol: chloroform, 25% H_2O) with 3 steel balls (Want et al., 2013). After room temperature ultrasound for 30 min and ice bath for 30 min, the samples were centrifuged at 12000 rpm and 4°C for the supernatant. The supernatant was then redissolved with 200 µl of 50% acetonitrile solution prepared with 2-amino-3-(2-chlorophenyl)-propionic acid (4 ppm; stored at 4°C) and filtered through a 0.22 µm membrane for Liquid chromatography-mass spectrometry (LC–MS) detection.

All samples were analyzed by LC–MS. Liquid chromatography (LC) analysis was performed on a Vanquish UHPLC System (Thermo Fisher Scientific, United States). Chromatography was carried out with an ACQUITY UPLC ® HSS T3 (150 × 2.1 mm, 1.8 µm; Waters, Milford, MA, United States). The column temperature was maintained at 40°C. The flow rate and injection volume were set at 0.25 ml/min and 2 µl, respectively. For LC-ESI (+)-MS analysis, the mobile phases consisted of (C) 0.1% formic acid in acetonitrile (v/v) and (D) 0.1% formic acid in water (v/v). Separation was conducted under the following gradient: 0 ~ 1 min, 2% C; 1 ~ 9 min, 2% ~ 50% C; 9 ~ 12 min, 50% ~ 98% C; 12 ~ 13.5 min, 98% C; 13.5 ~ 14 min, 98% ~ 2% C; 14 ~ 20 min, 2% C. For LC-ESI (−)-MS analysis, the analytes were carried out with (A) acetonitrile and (B) ammonium formate (5 mM). Separation was conducted under the following gradient: 0 ~ 1 min, 2% A; 1 ~ 9 min, 2% ~ 50% A; 9 ~ 12 min, 50% ~ 98% A; 12 ~ 13.5 min, 98% A; 13.5 ~ 14 min, 98% ~ 2% A; 14 ~ 17 min, 2% A. Mass spectrometric (MS) detection of metabolites was performed on a Q Exactive (Thermo Fisher Scientific, United States) with an ESI ion source. Simultaneous MS1 and MS/MS (Full MS-ddMS2 mode, data-dependent MS/MS) acquisition was used. The parameters were as follows: sheath gas pressure, 30 arb; aux gas flow, 10 arb; spray voltage, 3.50 kV and-2.50 kV for ESI(+) and ESI(−), respectively; capillary temperature, 325°C; MS1 range, m/z 81–1,000; MS1 resolving power, 70,000 FWHM; number of data-dependent scans per cycle, 10; MS/MS resolving power, 17,500 FWHM; normalized collision energy, 30%; dynamic exclusion time, automatic.

2.7. Statistical analysis

2.7.1. Behavior observation data analysis

The SFP frequencies were determined per individual cage per min and averaged at the cage level. IBM SPSS Statistic 26 software (IBM Corp., Armonk, NY) was used to compare the SFP frequencies by one-way analysis of variance (ANOVA). Multiple comparisons were conducted using the Duncan method. The data are presented as the means ± standard errors (SE). Significant differences were reported as those with $p < 0.05$.

2.7.2. Plasma stress and immune data analysis

The plasma stress and immune data were analyzed with independent sample T tests using IBM SPSS Statistic 26 software (IBM Corp., Armonk, NY). The data are presented as the means ± SE. Significant differences were reported as those with $p < 0.05$.

2.7.3. 16S rRNA gene sequencing data analysis

The 16S rRNA gene sequencing data were analyzed using the QIIME data analysis package (V1.9.1[1]) in R software (version 4.0.3) and R studio (version 1.3.1093). The forward and reverse reads were joined to form joined sequences. After removing barcode and primer sequences, the reads of each sample were spliced using FLASH V 1.2.7.[2] Quality filtering of the joined sequences was performed. Sequences that did not fulfill the following standards were discarded: sequence length < 200 bp, no ambiguous bases, and mean quality score ≥ 15. The remaining sequences were compared with the reference database (RDP Gold database) using the UCHIME algorithm[3] to detect chimeras. Sequences with chimeric sequences were removed from further analysis. Filtered sequences were grouped into operational taxonomic units (OTUs) using the clustering program VSEARCH (V2.15.1[4]) against the Silva 119 database at 97% sequence identity. The Ribosomal Database Program (RDP) classifier was used to assign taxonomic categories to all OTUs at a confidence threshold of 0.8. The α and β diversity analyzes were conducted using USEARCH (V10.0.240[5]).

1 http://qiime.org/scripts/split_libraries_fastq.html
2 http://ccb.jhu.edu/software/FLASH/index.shtml
3 https://www.drive5.com/usearch/manual/uchime_algo.html
4 https://github.com/torognes/vsearch/releases
5 https://www.drive5.com/usearch/download.html

Alpha diversity (α-diversity) indices, including the Shannon index for diversity[6] and the Chao1 index for richness,[7] were calculated by QIIME (1.9.1) from rarefied samples. Difference analyzes of the alpha diversity index, parametric tests and nonparametric tests were conducted. Because there were only two groups, the T test was used for the difference analysis of the alpha diversity index. Beta diversities were calculated using unweighted UniFrac distances. The unweighted pair group method with arithmetic mean was used to generate dendrograms from the beta diversity distance matrix. The principal coordinate analysis (PCoA) and the significance analysis of microbial structure were performed using the OmicStudio tools at https://www.omicstudio.cn/tool. The PCoA analysis was performed based on the unweighted unifrac distance and the *p* value was calculated using the analysis of similarity (ANOSIM). The key bacterial taxa responsible for discrimination between the two groups were identified using linear discriminant analysis (LDA) effective size (LEfSe).[8] The threshold of the logarithmic LDA score was 3.5.

2.7.4. Nontargeted metabolomic data analysis

The raw data were first converted to mzXML format by MSConvert in the ProteoWizard software package (v3.0.8789) and processed using XCMS for feature detection, retention time correction and alignment. The metabolites were identified by accuracy mass (< 30 ppm) and MS/MS data, which were matched with HMDB,[9] massbank,[10] LipidMaps,[11] mzclound[12] and KEGG.[13] The robust LOESS signal correction (QC-RLSC) was applied for data normalization to correct for any systematic bias. After normalization, only ion peaks with relative standard deviations (RSDs) less than 30% in QC were kept to ensure proper metabolite identification.

Ropls software was used for all multivariate data analyzes and modeling. Data were mean-centered using scaling. Models were generated on principal component analysis (PCA), orthogonal partial least-square discriminant analysis (PLS-DA) and partial least-square discriminant analysis (OPLS-DA). The metabolic profiles could be visualized as a score plot, where each point represents a sample. The corresponding loading plot and S-plot were generated to provide information on the metabolites that influence the clustering of the samples. All the models evaluated were tested for overfitting with permutation tests. The descriptive performance of the models was determined by R2X (cumulative; perfect model: R2X (*cum*) = 1) and R2Y (cumulative; perfect model: R2Y (*cum*) = 1) values, while their prediction performance was measured by Q2 (cumulative; perfect model: Q2 (*cum*) = 1) and a permutation test. The permuted model should not be able to predict classes: R2 and Q2 values at the Y-axis intercept must be lower than those of Q2 and the R2 of the nonpermuted model. OPLS-DA allowed the determination of discriminating metabolites using the variable importance on projection (VIP). The *p* value, variable importance projection (VIP) produced by OPLS-DA and fold change (FC) were applied to discover the contributable variables for classification. Finally, results with a *p* value < 0.05 and VIP values > 1 were considered to be statistically significant metabolites. Differential metabolites were subjected to pathway analysis by MetaboAnalyst,[14] which combines results from powerful pathway enrichment analysis with pathway topology analysis. The metabolites identified in the metabolomics analysis were then subjected to KEGG pathway analysis for biological interpretation of higher-level systemic functions. The metabolites and corresponding pathways were visualized using the KEGG Mapper tool.

3. Results and discussion

3.1. Twelve-week-old birds showed more serious SFP

The SFP frequencies of birds at different stages are shown in Figure 1. Pullets showed significantly higher SFP frequencies than chicks and adults ($p < 0.05$). Compared to adults, pullets were kept at a higher rearing density. A previous study identified that the high rearing density of laying hens results in FP at the pullet stage and not the adult stage in hens (30 weeks of age) on commercial farms (Bestman et al., 2009). In this study, in addition to evaluating rearing density, continuous inspection and isolation of victims during commercial farming were investigated and may have been a potential factor contributing to the decrease in SFP at the adult stage of laying hens. The pullet stage is the stage of fastest weight gain, with higher stress vulnerability and more serious SFP than in chicks (Rodenburg et al., 2004). Therefore, pullets were selected for further investigation in this study.

3.2. Stress-induced immunosuppression in peckers

After 4 consecutive days of behavioral observation, one pecker and one natural were chosen from each cage, resulting in a total of 16 hens, for further investigation. The plasma levels of stress and immune indices were measured by ELISA (Figure 2). The plasma levels of IgA, IL-1, IL-6 and TNF-α in the P group were significantly lower than those in the N group ($p < 0.05$), while the IgG level showed no significant difference. Plasma CORT and Norepinephrine (NE) (Supplementary Figure S6) levels, which are measured in bird stress indices, were increased in the P group ($p < 0.05$). The increased stress hormone levels measured in the P group suggest that the FP birds were in a state of stress, and stress hormone signaling has been identified as the final common pathway involved in regulating the pathophysiological status and behavior of animals (Dallman et al., 1994). Various unpredicted extreme or mild chronic stresses, such as noise, mixing chicken breeds, strengthening or weakening light intensity and chicken transfer, have been found to contribute to the

6 http://scikitbio.org/docs/latest/generated/skbio.diversity.alpha.shannon.html#skbio.diversity.alpha.shannon

7 http://scikit-bio.org/docs/latest/generated/skbio.diversity.alpha.chao1.html#skbio.diversity.alpha.chao1

8 http://huttenhower.sph.harvard.edu/galaxy/

9 http://www.hmdb.ca

10 http://www.massbank.jp/

11 http://www.lipidmaps.org

12 https://www.mzcloud.org

13 http://www.genome.jp/kegg/

14 https://www.metaboanalyst.ca/

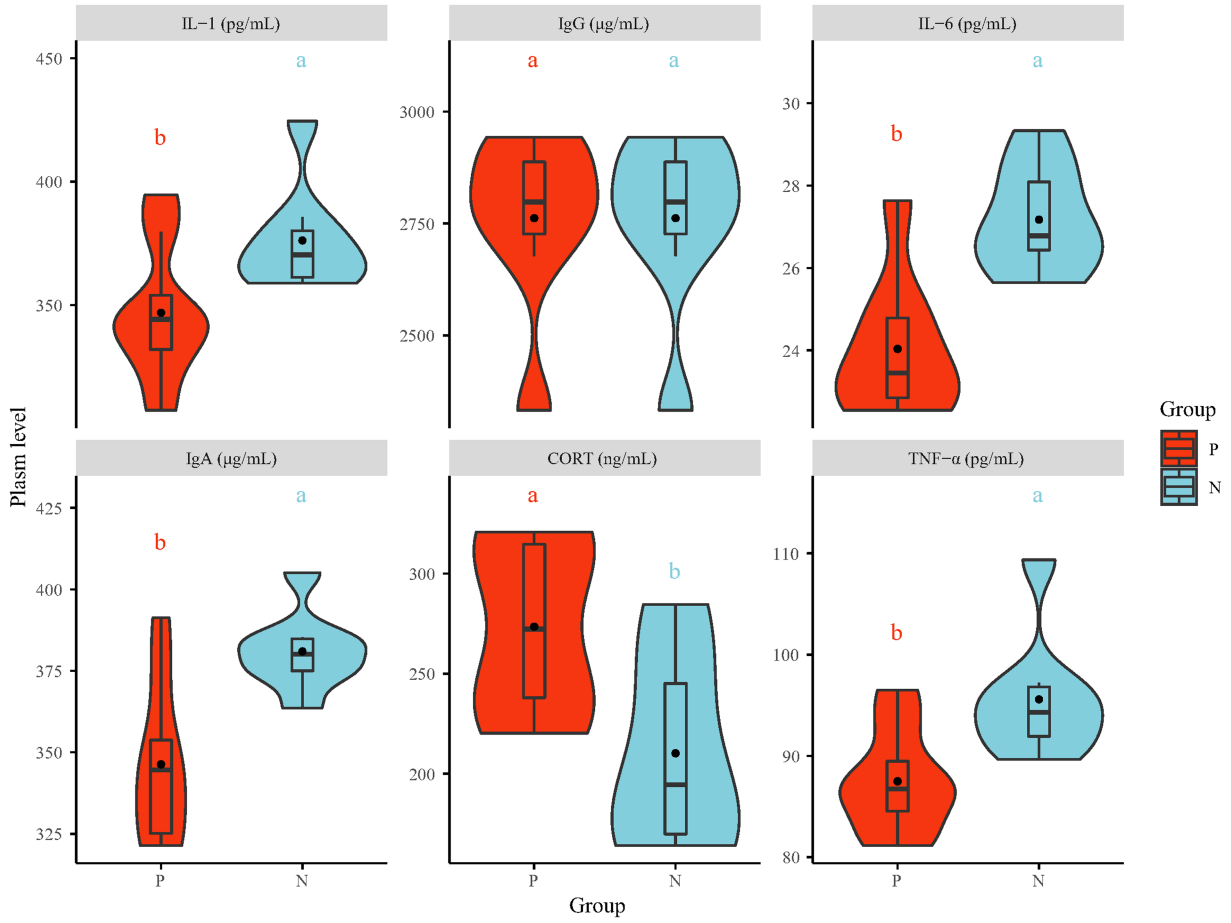

FIGURE 2
The plasma level of stress (CORT) and immune indices (IgA, IgG, IL-1, IL-6 and TNF-α). IgA= Immunoglobulin A, IgG=Immunoglobulin G, IL-1= interleukin-1, IL-6=interleukin-6, TNF-α=tumor necrosis factor-α, CORT=corticosterone. The values in the violin plot are the means ± SE (n=8). The statistically significant differences between the P and the N group are indicated as different letter (a, b; p<0.05). P=feather pecker, N=neutra.

development of FP in chickens (Maes et al., 2009). The outcomes from numerous experiments indicate that unpredicted stress is associated with many detrimental behaviors, including depression-like and anxiety-like behavior, *via* the immune system in both humans and animals (Ait-Belgnaoui et al., 2014). In this research, the suppressed immune system, which was associated with decreased levels of IgA and proinflammatory cytokines (IL-1, IL-6 and TNF-α), may have been the result of central anti-inflammatory cytokines (IL-10) expression caused by long-term stress (Mormède et al., 2003). Moreover, elevated stress hormone levels can lead to disrupted gut barrier function and altered commensal bacteria (Maes et al., 2013).

3.3. Changes in the intestinal microbiota community in peckers

In total, we obtained 967,536 high-quality reads across all cecal samples, and these reads were clustered into 509 OTUs at 97% sequence similarity. In the duodenum and ileum, 809,736 and 785,292 high-quality sequences and 788 and 817 OTUs were obtained, respectively. Most rarefaction curves tended to approach the saturation plateau, suggesting that the sequencing depth was sufficient to cover the whole bacterial diversity (Supplementary Figures S1A–C). Alpha diversity (α-diversity) showed that both the richness index (Chao 1) and diversity index (Shannon) of the duodenum, ileum and cecum did not differ between the P group and the N group (Figures 3A,B). To determine whether the microbial composition of birds with FP was substantially different from that of the N group, we carried out β-diversity analysis. Based on the unweighted UniFrac distance, PCoA revealed a significant difference in the cecal microbiota community ($p < 0.05$) but no significant difference in the duodenum and ileum ($p > 0.05$; Figure 3C; Supplementary Figures S2A,B). Therefore, we focused on the characteristics of the cecal microbial community in the rest of this study. At the phylum level, the top five phyla identified were *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Euryarchaeota* and *Fusobacteria* in the cecum of laying hens (Figure 4A). Among these phyla, *Firmicutes* and *Bacteroidetes* were dominant. At the genus level, the majority of 16S rRNA amplicons belonged to *Bacteroides*, *Faecalibacterium*, *Methanobrevibacter*, *Ruminococcus2*, *Alistipes* and *Lactobacillus* (Figure 4B). To further identify the cecal microbiota responsible for discriminating peckers from neutrals, we carried out LEfSe. This analysis identified 7 differential microorganisms responsible for the discrimination between the two groups at the genus level (Figures 4C,D). The relative

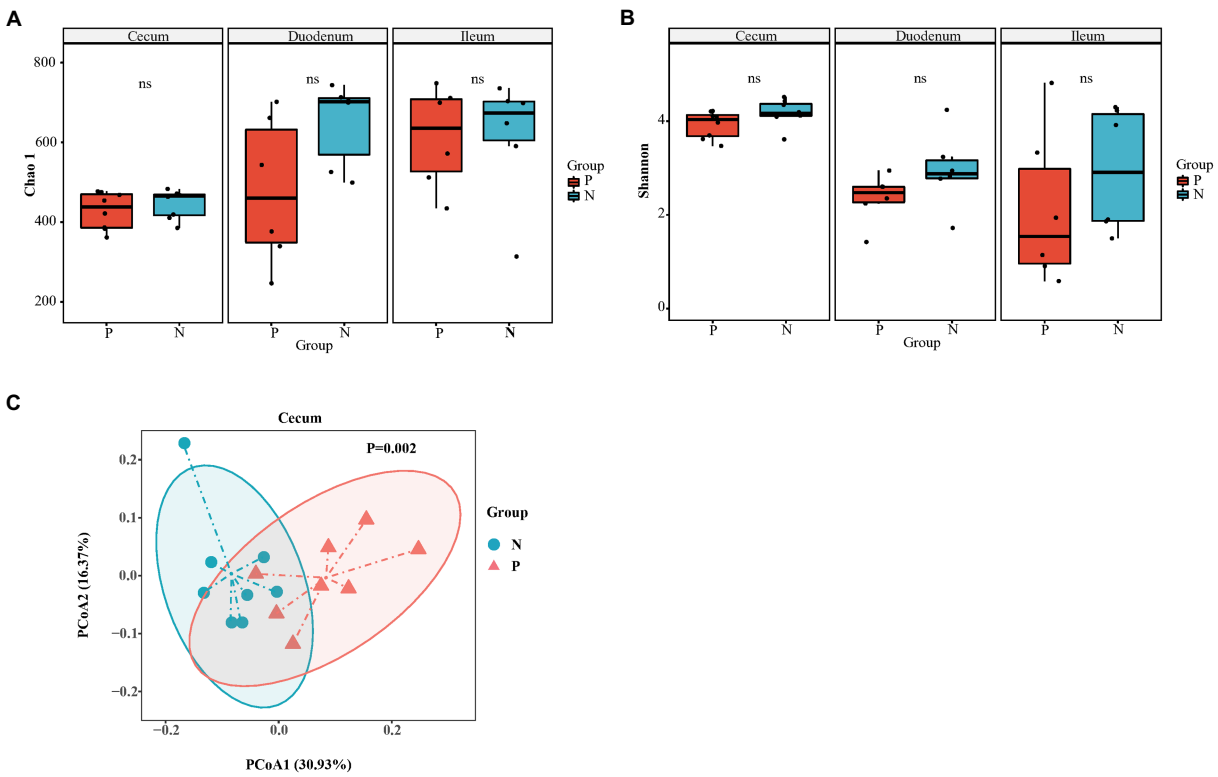

FIGURE 3
The diversity of gut microbiota of laying hen with different FP phenotype. **(A)** Chao 1 index of cecum (n=8), duodenum (n=6) and ileum (n=6); **(B)** Shannon index of cecum, duodenum and ileum; **(C)** The principal co-ordinates (PCoA) analysis of cecum based on the unweighted unifrac distance. P=feather pecker, N=neutral.

abundances of *Bacteroides* and *Gemmiger* were higher in the P group, while *Roseburia*, *Ruminococcus2*, *Anaerostipes*, *Lachnospiracea_incertae_sedis* and *Methanobrevibacter* were more enriched in the N group ($p < 0.05$). These data imply that the structure of the cecal microbiota was disordered in peckers.

The gut microbiota, known as the second brain, has been found to exert its effect on the brain and behavior by regulating catabolism or the nervous system based on the "gut-brain axis" (Zheng et al., 2019). To date, an increasing number of investigations have focused on whether gut microbiota affect feather pecking in laying hens. Early-life microbiota transplantation showed a long-term influence on depression-like and anxiety-like behavior related to FP in laying hens (van der Eijk et al., 2020). The gut microbiota analysis of LFP and HFP birds revealed significantly different diversity and composition, including increased abundance of *Lactobacillus* and decreased abundance of *Clostridiales* in HFP birds (van der Eijk et al., 2019c). Moreover, ingestion of *Lactobacillus rhamnosus* retards chronic stress-induced FP in chickens, suggesting the important role of gut microorganisms in relieving FP in birds (Mindus et al., 2021). The features of the gut microbiota found in this research showed a different pattern from those observed in previous studies, which could have been the result of multiple factors, including genetics, nutrition and stress (Maes et al., 2013; Tremblay et al., 2021). *Bacteroides*, *Ruminococcus2* and *Methanobrevibacter*, the main enriched microorganisms, were found to have a higher or lower relative abundance in peckers, which has a close association with depression-like and anxiety-like behavior (Chen et al., 2021; Zhang et al., 2022). Knowledge regarding the underlying associations between feather pecking and these differential microbes are vague and require further metabolomics analysis.

3.4. Changes in the metabolic profile in peripheral and central organisms in peckers

We further performed nontargeted metabolomics to determine whether the metabolic state reflected in the plasma and hippocampus were paralleled by an altered gut microbiota. To further explore the metabolic distinctions between the P and N groups, the multivariate statistical analysis was performed (Supplementary Figures S3–S5). Furthermore, a total of 94 metabolites were changed significantly in the P groups. The P group had 89 metabolites with higher levels and 5 metabolites with lower levels than in the N group ($p < 0.05$; Supplementary Figure S6; Supplementary Table S1). Tryptophan, as the precursor of the central major inhibitory neurotransmitter 5-HT, can pass through the blood brain barrier (BBB) and affect brain function and behavior (Hasebe et al., 2021). Tryptophan-5-HT deficiency has been identified to be involved in the development of many maladaptive behaviors in birds, such as aggression and FP (de Haas and van der Eijk, 2018). In the P group, however, the plasma concentration of L-tryptophan was significantly higher than that in the N group by 2.19-$\log_2$-fold ($p < 0.05$). Moreover, indole and quinolinic acid, downstream metabolites of tryptophan, also had

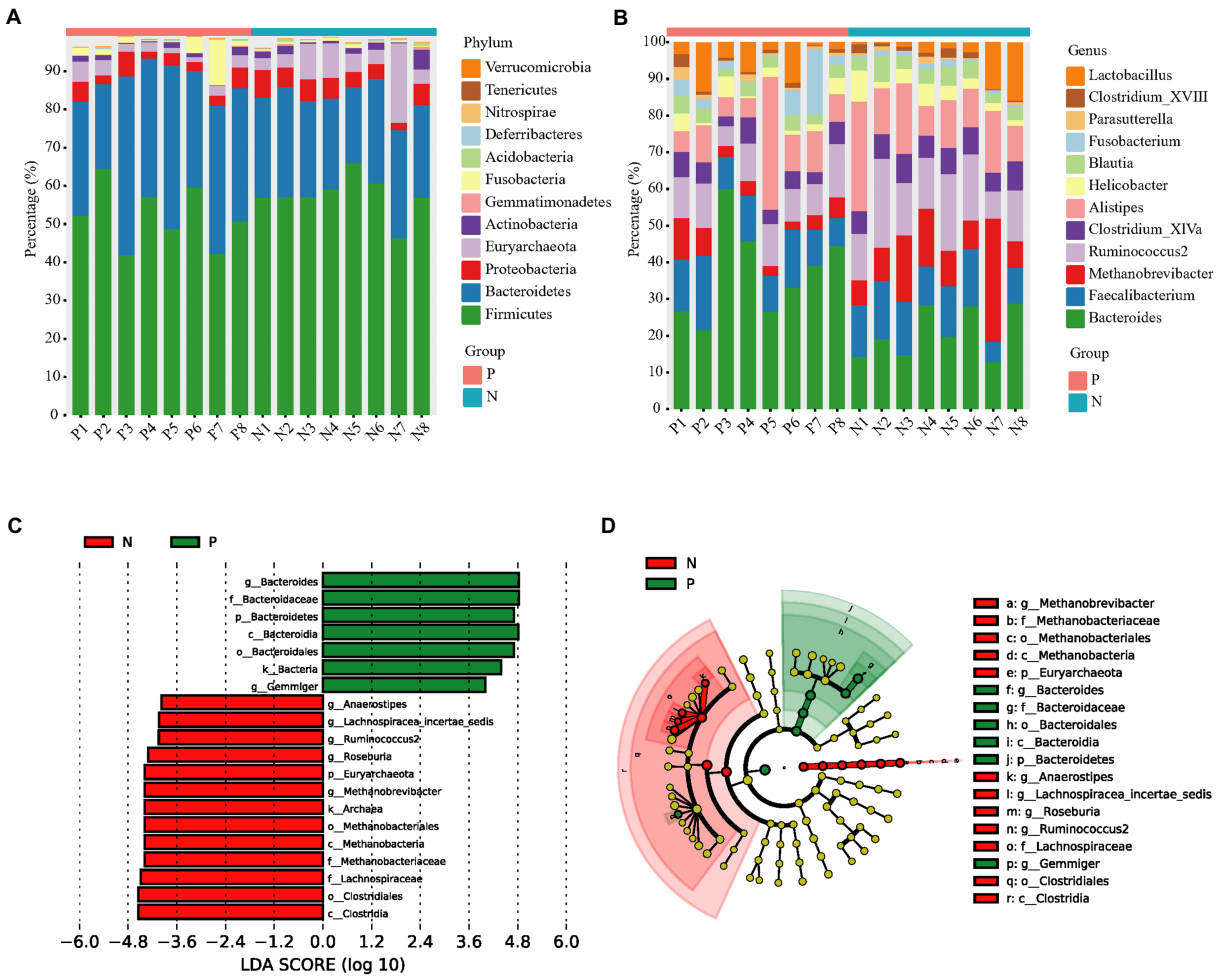

FIGURE 4
The relative abundance of cecal bacterial composition at the phylum and genus level and LefSe analysis of cecal microbiota. **(A)** The Relative abundance of cecal bacterial composition at phylum level; **(B)** The Relative abundance of cecal bacterial composition at genus level; **(C)** and **(D)** LEfSe analysis of cecal microbiota. The threshold of the logarithmic LDA score was 3.5. P=feather pecker, N=neutral.

log_2-fold increases of 1.78 and 2.36, respectively ($p < 0.05$). NE, which acts as a neurotransmitter and as a hormone, is usually activated after exposure to stress and has been found to have increased levels in the blood of HFP birds in response to manual restraint (Korte et al., 1997). In our research, the levels of NE and its precursor tyrosine were higher in the P group than in the N group ($p < 0.05$). Outcomes from various experiments suggest that cognition and memory function are tightly related to fluctuations in the histidine level in the peripheral system (Holeček, 2020). In the P group, the L-histidine concentration was more enriched in plasma than in the N group ($p < 0.05$). Notably, peripheral histidine and aromatic amino acids (AAA, tyrosine, phenylalanine and tryptophan), which had increased levels in P plasma, can be transported into the brain *via* large neutral amino acids (LNAAs) transporter and affect the CNS neurotransmitters histamine, 5-HT, glutamic acid and GABA (Oldendorf et al., 1988). To further investigate the characteristics of the metabolic profile in peckers, we conducted KEGG pathway enrichment analysis of differential metabolites *via* MetabAnaylst. Specifically, 9 metabolic pathways, including 'glycine, serine and threonine metabolism', 'alanine, aspartate and glutamate metabolism', 'beta-alanine metabolism', 'histidine metabolism' and 'tyrosine metabolism', were identified (Figure 5A; Supplementary Table S2). The peripheric change including glycine, serine and threonine metabolism', 'alanine, aspartate and glutamate metabolism', 'beta-alanine metabolism', 'histidine metabolism' and 'tyrosine metabolism' have previously reported to be associated with depression (Goto et al., 2017; Liu et al., 2020; Johnston et al., 2021; Solís-Ortiz et al., 2021). Next, the association between KEGG metabolic pathways and differential metabolites was exhibited by a network plot (Figure 5B). These enriched metabolic pathways exhibit a complicated interaction involving metabolites, including L-tryptophan, L-histidine and NE.

To determine the central effects of the changes in plasma metabolism and the cecal microbial community, hippocampal metabolomics analysis was performed. For subsequent differential metabolite screening and pathway enrichment analysis, multivariate statistical analysis was performed (Supplementary Figures S7–S9). According to the criteria: p value < 0.05 and VIP values > 1, 51 differential metabolites were detected in the metabolic profiles of the two groups. In the P group, the levels of 47 metabolites were higher and the levels of 4 metabolites were lower than in the N group (Supplementary Figure S10; Supplementary Table S3). It is worth noting that the L-glutamic acid concentration showed a log_2-fold increase of 1.51 in the hippocampus in group P ($p < 0.05$). Glutamic acid, which acts as the main excitatory neurotransmitter

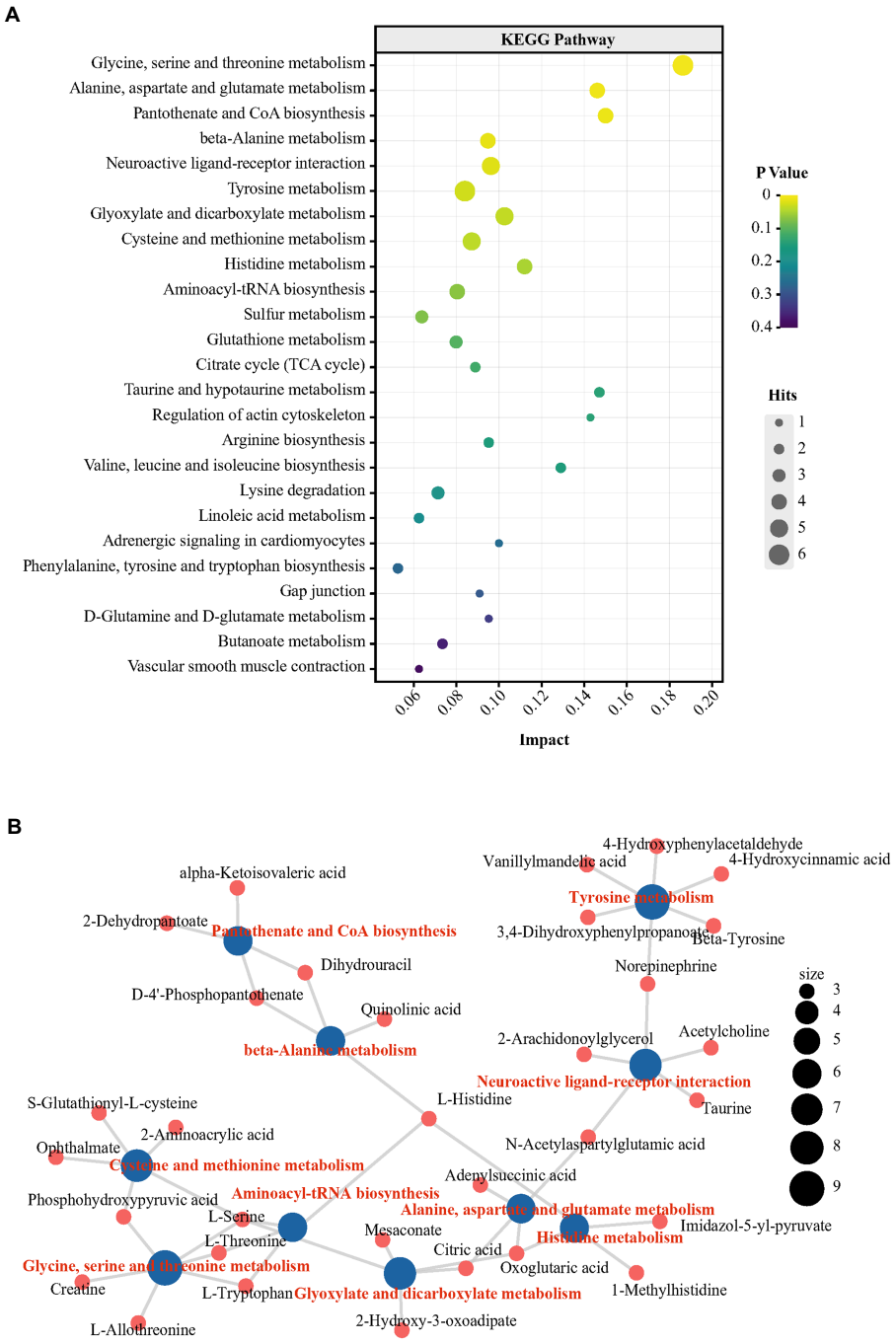

FIGURE 5
Changes in the metabolic profile in plasma obtained from FP birds. **(A)** Bubble chart using the top 25 KEGG pathways enriched by the differential metabolites in plasma; **(B)** Network plot using the top 10 KEGG pathways enriched from the differential metabolites in plasma.

in the CNS, plays an extensive and key role in the maintenance of brain functions, including emotion and cognition, therefore affecting numerous behaviors, such as aggression, depression and anxiety (Gerhard et al., 2016; Zheng et al., 2019). In the hippocampus of peckers, the concentration of L-tryptophan was also higher than that in the N group ($p < 0.05$). The levels of 5-HT, which is an important product of tryptophan and central neurotransmitters and plays an important role in regulating the onset of FP in laying hens (de Haas and van der Eijk, 2018), did not differ between the two groups. Instead, the levels of xanthurenic acid, one crucial metabolite in the kynurenine (KYN) pathway of tryptophan, significantly increased in the P group ($p < 0.05$). The KYN pathway is an alternate tryptophan breakdown pathway that, under physiological conditions, metabolizes tryptophan (> 95%) into KYN and an array of downstream neuroactive metabolites, including xanthurenic acid (Danielski et al., 2018). Xanthurenic acid, an endogenous kynurenine, is a known vesicular glutamic acid transport (VGLUT) inhibitor and has also been proposed as a

mGlu$_{2/3}$ receptor agonist (Neale et al., 2013). Previous studies have found stereoselective blood–brain barrier transport of histidine by *in vivo* or *in vitro* experiments (Nowak et al., 1997; Yamakami et al., 1998). Histamine, which is synthesized from the amino acid histidine through oxidative decarboxylation by histidine decarboxylase in the brain, exerts complicated effects in depression-like and anxiety-like behavior (Raber, 2005). In the present study, the hippocampal levels of L-histidine and histamine were increased in the P group ($p < 0.05$). The decreased NE concentration shown in group P ($p < 0.05$) may have been the result of the competition between peripheral tyrosine (the precursor of NE), other AAAs (phenylalanine and tryptophan) and histidine to LNAA carriers to be transported into the brain (Oldendorf et al., 1988). The KEGG pathway enrichment analysis of differential metabolites showed 9 significantly enriched metabolic pathways, including 'Histidine metabolism', 'Phenylalanine, tyrosine and tryptophan biosynthesis', 'Arginine and proline metabolism', 'Tryptophan metabolism' and 'Aminoacyl-tRNA biosynthesis' (Figure 6A; Supplementary Table S4). The central alteration, including 'Histidine metabolism', 'Phenylalanine, tyrosine and tryptophan biosynthesis', and 'Tryptophan metabolism' have reported to be associated with depression-like behaviors

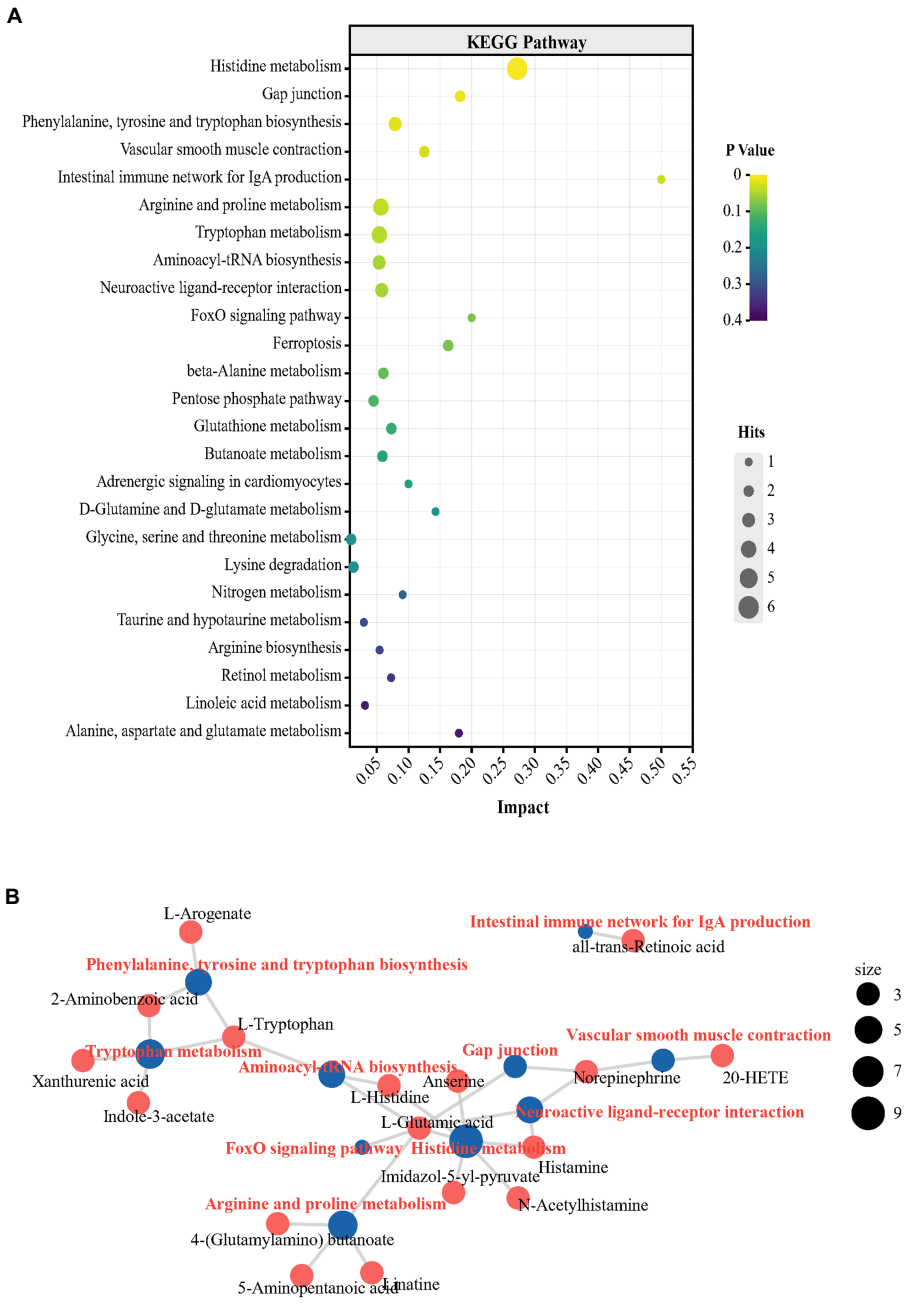

FIGURE 6
The turbulence of metabolic profile in hippocampus of FP bird. **(A)** Bubble chart from the top 25 KEGG pathways enriching with the differential metabolites in plasma; **(B)** The network plot from the top 10 KEGG pathways enriching with the differential metabolites in hippocampus.

(Yoshikawa et al., 2014; Wang et al., 2022). The network diagram showed the association between differential metabolites and enrichment pathways (Figure 6B). L-glutamic acid shows a complex relationship with multiple metabolic pathways, suggesting the potentially important role of L-glutamic acid in FP development.

3.5. Correlation analysis

To investigate potential associations among the gut microbiome, plasma physiological index, plasma metabolites and hippocampal metabolites, correlation analysis was conducted. Figure 7A shows the

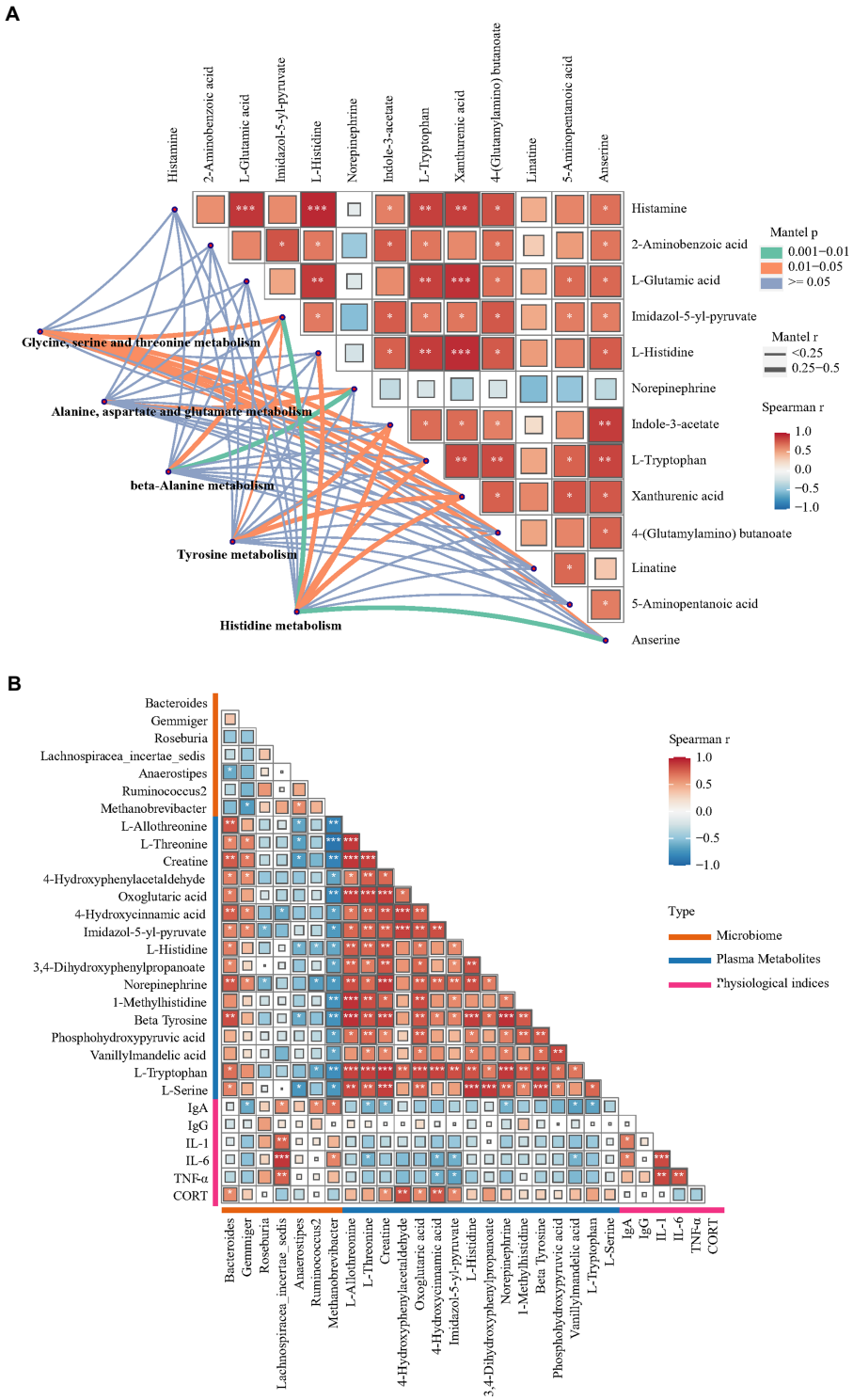

FIGURE 7
Correlation analysis between gut microorganisms, metabolites and plasma physiological indices. **(A)** Pairwise comparisons of hippocampus metabolites, with a color gradient denoting the Spearman's correlation coefficients. Plasma KEGG pathways enriched with differential metabolites was correlated with each hippocampus metabolites by partial Mantel tests. Curve width represents the significant correlation coefficients ($p<0.05$) of the partial Mantel tests. **(B)** Spearman's correlation analysis between differential microorganism, plasma differential metabolites and plasma physiological indices.

results of Spearman's correlation analysis performed using the levels of hippocampal differential metabolites and Mantel's test performed using the levels of hippocampal differential metabolites and plasma metabolic pathways. L-glutamic acid levels showed a significant positive correlation with L-histidine, histamine, L-tryptophan and xanthurenic acid levels in the hippocampus (Spearman $r > 0.6$, $p < 0.01$). A previous study reported an association between glutamic acid and histidine levels (Ritz et al., 2002). The main pathway of histidine catabolism begins with deamination catalyzed by histidase to urocanate and leads through 4-imidazolone-5-propionate and formiminoglutamate to glutamic acid, while the alternative pathways of histidine catabolism include transamination to imidazolepyruvate and decarboxylation to histamine. Increased L-histidine and histamine levels were also found in a rat model of glutamic acid excitotoxicity and other neurodegenerative disorders (Fang et al., 2014). The present study revealed an increase in xanthurenic acid concentration but not in 5-HT levels. Xanthurenic acid is a known VGLUT inhibitor and has also been proposed as a $mGlu_{2/3}$ receptor agonist (Neale et al., 2013). These results indicated that the development of FP is potentially correlated with changes in the glutamatergic system in the CNS. Furthermore, the levels of multiple differential metabolites, including L-histidine, histamine, L-tryptophan and xanthurenic acid, in the hippocampus were significantly correlated with 'glycine, serine and threonine metabolism', 'tyrosine metabolism' and 'histidine metabolism' in plasma. Collectively, these findings indicated that the disturbed glutamatergic system was potentially associated with the differential metabolites involved in 'glycine, serine and threonine metabolism' or 'histidine metabolism' in plasma. Therefore, a total of 16 differential metabolites, such as L-histidine, L-tryptophan and beta-tyrosine, which participate in 'glycine, serine and threonine metabolism', 'tyrosine metabolism' and 'histidine metabolism', were chosen to perform Spearman correlation analysis with cecal flora genera and plasma physiological indices (Figure 7B). *Bacteroides* and *Methanobrevibacter* abundance showed the closest association with the plasma metabolite profile. L-tryptophan, L-histidine and beta-tyrosine levels were both positively correlated with *Bacteroides* abundance but negatively correlated with *Methanobrevibacter* abundance. *Bacteroides*, the most prominent genus that was enriched in the P group, was associated with abnormal behaviors of the host. The gut microbiome from major depressive disorder patients was found to be enriched with the genus *Bacteroides*, and these microbes were found to be associated with increased anxiety and depression-like behavior and impaired hippocampal neurogenesis in rats subjected to fecal microbiota transplantation (Zhang et al., 2022). Outcomes from numerous experimental methods, including dietary tryptophan restriction and histidine supplementation, have revealed that *Bacteroides* play an important role in histidine metabolism and tryptophan metabolism (Zapata et al., 2018; Kang et al., 2020). More OTUs were enriched in the genus *Methanobrevibacter*, which had decreased abundance in the P group, and its function is closely related to anxiety and depression-like behavior (Chen et al., 2021). Previous research found a negative association between the abundance of *Methanobrevibacter* and dietary tryptophan levels (Rao et al., 2021). Elevated levels of stress hormones, including CORT and N, can also lead to disrupted gut barrier function and altered commensal bacteria (Maes et al., 2013). In this research, the increase in the levels of NE and CORT, which are stress hormones, was positively correlated with *Bacteroides* abundance. However, the potential association between NE, CORT and *Bacteroides* is unclear. To our knowledge, NE levels were found only to change with a significant negative correlation with *Bacteroides* abundance, which was reported in research on depression (Chen et al., 2021).

4. Conclusion

Taken together, our results demonstrated the different patterns of the gut microbiota, metabolism and immune system and revealed the potential association between FP, the gut microbiota and the glutamatergic neurotransmitter system. In this research, peckers were found to suffer from long-term stress with a suppressed immune system. Disturbances in the cecal microbiota, including increased *Bacteroides* abundance and decreased *Methanobrevibacter* abundance, were found in peckers. The abundances of the two microorganisms showed significant correlations with the plasma levels of L-tryptophan, beta-tyrosine and L-histidine, which may further affect the hippocampal levels of metabolites involved in the glutamatergic neurotransmitter system, including L-glutamic acid, L-tryptophan, xanthurenic acid, and L-histidine. In conclusion, the findings of this study have provided a new insight into developing novel biotherapeutic strategies for alleviating FP in laying hens.

Data availability statement

The data presented in the study are deposited in NCBI Sequence Read Archive (SRA) repository (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA933384), accession number PRJNA933384.

Ethics statement

The animal study was reviewed and approved by the Scientific Ethics Committee of South China Agricultural University.

Author contributions

CW contributed to the data analysis, investigation, and drafting the manuscript. YL, HW, and ML were responsible for behavioral observation, breeding and sampling. JR provided technical support. XL and YBW were responsible for supervision. YW contributed to the conceptualization, project administration and critical revision of the manuscript. All the authors have read and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1132866/full#supplementary-material

References

Ait-Belgnaoui, A., Colom, A., Braniste, V., Ramalho, L., Marrot, A., Cartier, C., et al. (2014). Probiotic gut effect prevents the chronic psychological stress-induced brain activity abnormality in mice. *Neurogastroenterol. Motil.* 26, 510–520. doi: 10.1111/nmo.12295

Bestman, M., Koene, P., and Wagenaar, J. P. (2009). Influence of farm factors on the occurrence of feather pecking in organic reared hens and their predictability for feather pecking in the laying period. *Appl. Anim. Behav. Sci.* 121, 120–125. doi: 10.1016/j.applanim.2009.09.007

Birkl, P., Bharwani, A., Kjaer, J. B., Kunze, W., McBride, P., Forsythe, P., et al. (2018). Differences in cecal microbiome of selected high and low feather-pecking laying hens. *Poult. Sci.* 97, 3009–3014. doi: 10.3382/ps/pey167

Birkl, P., Franke, L., Rodenburg, T. B., Ellen, E., and Harlander-Matauschek, A. (2017). A role for plasma aromatic amino acids in injurious pecking behavior in laying hens. *Physiol. Behav.* 175, 88–96. doi: 10.1016/j.physbeh.2017.03.041

Chen, Y., Xiao, N., Chen, Y., Chen, X., Zhong, C., Cheng, Y., et al. (2021). Semen sojae praeparatum alters depression-like behaviors in chronic unpredictable mild stress rats via intestinal microbiota. *Food Res. Int.* 150:110808. doi: 10.1016/j.foodres.2021.110808

Colombo, M., and Broadbent, N. (2000). Is the avian hippocampus a functional homologue of the mammalian hippocampus? *Neurosci. Biobehav. Rev.* 24, 465–484. doi: 10.1016/S0149-7634(00)00016-6

Daigle, C. L., Rodenburg, T. B., Bolhuis, J. E., Swanson, J. C., and Siegford, J. M. (2015). Individual consistency of feather pecking behavior in laying hens: once a feather pecker always a feather pecker? *Front. Vet. Sci.* 2:6. doi: 10.3389/fvets.2015.00006

Dallman, M. F., Akana, S. F., Levin, N., Walker, C. D., Bradbury, M. J., Suemaru, S., et al. (1994). Corticosteroids and the control of function in the hypothalamo-pituitary-adrenal (HPA) axis. *Ann. N. Y. Acad. Sci.* 746, 22–31. doi: 10.1111/j.1749-6632.1994.tb39206.x

Danielski, L. G., Giustina, A. D., Goldim, M. P., Florentino, D., Mathias, K., Garbossa, L., et al. (2018). Vitamin B6 reduces neurochemical and long-term cognitive alterations after polymicrobial sepsis: involvement of the kynurenine pathway modulation. *Mol. Neurobiol.* 55, 5255–5268. doi: 10.1007/s12035-017-0706-0

de Haas, E. N., and van der Eijk, J. A. (2018). Where in the serotonergic system does it go wrong? Unravelling the route by which the serotonergic system affects feather pecking in chickens. *Neurosci. Biobehav. Rev.* 95, 170–188. doi: 10.1016/j.neubiorev.2018.07.007

Desbonnet, L., Garrett, L., Clarke, G., Kiely, B., Cryan, J. F., and Dinan, T. (2010). Effects of the probiotic *Bifidobacterium infantis* in the maternal separation model of depression. *Neuroscience* 170, 1179–1188. doi: 10.1016/j.neuroscience.2010.08.005

Falker-Gieske, C., Mott, A., Preuß, S., Franzenburg, S., Bessei, W., Bennewitz, J., et al. (2020). Analysis of the brain transcriptome in lines of laying hens divergently selected for feather pecking. *BMC Genomics* 21, 595–514. doi: 10.1186/s12864-020-07002-1

Fang, Q., Hu, W. W., Wang, X. F., Yang, Y., Lou, G. D., Jin, M. M., et al. (2014). Histamine up-regulates astrocytic glutamate transporter 1 and protects neurons against ischemic injury. *Neuropharmacology* 77, 156–166. doi: 10.1016/j.neuropharm.2013.06.012

Gerhard, D. M., Wohleb, E. S., and Duman, R. S. (2016). Emerging treatment mechanisms for depression: focus on glutamate and synaptic plasticity. *Drug Discov. Today* 21, 454–464. doi: 10.1016/j.drudis.2016.01.016

Gilani, A. M., Knowles, T. G., and Nicol, C. J. (2013). The effect of rearing environment on feather pecking in young and adult laying hens. *Appl. Anim. Behav. Sci.* 148, 54–63. doi: 10.1016/j.applanim.2013.07.014

Goto, T., Tomonaga, S., and Toyoda, A. (2017). Effects of diet quality and psychosocial stress on the metabolic profiles of mice. *J. Proteome Res.* 16, 1857–1867. doi: 10.1021/acs.jproteome.6b00859

Gunnarsson, S. (1999). Effect of rearing factors on the prevalence of floor eggs, cloacal cannibalism and feather pecking in commercial flocks of loose housed laying hens. *Br. Poult. Sci.* 40, 12–18. doi: 10.1080/00071669987773

Hasebe, K., Kendig, M. D., and Morris, M. J. (2021). Mechanisms underlying the cognitive and behavioural effects of maternal obesity. *Nutrients* 13:240. doi: 10.3390/nu13010240

Holeček, M. (2020). Influence of histidine administration on ammonia and amino acid metabolism: a review. *Physiol. Res.* 69, 555–564. doi: 10.33549/physiolres.934449

Johnston, C. S., Jasbi, P., Jin, Y., Bauer, S., Williams, S., Fessler, S. N., et al. (2021). Daily vinegar ingestion improves depression scores and alters the metabolome in healthy adults: a randomized controlled trial. *Nutrients* 13:4020. doi: 10.3390/nu13114020

Kang, M., Yin, J., Ma, J., Wu, X., Huang, K., Li, T., et al. (2020). Effects of dietary histidine on growth performance, serum amino acids, and intestinal morphology and microbiota communities in low protein diet-fed piglets. *Mediat. Inflamm.* 2020:1240152, 1–7. doi: 10.1155/2020/1240152

Kops, M. S., de Haas, E. N., Rodenburg, T. B., Ellen, E. D., Korte-Bouws, G. A., Olivier, B., et al. (2013). Effects of feather pecking phenotype (severe feather peckers, victims and non-peckers) on serotonergic and dopaminergic activity in four brain areas of laying hens (*Gallus gallus domesticus*). *Physiol. Behav.* 120, 77–82. doi: 10.1016/j.physbeh.2013.07.007

Korte, S. M., Beuving, G., Ruesink, W. I. M., and Blokhuis, H. J. (1997). Plasma catecholamine and corticosterone levels during manual restraint in chicks from a high and low feather pecking line of laying hens. *Physiol. Behav.* 62, 437–441. doi: 10.1016/S0031-9384(97)00149-2

Lambton, S. L., Knowles, T. G., Yorke, C., and Nicol, C. J. (2010). The risk factors affecting the development of gentle and severe feather pecking in loose housed laying hens. *Appl. Anim. Behav. Sci.* 123, 32–42. doi: 10.1016/j.applanim.2009.12.010

Liu, T., Zhou, N., Xu, R., Cao, Y., Zhang, Y., Liu, Z., et al. (2020). A metabolomic study on the anti-depressive effects of two active components from *Chrysanthemum morifolium*. *Artif. Cells Nanomed. Biotechnol.* 48, 718–727. doi: 10.1080/21691401.2020.1774597

Maes, M., Kubera, M., Leunis, J. C., Berk, M., Geffard, M., and Bosmans, E. (2013). In depression, bacterial translocation may drive inflammatory responses, oxidative and nitrosative stress (O&NS), and autoimmune responses directed against O&NS-damaged neoepitopes. *Acta Psychiatr. Scand.* 127, 344–354. doi: 10.1111/j.1600-0447.2012.01908.x

Maes, M., Yirmyia, R., Noraberg, J., Brene, S., Hibbeln, J., Perini, G., et al. (2009). The inflammatory & neurodegenerative (I&ND) hypothesis of depression: leads for future research and new drug developments in depression. *Metab. Brain Dis.* 24, 27–53. doi: 10.1007/s11011-008-9118-1

Miller, B. J., Buckley, P., Seabolt, W., Mellor, A., and Kirkpatrick, B. (2011). Meta-analysis of cytokine alterations in schizophrenia: clinical status and antipsychotic effects. *Biol. Psychiatry* 70, 663–671. doi: 10.1016/j.biopsych.2011.04.013

Mindus, C., van Staaveren, N., Fuchs, D., Gostner, J. M., Kjaer, J. B., Kunze, W., et al. (2021). *L. Rhamnosus* improves the immune response and tryptophan catabolism in laying hen pullets. *Sci. Rep.* 11, 19538–19515. doi: 10.1038/s41598-021-98459-x

Mormède, C., Castanon, N., Médina, C., Moze, E., Lestage, J., Neveu, P. J., et al. (2003). Chronic mild stress in mice decreases peripheral cytokine and increases central cytokine expression independently of IL-10 regulation of the cytokine network. *Neuroimmunomodulation* 10, 359–366. doi: 10.1159/000071477

Neale, S. A., Copeland, C. S., Uebele, V. N., Thomson, F. J., and Salt, T. E. (2013). Modulation of hippocampal synaptic transmission by the kynurenine pathway member

xanthurenic acid and other VGLUT inhibitors. *Neuropsychopharmacology* 38, 1060–1067. doi: 10.1038/npp.2013.4

Nowak, J. Z., Zawilska, J. B., Woldan-Tambor, A., Sçk, B., Voisin, P., Lintunen, M., et al. (1997). Histamine in the chick pineal gland: origin, metabolism, and effects on the pineal function. *J. Pineal Res.* 22, 26–32. doi: 10.1111/j.1600-079X.1997.tb00299.x

Oldendorf, W. H., Crane, P. D., Braun, L. D., Gosschalk, E. A., and Diamond, J. M. (1988). pH dependence of histidine affinity for blood-brain barrier carrier transport systems for neutral and cationic amino acids. *J. Neurochem.* 50, 857–861. doi: 10.1111/j.1471-4159.1988.tb02991.x

Raber, J. (2005). Histamine receptors as potential therapeutic targets to treat anxiety and depression. *Drug Dev. Res.* 65, 126–132. doi: 10.1002/ddr.20015

Rao, Z., Li, J., Shi, B., Zeng, Y., Liu, Y., Sun, Z., et al. (2021). Dietary tryptophan levels impact growth performance and intestinal microbial ecology in weaned piglets via tryptophan metabolites and intestinal antimicrobial peptides. *Animals* 11:1. doi: 10.3390/ani11030817

Ritz, M. F., Schmidt, P., and Mendelowitsch, A. (2002). 17β-estradiol effect on the extracellular concentration of amino acids in the glutamate excitotoxicity model in the rat. *Neurochem. Res.* 27, 1677–1683. doi: 10.1023/A:1021695213099

Rodenburg, T. B., Buitenhuis, A. J., Ask, B., Uitdehaag, K. A., Koene, P., Van Der Poel, J. J., et al. (2004). Genetic and phenotypic correlations between feather pecking and open-field response in laying hens at two different ages. *Behav. Genet.* 34, 407–415. doi: 10.1023/B:BEGE.0000023646.46940.2d

Rodenburg, T. B., Van Krimpen, M. M., De Jong, I. C., De Haas, E. N., Kops, M. S., Riedstra, B. J., et al. (2013). The prevention and control of feather pecking in laying hens: identifying the underlying principles. *Worlds Poult. Sci. J.* 69, 361–374. doi: 10.1017/S0043933913000354

Solís-Ortiz, S., Arriaga-Avila, V., Trejo-Bahena, A., and Guevara-Guzmán, R. (2021). Deficiency in the essential amino acids l-isoleucine, l-leucine and l-histidine and clinical measures as predictors of moderate depression in elderly women: a discriminant analysis study. *Nutrients* 13:3875. doi: 10.3390/nu13113875

Tremblay, A., Lingrand, L., Maillard, M., Feuz, B., and Tompkins, T. A. (2021). The effects of psychobiotics on the microbiota-gut-brain axis in early-life stress and neuropsychiatric disorders. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 105:110142. doi: 10.1016/j.pnpbp.2020.110142

van der Eijk, J. A., de Vries, H., Kjaer, J. B., Naguib, M., Kemp, B., Smidt, H., et al. (2019c). Differences in gut microbiota composition of laying hen lines divergently selected on feather pecking. *Poult. Sci.* 98, 7009–7021. doi: 10.3382/ps/pez336

van der Eijk, J. A., Lammers, A., Kjaer, J. B., and Rodenburg, T. B. (2019a). Stress response, peripheral serotonin and natural antibodies in feather pecking genotypes and phenotypes and their relation with coping style. *Physiol. Behav.* 199, 1–10. doi: 10.1016/j.physbeh.2018.10.021

van der Eijk, J. A., Rodenburg, T. B., de Vries, H., Kjaer, J. B., Smidt, H., Naguib, M., et al. (2020). Early-life microbiota transplantation affects behavioural responses, serotonin and immune characteristics in chicken lines divergently selected on feather pecking. *Sci. Rep.* 10:2750, 2750–2713. doi: 10.1038/s41598-020-59125-w

van der Eijk, J. A., Verwoolde, M. B., de Vries Reilingh, G., Jansen, C. A., Rodenburg, T. B., and Lammers, A. (2019b). Chicken lines divergently selected on feather pecking differ in immune characteristics. *Physiol. Behav.* 212:112680:112680. doi: 10.1016/j.physbeh.2019.112680

Wang, D., Wu, J., Zhu, P., Xie, H., Lu, L., Bai, W., et al. (2022). Tryptophan-rich diet ameliorates chronic unpredictable mild stress induced depression-and anxiety-like behavior in mice: the potential involvement of gut-brain axis. *Food Res. Int.* 157:111289. doi: 10.1016/j.foodres.2022.111289

Want, E. J., Masson, P., Michopoulos, F., Wilson, I. D., Theodoridis, G., Plumb, R. S., et al. (2013). Global metabolic profiling of animal and human tissues via UPLC-MS. *Nat. Protoc.* 8, 17–32. doi: 10.1038/nprot.2012.135

Yamakami, J., Sakurai, E., Sakurada, T., Maeda, K., and Hikichi, N. (1998). Stereoselective blood-brain barrier transport of histidine in rats. *Brain Res.* 812, 105–112. doi: 10.1016/S0006-8993(98)00958-5

Yoshikawa, T., Nakamura, T., Shibakusa, T., Sugita, M., Naganuma, F., Iida, T., et al. (2014). Insufficient intake of L-histidine reduces brain histamine and causes anxiety-like behaviors in male mice. *J. Nutr.* 144, 1637–1641. doi: 10.3945/jn.114.196105

Zapata, R. C., Singh, A., Ajdari, N. M., and Chelikani, P. K. (2018). Dietary tryptophan restriction dose-dependently modulates energy balance, gut hormones, and microbiota in obesity-prone rats. *Obesity* 26, 730–739. doi: 10.1002/oby.22136

Zelena, E., Dunn, W. B., Broadhurst, D., Francis-McIntyre, S., Carroll, K. M., Begley, P., et al. (2009). Development of a robust and repeatable UPLC-MS method for the long-term metabolomic study of human serum. *Anal. Chem.* 81, 1357–1364. doi: 10.1021/ac8019366

Zhang, Y., Fan, Q., Hou, Y., Zhang, X., Yin, Z., Cai, X., et al. (2022). Bacteroides species differentially modulate depression-like behavior via gut-brain metabolic signaling. *Brain Behav. Immun.* 102, 11–22. doi: 10.1016/j.bbi.2022.02.007

Zheng, P., Zeng, B., Liu, M., Chen, J., Pan, J., Han, Y., et al. (2019). The gut microbiome from patients with schizophrenia modulates the glutamate-glutamine-GABA cycle and schizophrenia-relevant behaviors in mice. *Sci. Adv.* 5:eaau8317. doi: 10.1126/sciadv.aau8317

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Cold stress induces colitis-like phenotypes in mice by altering gut microbiota and metabolites

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Introduction: The modernized lifestyle has been paralleled by an epidemic of inflammatory bowel disease (IBD). Excessive consumption of cold beverages is especially common among the modern humans. However, whether cold stress contributes directly to the gut barrier and gut–brain axis is not clear.

Methods: We conducted a cold stress model induced by cold water. The mice were treated with 14 consecutive days of intragastric cold or common water administration. We observed changes in gut transit and gut barrier in the colon. We also employed RNA sequencing-based transcriptomic analysis to identify the genes potentially driving gut injury, and simultaneously examined the gut microbiota and metabolites in the feces.

Results: We found that cold stress disturbed the intestinal function and increased gut permeability. A set of core genes related to immune responses were consistently overexpressed in the cold stress group. Additionally, cold stress induced decreased bacterial diversity, ecological network, and increased pathogens mainly belonging to Proteobacteria. The dopamine signaling pathway-related metabolites were largely reduced in the cold stress group.

Conclusion: This study revealed that cold stress could trigger an IBD-like phenotype in mice, implying that cold stress is a possible risk factor for IBD development.

KEYWORDS

cold stress, inflammatory bowel disease, gut microbiota, gut–brain axis, animal research

Introduction

The modernized lifestyle has been paralleled by an epidemic of several gastrointestinal diseases worldwide beginning in the 1980s, including irritable bowel disease (IBD) characterized by gut barrier dysfunction (Ananthakrishnan, 2015; Khademi et al., 2021). Especially, the incidence of IBD is continuously rising among young people, even among children (Barnes and Kappelman, 2018). Patients with IBD present symptoms similar to those of patients with irritable bowel syndrome (IBS) to some extent, including anxiety and depression (Spiller and Major, 2016). These findings suggest a potential link between modernized lifestyle, gut barrier, and gut–brain interaction.

Excessive consumption of cold beverages is highly prevalent in modern population subsets, especially in the youth. Mounting evidence has shown that sweeteners, food additives, and other food ingredients in beverages play important roles in IBD (He et al., 2021). However, whether some other related factors (for instance, cold stress in the gut) might affect IBD occurrence or

not, is still not quite clear. It has shown that cold meal intake disturbs contractile activity of the gastric (Sun et al., 1995; Verhagen et al., 1998).

Interestingly, industrial and domestic refrigeration development is regarded as a key environmental factor in the etiology of IBD since it increases the chance for exposure of human populations to psychrotrophic bacteria (such as *Yersinia*), which exacerbate intestinal inflammation (Hugot et al., 2021). However, only approximately 10% of patients with IBD were found to harbor *Yersinia* in their gut (Le Baut et al., 2018); therefore, the theory of psychrotrophic bacteria cannot explain these conditions completely. Consequently, some other related factors (for instance, cold stress in the gut) might affect IBD occurrence.

To untangle the link between cold stress, gut barrier, and the gut–brain interaction, we mimicked excess cold beverage consumption in a mouse model. Metabolic syndrome is a potentially important confounder factor, which can indirectly affect the gut–brain axis by changing a myriad of physiological and endocrine systems (Sun et al., 2018). To uncouple the metabolic effects directly caused by excess calorie intake from the beverage rather than the cold stress, we treated mice with cold water by gavage for 14 consecutive days. We then evaluated the gastrointestinal physiology and stress-related behavior in mice. Moreover, we investigated whether cold stress could affect the microbiota and metabolites of fecal contents since gut microbiota and metabolites are essential for bidirectional interactions within the gut–brain axis. These findings could highlight a link between cold stress on gut barrier dysfunction, gut microbiota, and metabolite disturbance, providing new preventive strategies to quell the creeping up in the incidence of IBD.

Materials and methods

Animals

The C57BL/6 mice (8 weeks, male) were used in this study. Sixteen mice were divided into two groups: In the control group (n = 8), the mice were subjected to gavage with room-temperature water (20–25°C). In the cold stress group (n = 8), the mice were subjected to gavage with cold water (0–4°C).

Regular drinking water and food were available to the mice at all times. Additionally, each mouse was given a total of 1 ml cold or room-temperature water. Prior to use the water was autoclaved and handled using sterile vessels. The maximum dose given by gavage was 200 μl at a time, and we gave it five times. This treatment had lasted for 2 weeks. Gut function tests and behavioral tests were carried out after gavage for 2 weeks. The protocol was approved by the Animal Research Ethics Board of the Northwest University (Approve number: 20210204).

Abbreviations: IBD, irritable bowel disease; IBS, inflammatory bowel syndrome; OTU, operational taxonomic unit; TST, tail suspension test; OFT, open field test; LC–MS, liquid chromatography-mass spectrometry.

Fecal output, fecal moisture content evaluation, and Bristol score

All the mice were transferred into separate clean cages, and the feces were numbered and collected for 4 h between 8 am, and 12 am. All the feces during the procedure were collected and placed in tubes, then tubes were weighed and the wet weight were recorded, dried overnight at 60°C, then reweighed, and the dry weight were recorded. At last the water content was calculated. The criteria for Bristol score were evaluated as described in the previous study (Koh et al., 2010).

Histological analysis and disease activity index score

The mice were killed by cervical dislocation. Then, the mice were dissected and the whole intestines were removed from the abdomen. Several segments were taken from the ileum and colon, respectively, and rinsed with cold saline, fixed in 4% polyformaldehyde. The ileum and colon samples were prepared for histological examination of lesions. The resected segments were opened lengthwise, embedded in paraffin, and sectioned. Hematoxylin–eosin staining of the ileum and colon was performed. Intestinal inflammation was assessed by observing ulcer formation, epithelial cell changes (goblet cells), lymphocyte infiltration, and lymph node formation, according to a previous report (Zielinska et al., 2017). DAI score is evaluated according the stool consistency, blood and weight loss as previously described (Kim et al., 2012).

Gut peristalsis

In brief, 0.6 g of carmine was dissolved in 0.5% hydroxymethyl cellulose solution to obtain a 6% carmine solution. A 200 μl aliquot of the 6% carmine solution was administered to the mice *via* gavage. The animals were then placed in separate cages on a white sheet of paper (to facilitate recognition of red feces). Gut peristalsis activity was defined as the time between gavage and the first red bolus excreted (Zielinska et al., 2017).

Indirect calorimetry

In this study, we randomly selected 6 mice from the control group and 6 mice from the cold stress group, to perform the metabolic tests. Twelve mice were transferred into metabolic cages (Columbus Comprehensive Lab Animal Monitoring System, Columbus, OH, USA) and housed for 24 h with food and water provided *ad libitum*. After 24 h of adaptation, the oxygen volume (VO2), carbon dioxide volume (VCO2), respiratory exchange ratio (RER; VCO2/VO2), heat production, food intake, and drink intake were automatically measured during the following 24 h by the system.

Immunofluorescence

The immunofluorescence was performed on paraffin section from the colon tissue as described (Dinallo et al., 2019). The slides were

incubated overnight at 4°C with the primary antibodies E-cadherin (1:1000; BD Biosciences) and ZO-2 (1:1000; Cell Signaling Technology), after washing three times with PBS, slides were incubated for 1 h at room temperature with specific secondary antibodies coupled with Alexa Fluor Dyes (1:300; Servicebio). Coverslips were mounted on glass slides using ProLong Gold antifade reagent with DAPI to counterstain the DNA. Lastly, the images were acquired on fluorescence microscope.

Depression- and anxiety-like behaviors test

The tail suspension test (TST) was used to evaluate depression-like behaviors. As described previously (Steru et al., 1985), the mice were admitted to suspend for 6 min. All sessions were video-recorded. The time spent struggling in TST (The mice have obvious struggling movements) within a 6-min session was recorded and evaluated as behaviors for survival. The behaviors with reduced time for struggling were regarded as depression-like behaviors. Additionally, anxiety-like behaviors were examined using Open Field Test (OFT) as described previously (Walsh and Cummins, 1976). Their locomotor activity were monitored for 15 min. The time spent in the central area were recorded as indicators of exploratory behavior. The behaviors with reduced time in the center were regarded as anxiety-like behaviors.

Feces collection, 16S rDNA sequencing, and untargeted metabolomic analysis

Feces samples were collected between 8 am and 11 am. The feces samples collected were stored at −80°C. The feces DNA extraction, polymerase chain reaction, library construction, and sequencing were performed as previously described (Sun et al., 2019a). Diversity indices were calculated using QIIME2. Principal coordinate analysis was performed using Calypso online tools. The relative abundances, Spearman correlation coefficients, and heatmap were calculated and compared using T packages. Spearman's rank correlations at the genus level were calculated as our previous study (Sun et al., 2019b).

The liquid chromatography-mass spectrometry (LC–MS) was employed to analyze the fecal metabolome. We used the ACQUITY UPLC system (Waters Corporation, Milford, MA, United States) coupled with an AB SCIEX Triple TOF 6600 System (AB SCIEX, Framingham, MA, United States) in positive- and negative-ion modes as described previously (Su et al., 2020; Yan et al., 2020).

RNA sequencing and bioinformatics analysis

The TRIzol reagent (Invitrogen, Carlsbad, CA, United States) was used to extract the RNA, then assess the RNA integrity by Bioanalyzer 2,100 (Agilent, CA, United States). Poly(A) RNA was purified and fragmented into small pieces, and reverse-transcribed to synthesize cDNA, treated with U-labeled double-stranded DNAs with the heat-labile enzyme UDG, then amplified. Finally, the products were sequenced on an Illumina NovaSeq 6,000 platform (LC-Biotechnology Co., Ltd., Hangzhou, China).

We used the Fastp software for quality control, used HISAT2 to map the reads, and used StringTie to assemble them. The different mRNAs were selected as the following criteria: mRNAs with fold change of >2 or < 0.5 and $p < 0.05$ in a parametric F-test comparing nested linear models.

Statistical analysis

All data in gut function and behavioral tests were showed as the mean ± SEM. Statistical analysis was performed by using Student's t-test for comparisons between cold stress group and control group. Statistical analysis for bioinformatics data of RNA-seq, gut microbiota, and metabolites was performed using the R package vegan. The p value less than 0.05 was regarded as statistical significance.

Results

Cold stress disturbs intestinal function and increases gut permeability in mice

A total of 15 mice (8 in control group and 7 in cold stress group) were able to complete the experiment, as one mouse died from choking during gavage. There were no significant differences in heat production, food intake, drink intake, or respiratory exchange rate between the two groups (Supplementary Figures S1A–D). No changes were observed in the jejunum (Supplementary Figure S1E) or colon length (Supplementary Figure S1F) in the cold stress group. Assessment of gastrointestinal motility by carmine red administration showed that gastrointestinal propulsion was impaired in the cold stress group (Supplementary Figure S1G). In the cold stress group, lower fecal output ($t = 2.493$, $p = 0.027$; Figure 1A), lower fecal moisture percentage ($t = 4.815$, $p < 0.001$; Figure 1B), and lower Bristol scores ($t = 3.015$, $p = 0.010$; Figure 1C) were observed. These results suggest that cold stress disturbs the intestinal function. Altered fecal properties, bloody stools, and weight loss are the most important characteristics in IBD, then we used the DAI scores to evaluate the severity of the colitis. We found that the DAI scores in the cold stress group were significantly higher than in the control group ($t = -4.861$, $p < 0.001$; Figure 1D). Correspondingly, hematoxylin–eosin staining showed sparse intestinal villus, and edema in the intestinal villi of the jejunum (Figure 1E), a significant reduction in goblet cells, an enhanced inflammation response, and an elevated histological score in the colon ($t = -2.193$, $p = 0.047$; Figure 1F). As to the gut barrier, we found that the expression of E-cadherin and ZO-2 was lowered by immunofluorescence (Figure 1G), compared to that in the control group.

Cold stress exacerbates the inflammatory response of the intestinal tissue in mice

To further identify the changes in gene expression in the intestinal tissue after cold stress, we randomly selected out 6 mice from the two groups and investigated the gene expression profiles of the intestinal tissue using RNA sequencing (Figure 2A). A total of 432 genes with differential expression between groups were identified, with 330 genes

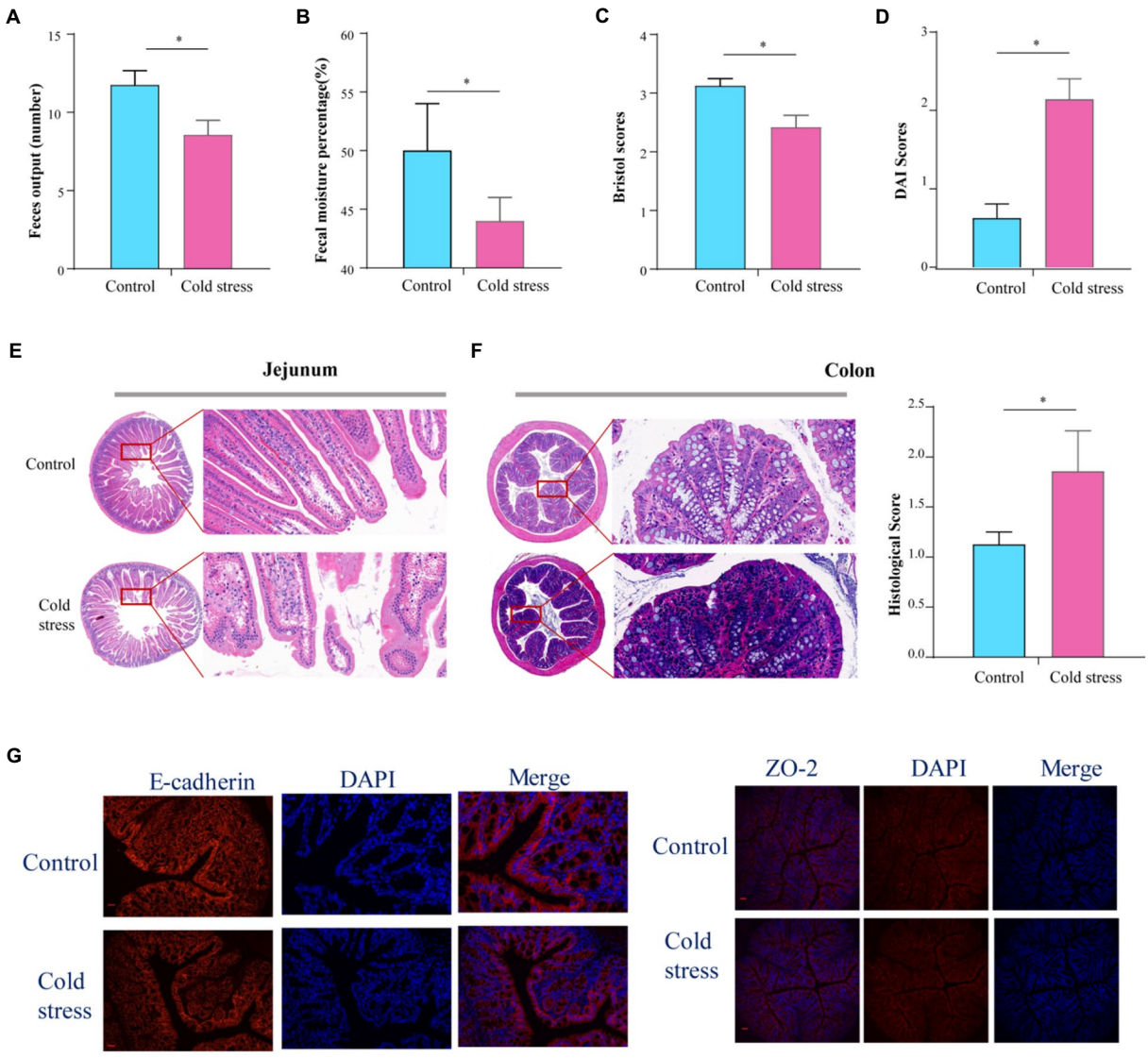

FIGURE 1
Cold stress disturbs intestinal function and increases gut permeability in mice. **(A)** Feces output in 4h. **(B)** Fecal moisture percentage. **(C)** Bristol scores. **(D)** DAI scores calculating using body weight and fecal characters. **(E,F)** Hematoxylin and eosin-stained jejunum and colon sections from control and cold stress-treated mice and the corresponding histological scores. **(G)** Representative images of markers of gut barrier in the colon, E-cadherin (left) and ZO-2(right) Scale bars=50μm. All the data are expressed as mean±SEM (n=8 in the control group; n=7 in the cold stress group). *p<0.05.

upregulated and 102 genes downregulated in the cold stress group when compared with the control group (Figure 2B). Gene Ontology analysis showed that all the genes most strongly enriched in immune function processes, including innate immune response and adaptive immune response, such as the B cell activation and B cell receptor complex, immunoglobulin production, immunoglobulin complex, complement activation, antigen binding, and defense response to a bacterium (Figure 2C). In accordance with these findings, KEGG pathway analysis also showed that the genes were enriched in immune activation in the colon, including the primary immunodeficiency, B cell receptor signaling, intestinal immune network for IgA production, NK cell-mediated cytotoxicity pathways, leukocyte transendothelial migration, Th1 and Th2 cell differentiation, Th17 cell differentiation, cytokine–cytokine receptor interaction, and chemokine and Fc gamma R-mediated phagocytosis, all of which are closely related to the immune response in the colon (Figure 2D).

To further reveal the effects of cold stress on the colon, we used gene set enrichment analysis to analyze the genes which revealed substantial upregulation of genes involved in the B and T cell receptor signaling pathway, leukocyte transendothelial migration, FC epsilon RI signaling pathway, chemokine signaling pathway, and cytokine–cytokine receptor interaction (Figure 2E). These findings suggested that cold stress triggers an IBD-like phenotype in mice.

Cold stress leads to low bacterial diversity and a fragile ecological network in the gut microbiota

In general, IBD is considered to occur when the immune system overreacts to the resident gut microbiota, inducing a chain of inflammatory events that can destroy the gut barrier (Eisenstein,

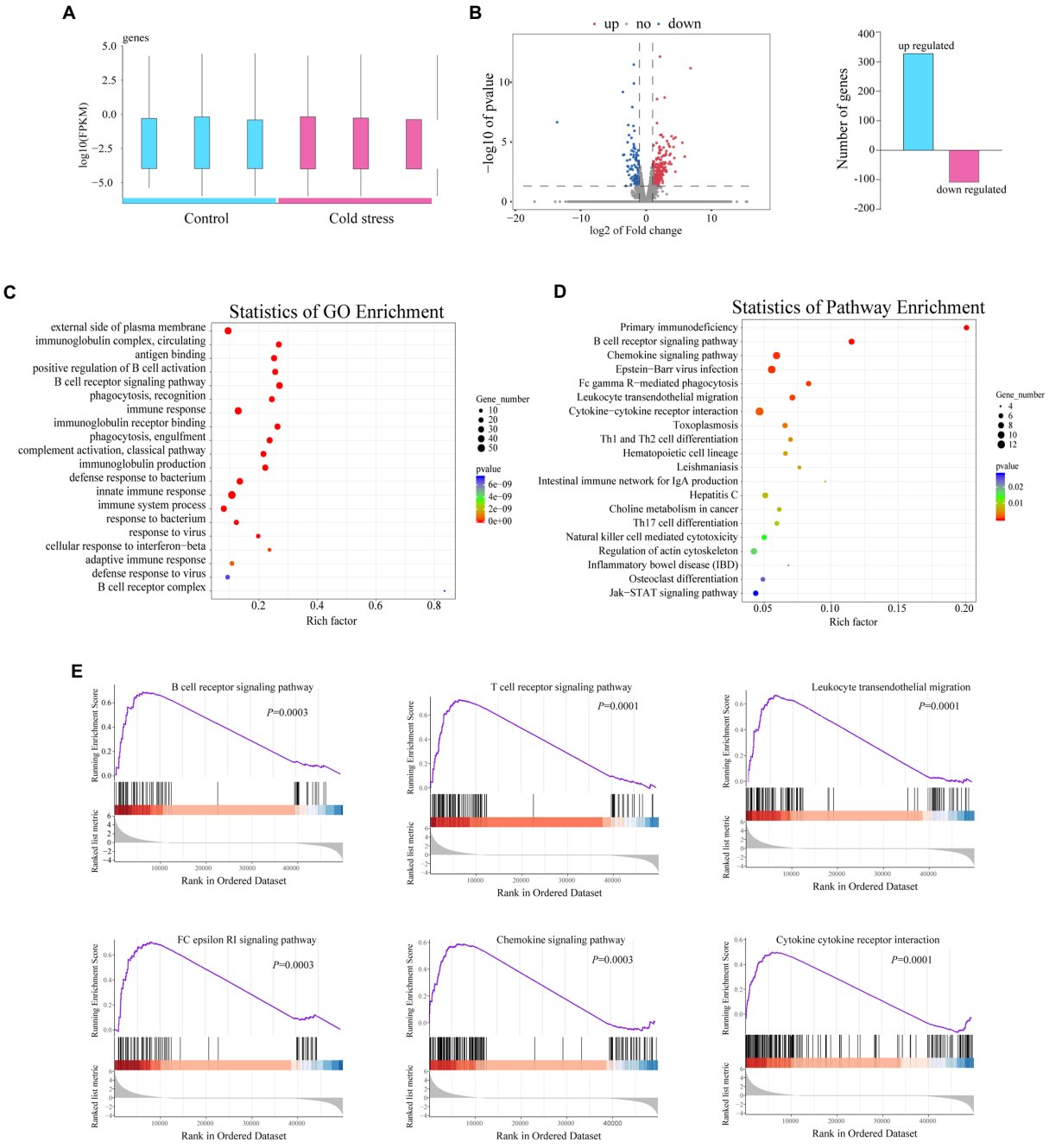

FIGURE 2
Cold stress exacerbates the inflammatory response of the intestinal tissue in mice. **(A)** Number of genes upregulated and downregulated in the cold water-treated group. **(B)** Volcano plot of genes differentially expressed between the two groups. **(C,D)** GO and KEGG pathway enrichment analysis between the two groups. **(E)** Gene set enrichment analysis of the cold stress group compared to the control group. Data are representative of three biological repeats (n=3).

2016). These findings prompted us to further investigate whether changes in microbiota or bacterial metabolites from the feces under cold stress stimulation may regulate the gut barrier and gut–brain interactions.

Eight individual fecal samples from the control group and seven individual fecal samples from the cold stress group were collected and sequenced. The principal component plots with unweighted UniFrac distances showed a clear separation between the cold stress and control groups (Figure 3A), which suggested that cold stress led to a significant alteration in the gut microbiota composition. The Shannon index showed no difference between the two groups (Figure 3B). However, at the operational taxonomic unit (OTU) level, the microbiota were significantly differed (Figure 3C). The abundances of *OTU198* (*Lachnospiraceae_unclassified*), *OTU337* (*Clostridiales_Incertae_unclassified*), *OTU156* (*Muribaculaceae_unclassified*), *OTU254* (*Erysipelotrichaceae_unclassified*), *OTU88* (*Duncaniella*), *OTU334* (*Muribaculaceae_unclassified*), *OTU13* (*Paramuribaculum*), *OTU433* (*Proteobacteria_unclassified*), *OTU215*

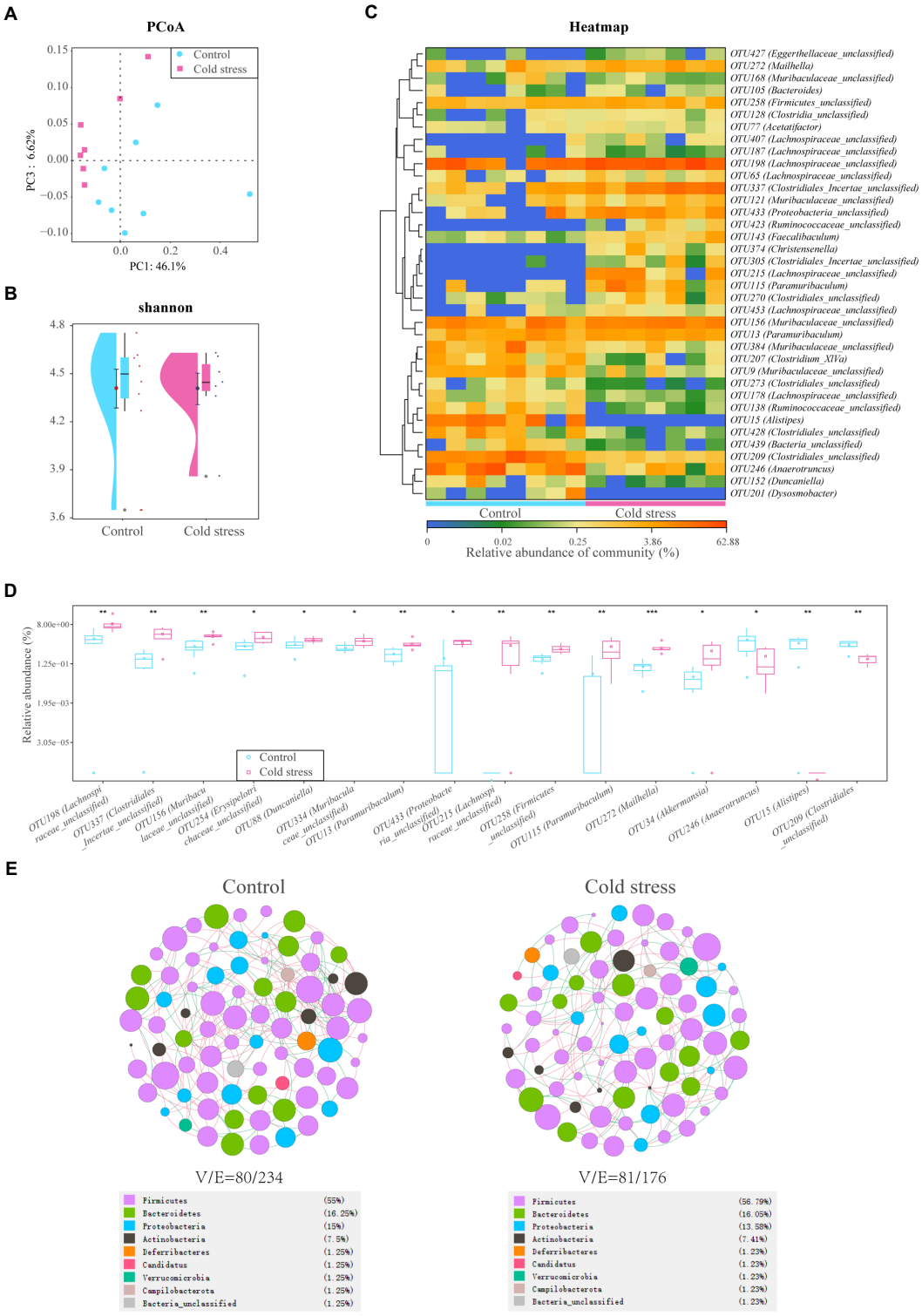

FIGURE 3
Cold stress leads to low bacterial diversity and a fragile ecological network in the gut microbiota. **(A)** Principal component analysis (PCoA) plots of unweighted UniFrac distances between the two groups. **(B)** Shannon diversity scores. **(C)** Heatmap of key operational taxonomic units (OTUs). **(D)** Gut bacteria with different abundances at the OTU level. **(E)** Network analysis at the genus level. V: number of nodes. E: number of edges. (n=8 in control group and n=7 in cold stress group).

(*Lachnospiraceae_unclassified*), *OTU258* (*Firmicutes_unclassified*), *OTU115* (*Paramuribaculum*), *OTU272* (*Mailhella*), and *OTU34* (*Akkermansia*) were significantly increased in the cold stress group. Additionally, the abundances of *OTU246* (*Anaerotruncus*), *OTU15* (*Alistipes*), and *OTU209* (*Clostridiales_unclassified*) decreased under cold stress (Figure 3D). The detailed information has been shown in

Supplementary Table S1. Linear discriminant analysis of effect size further showed that the bacteria with increased abundance in the cold stress group mainly belong to Proteobacteria (Supplementary Figure S2). To determine the pattern of bacteria, we constructed their networks in the two group respectively, we found that the network of the cold stress-treated mice had a simpler property (nodes/edges = 81/176) than the control group (nodes/edges = 80/234), indicating that cold stress may induce vulnerability to environmental stress in the gut microbiota (Figure 3E).

Cold stress downregulates metabolites of the dopamine-related pathway in the intestinal flora

As a microbe–host bridge, some metabolites of the intestinal flora can affect host physiology by entering the bloodstream. Therefore, we analyzed fecal metabolites using LC–MS. We found that the metabolic data clusters of the control and cold stress groups were separated from each other in both positive- and negative-ion modes by partial least-squares discriminant analysis (Figures 4A,B). The heatmap also showed that cold stress led to significant alterations in fecal metabolite levels (Figure 4C); 1,179 metabolites were upregulated, and 1896 metabolites were downregulated with significant changes (Figure 4D). The most strongly impacted metabolic pathways included cocaine addiction, dopaminergic synapse, amphetamine addiction, and alcoholism addiction (Figure 4E), all of which were accompanied by a significant reduction in levels of dopamine, L-dopamine, L-tyrosine, and homovanillic acid (Figure 4F). As one of the most important neurotransmitters, decreased dopamine levels may contribute to anxiety-like and depression-like behaviors in the cold stress mice. Additionally, patients with IBD present some similar symptoms to patients with IBS, including anxiety and depression. Therefore, these behaviors were further evaluated.

Cold stress increases depression-like behaviors in mice

In the tail suspended test, the struggling time was significantly decreased in the cold stress group ($t = 2.347$, $p = 0.035$; Figure 5A), suggesting an increase in depression-like behaviors in mice exposed to cold stress. Furthermore, in the open field test, the center time ($t = 2.451$, $p = 0.029$) was reduced in the cold stress group (Figure 5B), implying a tendency of decreased exploratory behavior and increased anxiety-like behavior in the cold stress group.

Correlations of gut microbiota and metabolic changes

Finally, to explore the functional significance of the metabolite perturbations in the gut microbiota of the cold stress-treated group, the 97 annotated metabolites with significant differences were selected, and their Spearman correlation coefficients with different bacteria were calculated. Significant correlations were observed between the gut microbiota and metabolites (Supplementary Figure S3A), and also observed between metabolites and gut function (Supplementary Figure S3B).

Discussion

Increased beverage consumption has long been recognized to play an important role in the pathogenesis of metabolic diseases and colorectal cancer (Yoshida and Simoes, 2018; Goncalves et al., 2019; Hur et al., 2021). Although food additives such as emulsifiers, food colorants, titanium dioxide, and aluminum have largely contributed to the development of these conditions, recent evidence have also identified that food additives are pathogenic factors for colitis (He et al., 2021). In this study, we focused on the potential contribution of cold water to IBD development. These findings suggest that exposure to cold stress in the gut is also a novel risk factor for IBD in humans and a potential trigger for establishing experimental IBD in mice.

Dysregulation of the gut barrier in IBD is caused by environmental factors and genetic predisposition (Graham et al., 2020); however, identifying specific environmental factors has been difficult. Specific food additives are identified as environmental risk factors for IBD (Yang et al., 2018). Here, we aimed to identify whether the low temperature directly affects the development of colitis. Similar to observations in humans, the administration of cold water to mice attenuated gut transit; additionally, the mice exhibited a colitis-like phenotype of gut barrier injury. The fact that increased cold beverage consumption is related to some gastrointestinal symptoms seemingly suggests a link between cold stress and intestinal disorders. Indeed, our results indicate that exposure to cold water not only induces gut transit disturbances but also produces low-grade inflammation. In line with these considerations, cold stress exposure expectedly led to activation of ubiquitous inflammatory signaling pathways, suggesting local mucosal immune cell activation and immune cell trafficking. These are also the characteristics of IBD patients (Neurath, 2019).

Low-grade inflammation in colitis is associated with and may be promoted by gut microbiota dysbiosis (Frank et al., 2007). The low bacterial diversity and altered gut microbiota observed in the cold stress group in this study are extremely similar to those observed in IBD. For example, microbial diversity studies have demonstrated the overgrowth of Proteobacteria in IBD patients (Zhou et al., 2018). Under normal homeostasis, epithelial cells, tight junctions, and the local immune system prevent the translocation of pathogens in the gut. However, in genetically susceptible individuals, Proteobacteria expand to colonize the lumen and invade the lamina propria, further aggravating disruption of the gut barrier (Mukhopadhya et al., 2012). The host recognizes Proteobacteria *via* nucleotide oligomerization domain-like receptors, Toll-like receptors, and retinoic acid-inducible gene I-like receptors. Moreover, microbiota-derived products such as lipopolysaccharide, peptidoglycan, and flagellin from Proteobacteria trigger the activation of immune responses in the mucosa. Our study suggests that the increased abundance of pathogens accompanied by cold stress may contribute to gut barrier disruption in mice by accelerating inflammation in the gut.

Apart from the microbiota-derived products, the metabolites of the gut microbiota are also key actors in the development and exacerbation of IBD. Accumulating evidence suggests that signals from microbial metabolites affect mucosal integrity and immune homeostasis. Moreover, in IBD patients, the metabolites composition

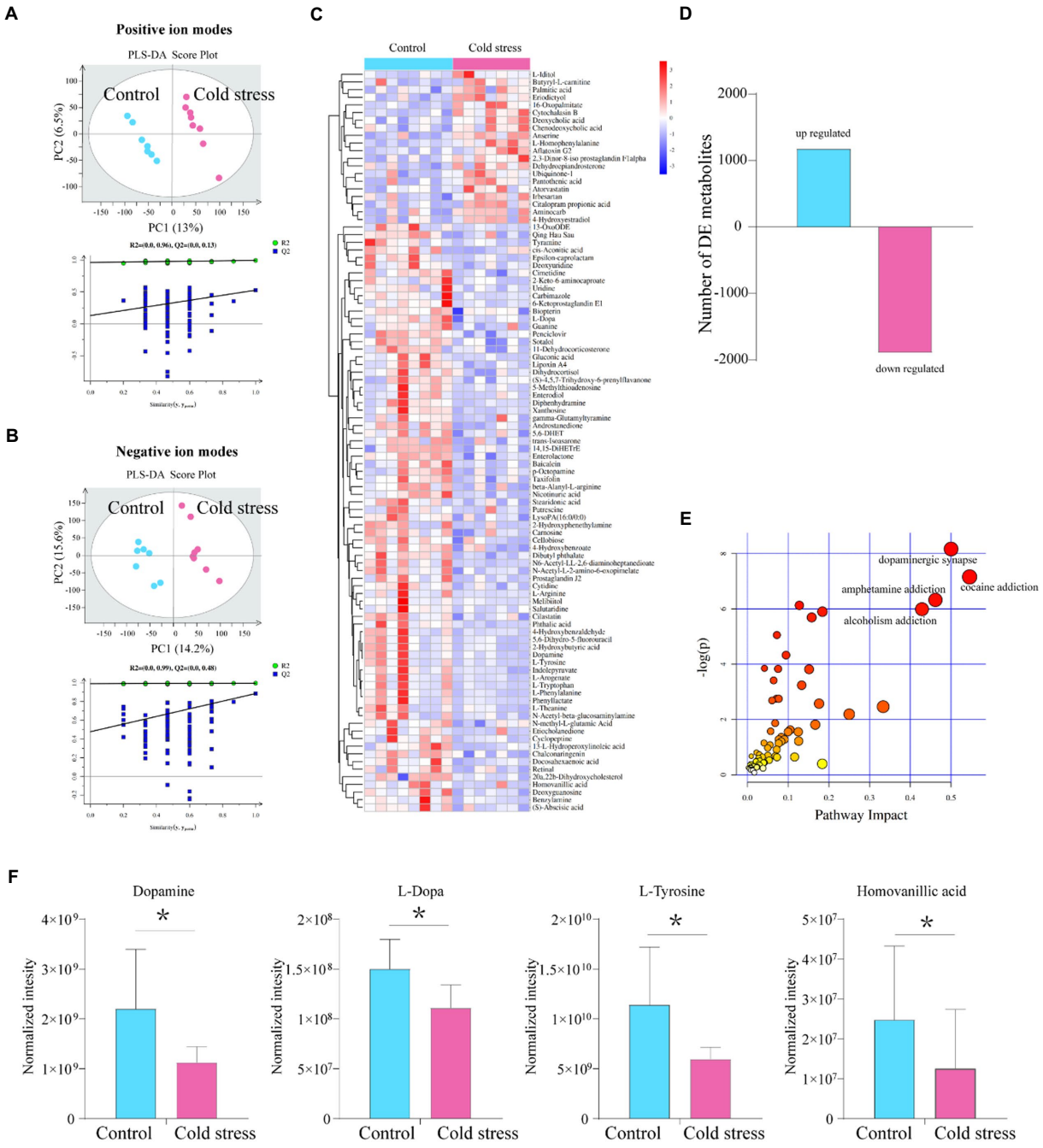

FIGURE 4
Metabolites in the dopamine-related pathway are downregulated by cold stress treatment. **(A)** Partial least-squares discriminant analysis (PLS-DA) scores of all peak features in positive-ion mode. **(B)** PLS-DA scores of all peak features in negative-ion mode. **(C)** Heatmap of the metabolites that altered in the cold stress group. **(D)** Number of differentially expressed (DE) metabolites between the two groups. **(E)** Pathways impacted. **(F)** Metabolites in dopamine-related pathways (dopamine, L-dopamine, L-tyrosine, and homovanillic acid). *p<0.05 (n=8 in control group and n=7 in cold stress group).

and function are disturbed seriously, including bile acids, short-chain fatty acids, and tryptophan (Lloyd-Price et al., 2019; Lavelle and Sokol, 2020). Significant alteration in the gut microbiota metabolites was also identified under cold stress exposure in this study. Interestingly, we found a remarkable reduction in the levels of several metabolites in the dopamine-related pathway.

Dopamine is a critical catecholaminergic neurotransmitter that present in the peripheral tissues and central nervous system simultaneously, which regulates blood pressure, sodium balance, glucose homeostasis, cognition, memory, the sympathetic nervous system, and mood (Pinoli et al., 2017). Although the dopamine is mainly synthesized in the brain, T cells, dendritic cells, and as well as by gut commensals such as members of the genus Clostridium (Vidal and Pacheco, 2020), recently, it has recently recognized as an important regulator of the immune system. Disturbance of the dopamine pathway affects both innate and adaptive immunity

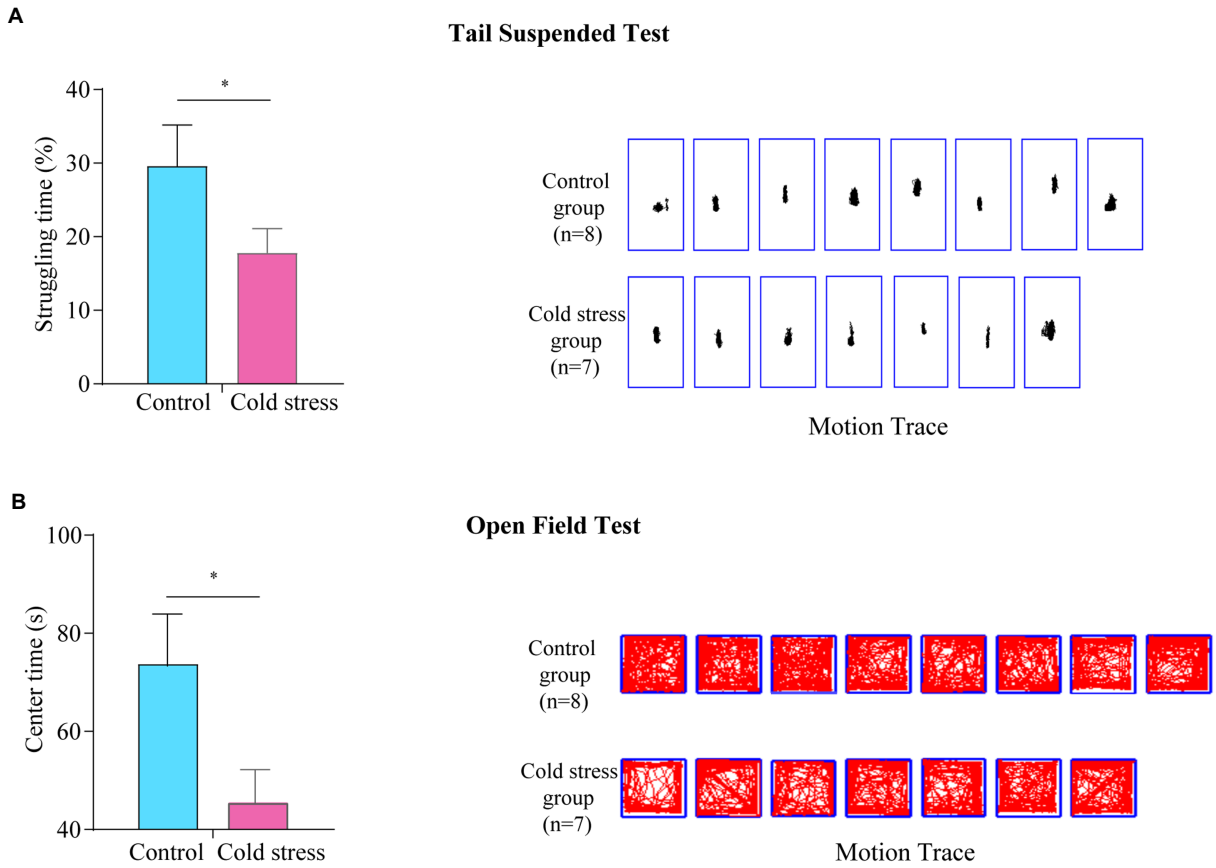

FIGURE 5
Cold stress increases anxiety- and depression-like behaviors. **(A)** Struggling time (left) and motion tracking (right) in the tail suspended test. (B) Center time and motion tracking in the open field test. *p<0.05. (n=8 in control group and n=7 in cold stress group).

largely, causing the development of inflammatory pathologies (Vidal and Pacheco, 2020). A significant proportion of patients with gut barrier injury suffer from anxiety and depression (Byrne et al., 2017), implying a rational connection between the gut and the brain. Consistently, we examined their behaviors and found that cold stress leads to a tendency to depression. Our findings suggest that cold water stress results in reduced neuro-activities in gut microbiota and enhanced pro-inflammatory activity that promotes anxiety and depression, this is may be related to the dopamine pathway disturbance.

Nevertheless, our study has several limitations. We handled mice with cold water to explore the effect of cold stress to the gut barrier, microbiota, and metabolites. These findings acknowledge the important role of cold stress; however, it cannot mimic the condition of cold beverage consumption completely in humans because of the variety of beverages used and the differences between human and mouse physiology. In addition, although we find a significant overgrowth of pathogens and decreased dopamine-related metabolites in cold stress-treated mice, we cannot completely identify that the alteration in gut microbiota causes the gut barrier injury. Further studies to investigate the role of microbiota and their metabolites with the effect on gut barrier and behavior are also needed to deeply elucidate their causal or accompanied relationships. This is a novel attempt to describe the systematic and detailed phenotypes of this model, and the sample size is somewhat too small, more studies are needed to assure the conclusions furtherly.

Conclusion

In summary, our study shows that exposure to cold stress promotes the development of colitis in mice. Our results may also have implications for human beings, as habit-forming consumption of cold beverages or food is implicated in IBD development.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.jianguoyun.com/p/DRel6OYQuaiFChi1vOoEIAA.

Ethics statement

The animal study was reviewed and approved by Animal Research Ethics Board of the Northwest University.

Author contributions

JZ designed the study. LS, XW, YZ, and PL performed the research and wrote the manuscript. YH analyzed the data. CL and JL contributed the methods and models. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1134246/full#supplementary-material

References

Ananthakrishnan, A. N. (2015). Epidemiology and risk factors for ibd. *Nat. Rev. Gastroenterol. Hepatol.* 12, 205–217. doi: 10.1038/nrgastro.2015.34

Barnes, E. L., and Kappelman, M. D. (2018). Editorial: increasing incidence of pediatric inflammatory bowel disease in France: implications for etiology, diagnosis, prognosis, and treatment. *Am. J. Gastroenterol.* 113, 273–275. doi: 10.1038/ajg.2017.431

Byrne, G., Rosenfeld, G., Leung, Y., Qian, H., Raudzus, J., Nunez, C., et al. (2017). Prevalence of anxiety and depression in patients with inflammatory bowel disease. *Can. J. Gastroenterol. Hepatol.* 2017:6496727. doi: 10.1155/2017/6496727

Dinallo, V., Marafini, I., di Fusco, D., Laudisi, F., Franzè, E., di Grazia, A., et al. (2019). Neutrophil extracellular traps sustain inflammatory signals in ulcerative colitis. *J. Crohns Colitis* 13, 772–784. doi: 10.1093/ecco-jcc/jjy215

Eisenstein, M. (2016). Biology: a slow-motion epidemic. *Nature* 540, S98–S99. doi: 10.1038/540S98a

Frank, D. N., St Amand, A. L., Feldman, R. A., Boedeker, E. C., Harpaz, N., and Pace, N. R. (2007). Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. *Proc. Natl. Acad. Sci. U. S. A.* 104, 13780–13785. doi: 10.1073/pnas.0706625104

Goncalves, M. D., Lu, C., Tutnauer, J., Hartman, T. E., Hwang, S. K., Murphy, C. J., et al. (2019). High-fructose corn syrup enhances intestinal tumor growth in mice. *Science* 363, 1345–1349. doi: 10.1126/science.aat8515

Graham, D. B., and Xavier, R. J. (2020). Pathway paradigms revealed from the genetics of inflammatory bowel disease. *Nature* 578, 527–539. doi: 10.1038/s41586-020-2025-2

He, Z., Chen, L., Catalan-Dibene, J., Bongers, G., Faith, J. J., Suebsuwong, C., et al. (2021). Food colorants metabolized by commensal bacteria promote colitis in mice with dysregulated expression of interleukin-23. *Cell Metab.* 33, 1358–1371.e5. doi: 10.1016/j.cmet.2021.04.015

Hugot, J. P., Dumay, A., Barreau, F., and Meinzer, U. (2021). Crohn's disease: is the cold chain hypothesis still hot? *J. Crohns Colitis* 15, 678–686. doi: 10.1093/ecco-jcc/jjaa192

Hur, J., Otegbeye, E., Joh, H. K., Nimptsch, K., Ng, K., Ogino, S., et al. (2021). Sugar-sweetened beverage intake in adulthood and adolescence and risk of early-onset colorectal cancer among women. *Gut* 70, 2330–2336. doi: 10.1136/gutjnl-2020-323450

Khademi, Z., Milajerdi, A., Larijani, B., and Esmaillzadeh, A. (2021). Dietary intake of total carbohydrates, sugar and sugar-sweetened beverages, and risk of inflammatory bowel disease: a systematic review and meta-analysis of prospective cohort studies. *Front. Nutr.* 8:707795. doi: 10.3389/fnut.2021.707795

Kim, J. J., Shajib, M. S., Manocha, M. M., and Khan, W. I. (2012). Investigating intestinal inflammation in dss-induced model of ibd. *J. Vis. Exp.* 60:3678. doi: 10.3791/3678

Koh, H., Lee, M. J., Kim, M. J., Shin, J. I., and Chung, K. S. (2010). Simple diagnostic approach to childhood fecal retention using the leech score and Bristol stool form scale in medical practice. *J. Gastroenterol. Hepatol.* 25, 334–338. doi: 10.1111/j.1440-1746.2009.06015.x

Lavelle, A., and Sokol, H. (2020). Gut microbiota-derived metabolites as key actors in inflammatory bowel disease. *Nat. Rev. Gastroenterol. Hepatol.* 17, 223–237. doi: 10.1038/s41575-019-0258-z

Le Baut, G., O'Brien, C., Pavli, P., Roy, M., Seksik, P., Tréton, X., et al. (2018). Prevalence of yersinia species in the ileum of crohn's disease patients and controls. *Front. Cell. Infect. Microbiol.* 8:336. doi: 10.3389/fcimb.2018.00336

Lloyd-Price, J., Arze, C., Ananthakrishnan, A. N., Schirmer, M., Avila-Pacheco, J., Poon, T. W., et al. (2019). Multi-omics of the gut microbial ecosystem in inflammatory bowel diseases. *Nature* 569, 655–662. doi: 10.1038/s41586-019-1237-9

Mukhopadhya, I., Hansen, R., El-Omar, E. M., and Hold, G. L. (2012). Ibd-what role do proteobacteria play? *Nat. Rev. Gastroenterol. Hepatol.* 9, 219–230. doi: 10.1038/nrgastro.2012.14

Neurath, M. F. (2019). Targeting immune cell circuits and trafficking in inflammatory bowel disease. *Nat. Immunol.* 20, 970–979. doi: 10.1038/s41590-019-0415-0

Pinoli, M., Marino, F., and Cosentino, M. (2017). Dopaminergic regulation of innate immunity: a review. *J. Neuroimmune Pharmacol.* 12, 602–623. doi: 10.1007/s11481-017-9749-2

Spiller, R., and Major, G. (2016). Ibs and ibd - separate entities or on a spectrum? *Nat. Rev. Gastroenterol. Hepatol.* 13, 613–621. doi: 10.1038/nrgastro.2016.141

Steru, L., Chermat, R., Thierry, B., and Simon, P. (1985). The tail suspension test: a new method for screening antidepressants in mice. *Psychopharmacology* 85, 367–370. doi: 10.1007/BF00428203

Su, X., Yin, X., Liu, Y., Yan, X., Zhang, S., Wang, X., et al. (2020). Gut dysbiosis contributes to the imbalance of treg and th17 cells in graves' disease patients by propionic acid. *J. Clin. Endocrinol. Metab.* 105, 3526–3547. doi: 10.1210/clinem/dgaa511

Sun, L., Ma, L., Ma, Y., Zhang, F., Zhao, C., and Nie, Y. (2018). Insights into the role of gut microbiota in obesity: pathogenesis, mechanisms, and therapeutic perspectives. *Protein Cell* 9, 397–403. doi: 10.1007/s13238-018-0546-3

Sun, L., Ma, L., Zhang, H., Cao, Y., Wang, C., Hou, N., et al. (2019a). Fto deficiency reduces anxiety- and depression-like behaviors in mice via alterations in gut microbiota. *Theranostics* 9, 721–733. doi: 10.7150/thno.31562

Sun, L., Zhang, H., Cao, Y., Wang, C., Zhao, C., Wang, H., et al. (2019b). Fluoxetine ameliorates dysbiosis in a depression model induced by chronic unpredicted mild stress in mice. *Int. J. Med. Sci.* 16, 1260–1270. doi: 10.7150/ijms.37322

Sun, W. M., Penagini, R., Hebbard, G., Malbert, C., Jones, K. L., Emery, S., et al. (1995). Effect of drink temperature on antropyloroduodenal motility and gastric electrical activity in humans. *Gut* 37, 329–334. doi: 10.1136/gut.37.3.329

Verhagen, M. A., Luijk, H. D., Samsom, M., and Smout, A. J. (1998). Effect of meal temperature on the frequency of gastric myoelectrical activity. *Neurogastroenterol. Motil.* 10, 175–181. doi: 10.1046/j.1365-2982.1998.00089.x

Vidal, P. M., and Pacheco, R. (2020). Targeting the dopaminergic system in autoimmunity. *J. Neuroimmune Pharmacol.* 15, 57–73. doi: 10.1007/s11481-019-09834-5

Walsh, R. N., and Cummins, R. A. (1976). The open-field test: a critical review. *Psychol. Bull.* 83, 482–504. doi: 10.1037/0033-2909.83.3.482

Yan, X., Jin, J., Su, X., Yin, X., Gao, J., Wang, X., et al. (2020). Intestinal flora modulates blood pressure by regulating the synthesis of intestinal-derived corticosterone in high salt-induced hypertension. *Circ. Res.* 126, 839–853. doi: 10.1161/CIRCRESAHA.119.316394

Yang, H., Wang, W., Romano, K. A., Gu, M., Sanidad, K. Z., Kim, D., et al. (2018). A common antimicrobial additive increases colonic inflammation and colitis-associated colon tumorigenesis in mice. *Sci. Transl. Med.* 10:eaan4116. doi: 10.1126/scitranslmed.aan4116

Yoshida, Y., and Simoes, E. J. (2018). Sugar-sweetened beverage, obesity, and type 2 diabetes in children and adolescents: policies, taxation, and programs. *Curr. Diabetes Rep.* 18:31. doi: 10.1007/s11892-018-1004-6

Zhou, Y., Xu, Z. Z., He, Y., Yang, Y., Liu, L., Lin, Q., et al. (2018). Gut microbiota offers universal biomarkers across ethnicity in inflammatory bowel disease diagnosis and infliximab response prediction. *mSystems* 3:e00188. doi: 10.1128/mSystems.00188-17

Zielinska, M., Jarmuz, A., Wasilewski, A., Cami-Kobeci, G., Husbands, S., and Fichna, J. (2017). Methyl-orvinol-dual activity opioid receptor ligand inhibits gastrointestinal transit and alleviates abdominal pain in the mouse models mimicking diarrhea-predominant irritable bowel syndrome. *Pharmacol. Rep.* 69, 350–357. doi: 10.1016/j.pharep.2016.12.001

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Beyond cellulose: pharmaceutical potential for bioactive plant polysaccharides in treating disease and gut dysbiosis

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Polysaccharides derived from plants, algae, or fungi serve as the major components of some human diets. Polysaccharides have been shown to exhibit diverse biological activities in improving human health, and have also been proposed to function as potent modulators of gut microbiota composition, thus playing a bi-directional regulatory role in host health. Here, we review a variety of polysaccharide structures potentially linked to biological functions, and cover current research progress in characterizing their pharmaceutical effects in various disease models, including antioxidant, anticoagulant, anti-inflammatory, immunomodulatory, hypoglycemic, and antimicrobial activities. We also highlight the effects of polysaccharides on modulating gut microbiota *via* enrichment for beneficial taxa and suppression of potential pathogens, leading to increased microbial expression of carbohydrate-active enzymes and enhanced short chain fatty acid production. This review also discusses polysaccharide-mediated improvements in gut function by influencing interleukin and hormone secretion in host intestinal epithelial cells.

KEYWORDS

polysaccharides, gut microbiota, structure, bioactivities, prebiotics

1. Introduction

Polysaccharides, formed by α- or β-glycosidic bond of identical or various monosaccharide monomers with10 or more polymerization, are naturally produced in large quantities by plants and fungi (Tan et al., 2017). To date, numerous polysaccharides have been shown to exhibit a range of biological activities, including anticoagulation, antiviral, antitumor, antioxidant, hypoglycemic, and immunomodulatory effects (Zeng et al., 2019; Zhang et al., 2019; Wang et al., 2019b; Kalinina et al., 2020; Chaisuwan et al., 2021; Chen C. et al., 2021; Kiddane and Kim, 2021; Liang Q. et al., 2021; Surayot et al., 2021; Figure 1 and Table 1). In addition, many studies have proposed that some polysaccharides also contribute to shaping the structure, diversity and function of gut microbiota and thus play a role in enhancing human health (Koropatkin et al., 2012; Chang et al., 2015; Lin et al., 2018; Zhang L. et al., 2018; Zhang X. et al., 2018; Chen G. et al., 2019; Ding et al., 2019; Wang M. et al., 2019; Wang Y. et al., 2020; Wang et al., 2020b; Guo et al., 2021a).

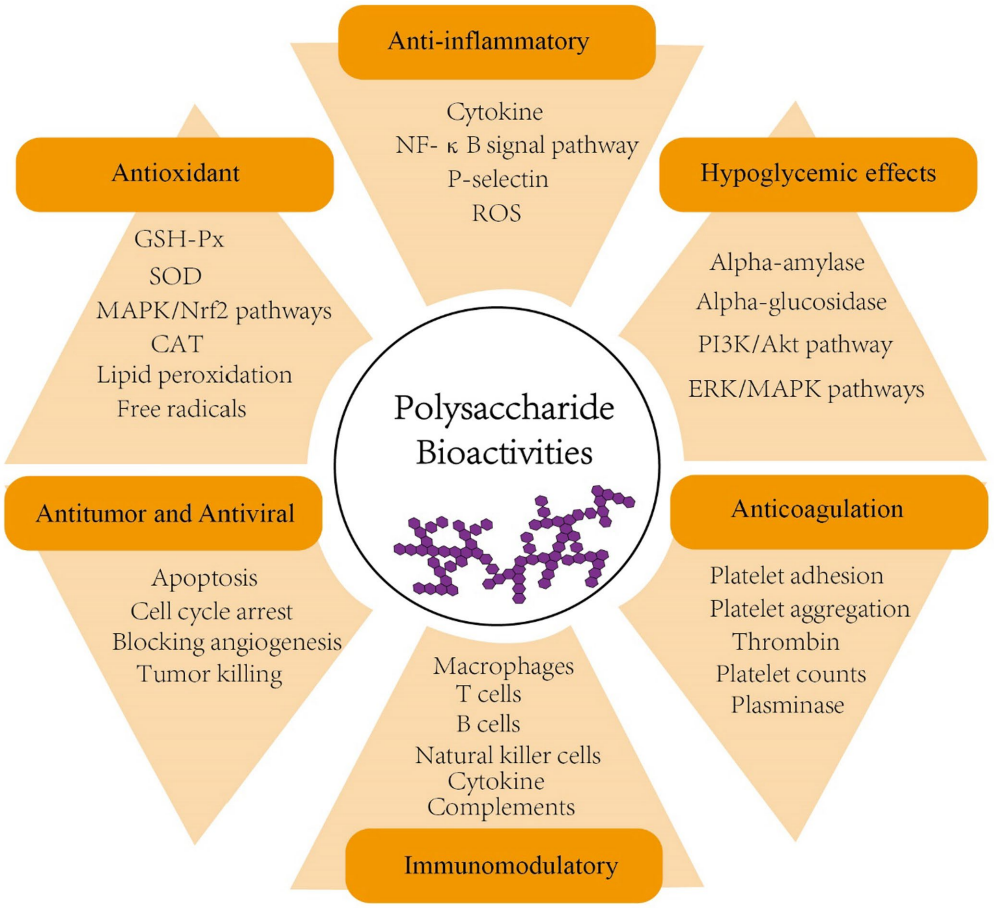

FIGURE 1
Bioactivities of polysaccharides (anticoagulation, antiviral, antitumor, antioxidant, hypoglycemic, anti-inflammatory and immunomodulatory). PI3K, phosphoinositide 3-kinase; AKT, serine/threonine-specific protein kinase; ERK, extracellular-signal-regulated kinase; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-κB; ROS, reactive oxygen species; GSH-Px, glutathione peroxidase; SOD, superoxide dismutase; CAT, catalase; Nrf2, nuclear factor erythroid 2–related factor 2.

Gut microbes share a symbiotic lifestyle with human hosts, colonizing the gastrointestinal tract, and a large number of studies have highlighted the significance of these microbes, collectively termed microbiota, in mediating interactions that determine human health or disease states involving the gastrointestinal, cardiovascular, orthopedic and even neurological systems (Björkstén et al., 2001; Li et al., 2019). Recently, naturally occurring undigestable polysaccharides in plants and foods were found to exert regulatory effects on gut microbiota by selecting for beneficial microorganisms in the gut while inhibiting the growth of pathogenic bacteria. This activity reinforces the structural and functional integrity of the intestinal mucosal barrier, and enhances the intestinal immune system *via* modulation of cytokine expression levels (de Vrese and Schrezenmeir, 2008; Chiu et al., 2014; Fuke et al., 2019; Han et al., 2020; Zhang et al., 2020; Zhao et al., 2020; Guo et al., 2021b). For example, Hawthorn HAW1-2 Polysaccharide (Guo et al., 2021b), *Ziziphus Jujuba* Polysaccharide (Han et al., 2020), glycyrrhiza polysaccharide (Zhang X. et al., 2018), and *Lycium barbarum* polysaccharide (Ding et al., 2019) were all reported to act as prebiotics by affecting gut microbiota structure and diversity. In addition, fungal polysaccharides were also found to play an important regulatory role as prebiotics through mechanisms similar to that of plant polysaccharides (Liang J. et al., 2021). However, studies of these various prebiotic effects of polysaccharides have yet to fully uncover the full range of interaction mechanisms between polysaccharides and gut microbiota. Thus, a summary of the different plant sources and structures of known bioactive polysaccharides can facilitate ongoing research efforts, especially their prebiotic effects and modulation of microbiota in the context of human health. It should be noted that progress toward understanding the diversity of prebiotic polysaccharide functions requires the integration of plant phenotypic data with multi-omics analyses to identify tripartite host-polysaccharide-microbiota interactions.

2. Overview of polysaccharides and their bioactivities

2.1. Structure and classification of polysaccharides

To provide an overview of higher order polysaccharide structures, polysaccharides are first categorized by their primary structure, determined by connection types, the organization and composition of

TABLE 1 The action of some bioactive polysaccharides in disease prevention and treatment.

Source	Polysaccharide	Disease	Action	References
Laminaria japonica	Fucoidan	Parkinson's disease (PD)	Reversed the loss of nigral dopaminergic neurons and striatal dopaminergic fibers	Zhang L. et al. (2018) and Zhang X. et al. (2018)
Inonotus obliquus	Polysaccharide	Alzheimer's disease (AD)	Improved the pathological behaviors correlated with memory and cognition, upregulated Nrf2 expression and its downstream proteins, decreased β-amyloid peptides deposition and neuronal fiber tangles	Han et al. (2019)
Walnut green husk	Polysaccharide	Inflammatory bowel disease (IBD)	Enhanced the Production of SCFAs through fermentation in the colon which help on allivating inflammatory damage and protecting integrity of the intestinal barrier function	Wang et al. (2021)
Walnut green husk	Polysaccharide	Obesity	Relieved the oxidative stress in the liver by modulating the MAPK/Nrf2 pathway, and promoted the browning of inguinal white adipose tissue and thermogenesis in brown adipose tissue.	Wang et al. (2021)
Hypsizygus ulmarius	Polysaccharide	Diabetes mellitus (DM)	Exhibited moderate inhibition activity against α-amylase and α-glucosidase enzyme in a concentration-dependent manner	Govindan et al. (2023)
Momordica charantia L	Selenylated polysaccharide (Se-MCPIIa-1)	DM	Reduced fasting blood glucose levels and increased insulin levels	Ru et al. (2020)
Hawthorn (*Crataegus.*)	Polysaccharide	Colon cancer	Arrested the cell cycle in the S and G2/M phases, increased the rate of apoptosis, downregulated the expression of Cyclin A1/D1/E1 and CDK-1/2	Ma et al. (2020)
Crataegus pinnatifida	Polysaccharide	Colitis	Restored the pathological lesions in colon, decreased the expression of inflammatory cytokines (IL-1β, IL-6, and TNF-α)	Guo et al. (2021b)
Lactobacillus plantarum YW11	Exopolysaccharide	IBD	Inhibited inflammatory cytokines (TNF-α, IL-1β, IL-6, IFN-γ and IL-12) and enhanced the anti-inflammatory cytokine IL-10.	Min et al. (2020)
Bacillus subtilis	Exopolysaccharide	Bacterial infections	Limited superantigens-T cell activation by *S. aureus* and abrogated systemic induction of gamma interferon.	Paik et al. (2019)
Red seaweed *Gelidium pacificum* Okamura	Sulfated polysaccharide	Antibiotic-associated diarrhea (AAD)	Promoted the recovery of the gut microbiota and improved mucosal barrier function, downregulated the levels of inflammatory cytokines and enhanced the production of SCFAs.	Cui et al. (2020)

*Nrf2, nuclear factor erythroid 2–related factor 2; SCFA, short-chain fatty acids; MAPK, mitogen-activated protein kinase; CDK1/2, cyclin dependent kinase-1/2; IL, interleukin; IFN, interferon; TNF, tumor necrosis factor.

sugar residues, the configuration of glycosidic bonds, and the conformation of sugar rings (Yang et al., 2020). The secondary structure of oligosaccharides, i.e., their regular conformation resulting from hydrogen bonding (the most common secondary bonds between main chains), is determined by the dihedral Φ, Ψ or ω angles of the polymer backbone (Hatcher et al., 2011). Based on the secondary structure of polysaccharides, the tertiary structure is formed by non-covalent interactions among carboxyl groups, hydroxyl groups, sulfate groups, and/or amino groups on the sugar units. The polysaccharide quaternary structure refers to aggregates formed by non-covalent bonding between polymer chains (Lafond et al., 2016). In addition, polysaccharides can be classified as either homopolysaccharides or heteropolysaccharides, homo-polysaccharides are composed of a single type of monosaccharide and hetero-polysaccharides are composed of different types of monosaccharides (Sinha and Kumria, 2001; Liu et al., 2008). For example, glucans are glucose homopolysaccharides, while mannans are mannose homopolysaccharides (d'Ayala et al., 2008). Overall, polysaccharides are categorized as glucans, mannans, pectin polysaccharides, arabinogalactans, galactans, fucoidan, fructan, and polyxylose, among others, based on their monosaccharide composition and linkage types (Tan et al., 2017; Maji, 2019; Figure 2).

Different monosaccharide contents and branching exhibit various bioactivities. Wu et al. (2020) isolated polysaccharide from the seeds of

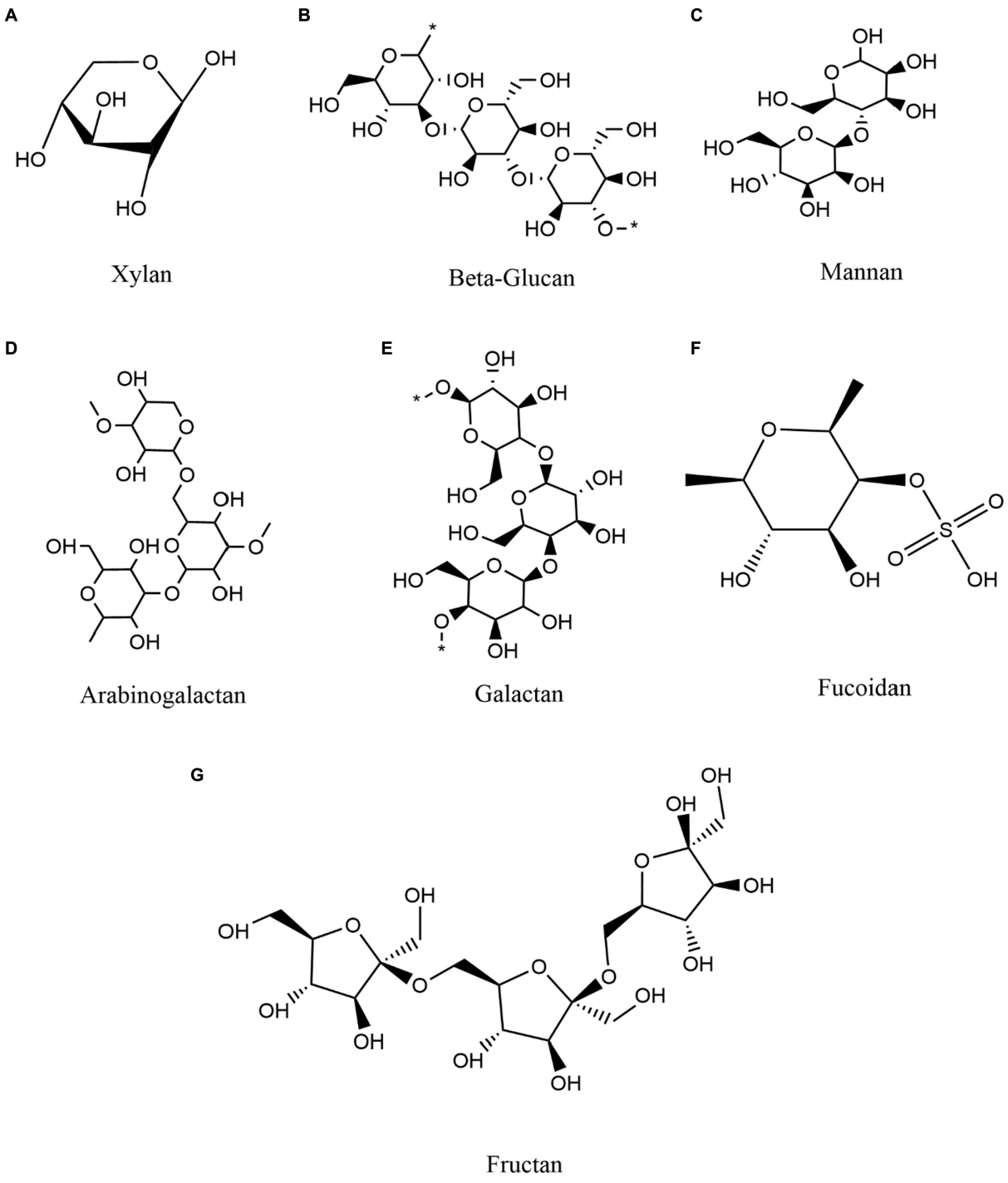

FIGURE 2
Classification and composition of some representative polysaccharides and commonly polymerized monosaccharides. **(A)** Xylan **(B)** Beta-Glucan **(C)** Mannan **(D)** Arabinogalactan **(E)** Galactan **(F)** Fucoidan **(G)** Fructan.

Litchi chinensis Sonn. and reported reduction of antitumor activity of branched backbone of polysaccharides with more than four monosaccharide units. The monosaccharide contents of polysaccharides could also influence their anti-inflammatory activity (Chen Y. C. et al., 2019). In addition, polysaccharides contain many functional groups, including hydroxyl groups and a hemiacetal reducing end that has the potential to reduce precursor salts (Park et al., 2011). Studies have revealed that the oxidation of polysaccharide hydroxyl groups to carbonyl groups play a significant role in reducing gold salts (Mata et al., 2009). Alternatively, the reducing end of polysaccharides has been used to introduce an amino functional group capable of forming complexes with and stabilizing metallic nanoparticles (Nadkarni et al., 1994). Carbohydrates with these amino groups bind strongly to the surface of gold or silver nanoparticles to

provide a hydrophilic surface (Kemp et al., 2009). The introduction of specific functional groups will change a polysaccharide's molecular weight, structure, types, position and number of substituents groups, and may alter the physiochemical and functional properties of that polysaccharide (Li et al., 2016). More specifically, various modifications, including sulfation, phosphorylation, carbomethylation, benzoylation or acetylation have all been shown to enhance the biology activity of some specific polysaccharides (Xu Y. et al., 2019). For instance, in heparan sulfate (HS) polysaccharides, negatively charged sulfate and carboxylate decorations can be organized into a variety of different so-called HS S-domains through a tightly controlled biosynthetic pathway that enables remarkable structural variability (Li and Kusche-Gullberg, 2016). Using standard DPPH and ABTS assays, Cao et al. (2020) characterized the antioxidant properties of sulfated polysaccharides from *Amana edulis* (Cao et al., 2020). Another study illustrated the positive effects of phosphorylation- based modifications on increasing the antioxidant activity of polysaccharides isolated from *Momordica charantia* (Chen F. et al., 2019). Recently, nanocellulose incorporated polysaccharides were reported to extent nanoparticle application in health promotion. Incorporation of chitosan and nanocellulose could improve their antimicrobial activity (Tomé et al., 2013), as well as exerting roles in wound healing (Hasan et al., 2017). These different combinations of functional groups of polysaccharides from diverse sources, along with monosaccharide composition, linkage types, and chemical modifications can thus result in different biological activities.

2.2. Polysaccharide bioactivities

2.2.1. Anticoagulation and anti-inflammatory effects

Polysaccharides are widely reported to exhibit anticoagulation properties by inhibiting thrombin activity, decreasing platelet counts, inhibiting platelet adhesion and aggregation, enhancing plasminase activity, and promoting the dissolution of fibrin (Matsubara et al., 2001; Pawlaczyk et al., 2011; Xue et al., 2018). Souza et al. (2015) identified polysaccharides from spinosa bark that could provide anticoagulant, antiplatelet and antithrombotic effects without increasing inducing a greater likelihood of hemorrhage (Souza et al., 2015). Similarly, an acidic polysaccharide was extracted from the edible mushroom *Auricularia auricula* which contained mannose, glucose, glucuronic acid and xylose oligosaccharides, but no sulfate ester links. This polysaccharide displayed potent anticoagulation effect by inhibiting thrombin *via* antithrombin activation (Yoon et al., 2003). Sulfated galactans produced by the seaweed *Hypnea musciformis* were also found to have antioxidant, anticoagulant, and immunostimulatory properties, depending on the method of their extraction (Gabriela das Chagas Faustino Alves et al., 2016).

Other than anticoagulant effects, polysaccharides are also known to exert anti-inflammatory activities. Fu et al. (2022) studied the structure of polysaccharides from Chinese aconite (*Aconitum carmichaelii*) leaves and demonstrated that these polysaccharides have immunomodulatory and anti-inflammatory effects on lipopolysaccharide (LPS)-induced inflammation in intestinal epithelial cells (Fu et al., 2022). In addition, *in vitro* investigation of the bioactivity of polysaccharides from *Typha angustifolia* using RAW264.7 cell provided evidence indicating that these polysaccharides can significantly suppress inflammatory cytokine production, nuclear factor-κB (NF-κB) signal pathway activation, and reactive oxygen species (ROS) production (Wei et al., 2021). Study on *Sargassum fusiforme* fucoidan has shown that polysaccharides can inhibit selectin-mediated leukocyte migration and infiltration by blocking interactions between P-selectin and its ligands on leukocytes, ultimately reducing the levels of IL-6, IL-8, TNF, and CRP cytokines to ameliorate systemic inflammation (Wu S. et al., 2019). Guo et al. (2018) studied the anti-inflammatory activity and related mechanism of polysaccharides isolated from *Sargentodoxa cuneata.* Their findings demonstrated that these polysaccharides could markedly inhibit carrageenan-induced edema in the hind paw of rats by decreasing malondialdehyde and prostaglandin E2 levels in the hind paw, serum and liver, while promoting SOD activity in serum and liver (Guo et al., 2018).

2.2.2. Immunomodulatory effects

Numerous polysaccharides from fungi and plants can reportedly provide various dietary and medicinal benefits, including marked effects on immune system function. Polysaccharides have also been shown to function as immunomodulators through a variety of mechanisms (Mousavi et al., 2015), such as activating macrophages, T cells, B lymphocytes, or natural killer cells, or by activating complements and promoting cytokine production (Kouakou et al., 2013). This regulation of innate immune response can substantially impact the host's ability to rapidly respond to pathogens. As an essential component of the host immune system, macrophages collaborate with other cell types, such as neutrophils, to resist the adverse effects of biotic and abiotic stresses (Schepetkin and Quinn, 2006; Shen et al., 2014). To augment this function, some polysaccharide signal molecules can activate a macrophage-mediated immune response *via* binding with different receptors on macrophages, such as Toll-like receptor 4, complement receptor 3, scavenger receptor, mannose receptor, and Dectin-1, consequently initiating one or more intracellular signaling cascades that ultimately result in production of inflammation-related cytokines (Schepetkin and Quinn, 2006; Liao et al., 2015; Bunyatyan et al., 2017; Gong et al., 2017; Zhou et al., 2020). These macrophage-associated immunomodulatory effects of plant polysaccharides are largely mediated by increased ROS generation, cytokine secretion, cell proliferation, and phagocytic activity of macrophages (Gong et al., 2017) One earlier study described a novel polysacchaide obtained from the fruiting body of *Dictyophora indusiate* that could significantly promote macrophage secretion of NO, TNF-α, and IL-6 *via* complement receptor 3 in mouse RAW 264.7 cells (Liao et al., 2015). As organic selenium compound with complex chemical structure and diverse sources, selenium polysaccharide has been widely studied as its biological activities. A wide variety of non-specific immune cells, such as natural killer cells and macrophages display significantly improved immune function in the presence of selenium polysaccharides (Zhou et al., 2020). These advances in understanding the scope of polysaccharide activity suggest that many more plant polysaccharides with immunomodulatory effects have yet to be identified through extensive screening and research.

2.2.3. Hypoglycemic effects

Diabetes is a chronic, metabolic disease with typical hyperglycemia symptoms which is characterized by insulin resistance and a relative/

absolute insulin insufficiency (Bunyatyan et al., 2017). Plant polysaccharides can also stimulate insulin secretion, modulate the activity of glucose metabolizing enzymes, inhibit the gluconeogenesis pathway, and promote glucose utilization in peripheral tissues, thus performing important functions in the prevention and treatment of diabetes (Wu et al., 2016). Tea made with guava (*Psidium guajava* L., Myrtaceae) leaves has long been used as a traditional herbal treatment for diabetes in Asia and North America (Oh et al., 2005). Polysaccharides from guava leaf have been shown to exhibit potent free-radical scavenging activity toward DPPH, OH, and ABTS, and can significantly lower fasting blood sugar, total cholesterol, total triglyceride, glycated serum protein, creatinine, and malonaldehyde levels (Luo et al., 2019). In addition, treatment with these polysaccharides can significantly increase total antioxidant activity and superoxide dismutase (SOD) enzyme activity in diabetic mice, consequently ameliorating damage to the liver, kidney, and pancreas (Luo et al., 2019). Recently, the application of bitter gourd (*Momordica charantia*) as herbal medicine/vegetable in the treatment against diabetes has been widely reported. One study investigating the hypoglycemic effects of *Momordica charantia* polysaccharides in alloxan-induced diabetic mice model showed that the polysaccharide treatment led to significantly lower fasting blood glucose levels and improved glucose tolerance, thus proposed dose-dependent anti-diabetic activity (Xu et al., 2015). In order to obtain better antidiabetic activities, a selenylated polysaccharide from *Momordica charantia* has been applied to diabetic mice and was reported to prevent pancreatic islets, liver and kidney damage from diabetes by reduction of fasting blood glucose levels, enhancement of insulin levels and antioxidant enzyme activities (Ru et al., 2020). The pumpkin polysaccharides also demonstrated a significant glucose tolerance effect, and effectively alleviated insulin resistance in addition to providing cytoprotective benefits on type II diabetes mellitus (T2DM) mice (Chen X. et al., 2019). In particular, glucomannan and glucogalactan have been shown to exhibit significant antidiabetic properties by inhibiting alpha-amylase and alpha-glucosidase activity to promote pancreatic beta cell proliferation and stimulate insulin sensitivity and secretion (Mirzadeh et al., 2021).

Mechanistically, these effects are mediated by phosphorylated tyrosine residues present in the intracellular substrates of the insulin receptor (IRS) (Mirzadeh et al., 2021). These substrates could activate the phosphoinositide 3-kinase (PI3K), phosphoinositide-dependent protein kinase 1 and 2 (PDK1/2) and then activates serine/threonine-specific protein kinase (AKT) pathways (Ganesan and Xu, 2019). AKT activates the phosphorylation of glycogen synthase kinase 3 (GSK3), resulting in upregulation of glycogen synthesis in the liver and skeletal muscle (Mirzadeh et al., 2021). Moreover, AKT can also stimulate the translocation of glucose transporter 4 (GLUT4) to the plasma membrane which consequently enhance glucose uptake (Wang et al., 2012). Study also found that AkT is a main mediator in activating the extracellular-signal-regulated kinase (ERK) /mitogen-activated protein kinase (MAPK) pathways thereby triggering several physiological and biochemical mechanisms, such as cell differentiation, proliferation, apoptosis, and cell endurance (Jayachandran et al., 2019). IRS studies on *Ophiopogon japonicus* polysaccharide and glucopyranose-rich heteropolysaccharide from *Catathelasma ventricosum* demonstrated that these compounds could trigger the PI3K/AKT signaling pathway through IRS1, PI3K-p85, and phosphorylated AKT to promote insulin sensitivity and improve diabetes-associated renal disease (Wang et al., 2012; Liu et al., 2016a,b). Collectively, polysaccharide could regulate glucose uptaking, glycogen synthesis and β-cell activity through PI3K/Akt pathway and ERK/MAPK pathways and resulting in playing an anti-diabetic role (Figure 3).

2.2.4. Antioxidant effects

Oxidative stress can be link to a variety of diseases including cancer, cardiovascular diseases, diabetes, respiratory diseases, immune deficiency and neurodegenerative disorders, while antioxidants could protect cells against free radicals and reduce the risk of many diseases (Fridovich, 1999; Fang et al., 2002). Plant polysaccharides have been shown to directly eliminate free radicals by inhibiting lipid peroxidation, scavenging hydroxyl free radicals, and clearing superoxide anion free radicals. They also act on free radicals indirectly by enhancing the activities of SOD, catalase (CAT), and glutathione peroxidase to maintain a balance of free radicals, which can collectively diminish or avert the occurrence of disease (Xie et al., 2016). Lin et al. (2022) used enzymatic and microwave extraction methods to obtain polysaccharides from Purple-Heart Radish (*Raphanus sativus*) that displayed high antioxidant effects by inhibiting lipid peroxidation (Lin et al., 2022). Studies of polysaccharides from yerba mate (*Ilex paraguariensis*) tea reported IC50 values of 3.36 ± 0.31 mg/mL for ·OH scavenging activity, suggesting a strong antioxidant capacity (Chen X. et al., 2019). Xu et al. (2012) characterized polysaccharides from flowers of *Camellia sinensis* and found a high, dose-dependent capacity for scavenging superoxide anion free radicals (Xu et al., 2012). Study of polysaccharides from *Astragalus membranaceus* (Fisch.) has shown that ROS levels decrease, SOD activity increases, and superoxide dismutase free radical scavenging is enhanced, which can alleviate tissue damage and delay senescence (Song et al., 2019). Shan et al. (2011) found that administering *Lycium barbarum* polysaccharides led to significantly higher SOD and glutathione peroxidase (GPX) levels in rats with exercise-induced oxidative stress, indicating that these polysaccharides played a significant role in preventing oxidative stress after exhaustive exercise (Shan et al., 2011). Investigation of alfalfa polysaccharides illustrated their antioxidant effects on immune response in preventing H_2O_2-induced oxidative damage by activating mitogen-activated protein kinase (MAPK)/nuclear factor erythroid 2–related factor 2 (Nrf2) signaling pathways while suppressing NF-κB signaling in mouse embryonic fibroblasts (Wang et al., 2019a).

In addition to naturally occurring polysaccharides, chemical modification can also enhance the antioxidant effects of some polysaccharides. For example, findings by Gao et al. (2017) showed that selenylation of *Angelica sinensis* polysaccharide could enhance its antioxidant and hepatoprotective activity through inhibition of p-ERK and p-JNK signaling in mice with hepatic injury (Gao et al., 2017). Collectively, polysaccharides from diverse sources have been applied as functional antioxidant components in many pharmaceutical/nutraceutical products due to their capacity for modulating ROS levels, enzymatic and non-enzymatic antioxidant defense responses (e.g., SOD, CAT, GPX), and oxidative stress-induced signaling pathways (e.g., MAPK ERK, and JNK).

2.2.5. Antitumor and antiviral capacities

Cancer remains one of the greatest threats to public health worldwide, and is a long-standing focus of research attention and drug

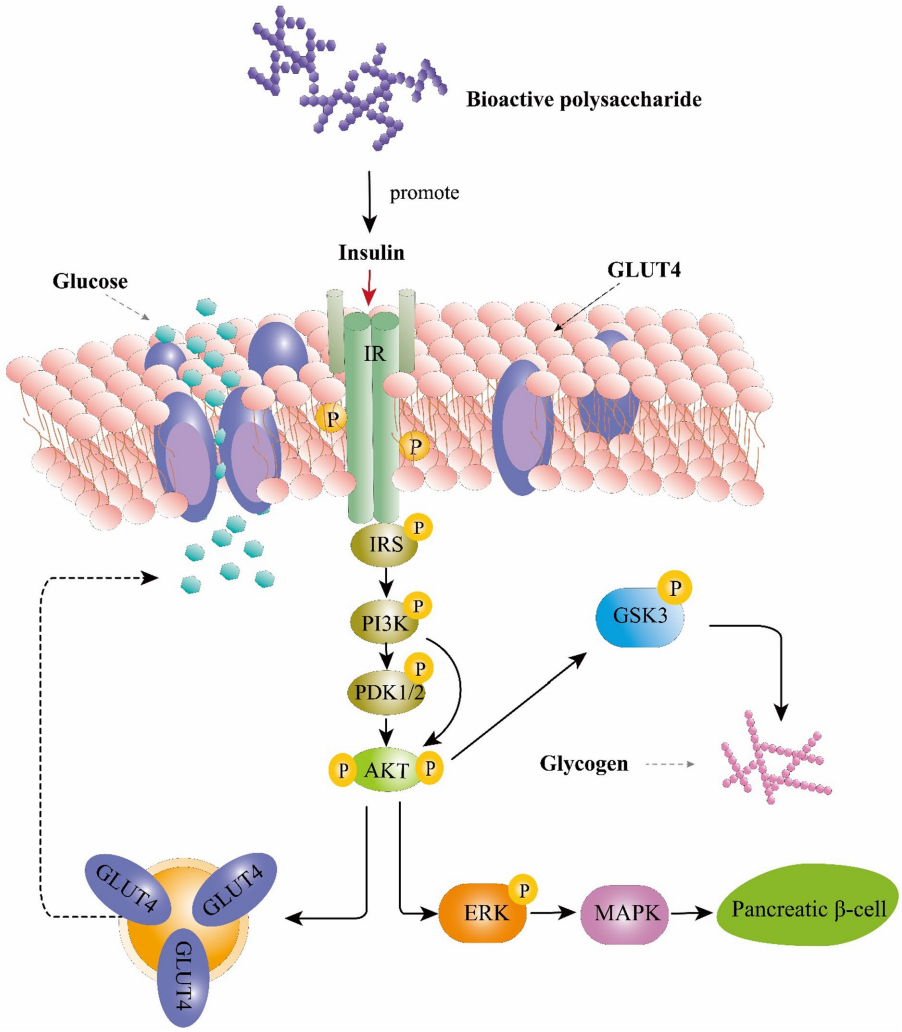

FIGURE 3
The hypoglycemic function of bioactive polysaccharides on the insulin signaling pathway. IRS, insulin receptor substrate; PI3K, phosphoinositide 3-kinase; PDK1/2, phosphoinositide-dependent protein kinase 1 and 2; AKT, serine/threonine-specific protein kinase; GSK-3, glycogen synthase kinase-3; GLUT4, glucose transporter type 4; ERK, extracellular-signal-regulated kinase; MAPK, mitogen-activated protein kinase.

development, especially those with reduced side-effects. Bioactive polysaccharides have been identified that exert anti-tumor activity toward a variety of tumor cells without inducing cytotoxicity in normal cells (You et al., 2013). In the cactus (*Opuntia dilleniid*), polysaccharides were found that can induce S-phase arrest and block the growth of SK-MES-1 cells, possibly through increased levels of P53 and phosphatase and tensin homolog deleted on chromosome 10 protein (PTEN) (Li et al., 2014). The screening and discovery of new anti-tumor polysaccharides represents an ongoing pursuit for many research groups, with the most common direct tumor-killing mechanisms of anti-tumor polysaccharides involving cell cycle arrest, blocking angiogenesis, and inducing apoptosis. In addition, some polysaccharides act through immunomodulation to indirectly induce tumor killing (Khan T. et al., 2019; Lu et al., 2020).

Several studies have described antiviral effects of both natural and chemically modified polysaccharides. For example, Witvrouw et al. (1994) observed antiviral activity by a sulfated polysaccharide isolated from the red seaweed (*Aghardhiella tenera*) *in vitro* and found that it could inhibit the cytopathic effects of human immunodeficiency virus type 1 (HIV-1) and type 2 (HIV-2), as well as against other enveloped viruses. Examination of the inhibitory effects of *Glycyrrhiza* polysaccharide (GPS) on bovine immunodeficiency virus (BIV), adenovirus type III (AdVIII), and coxsackie virus (CBV3) revealed that GPS could inhibit BIV to some extent, but showed obvious inhibition or direct inactivation of AdVIII and CBV3 (Wang Y. et al., 2020). Further technological advances in plant polysaccharide research will enable more comprehensive screening for effective anti-tumor and antiviral polysaccharide drugs.

3. Prebiotic effects of various sources of polysaccharides on gut microbiota

The adult human intestinal tract harbors an estimated stable community of 39 trillion microbial cells, which has been recognized as a diverse and dynamic ecosystem containing bacteria, fungi, protozoa, and viruses (Rajakovich and Balskus, 2019). Gut bacterial communities are generally comprised of six major phyla, including

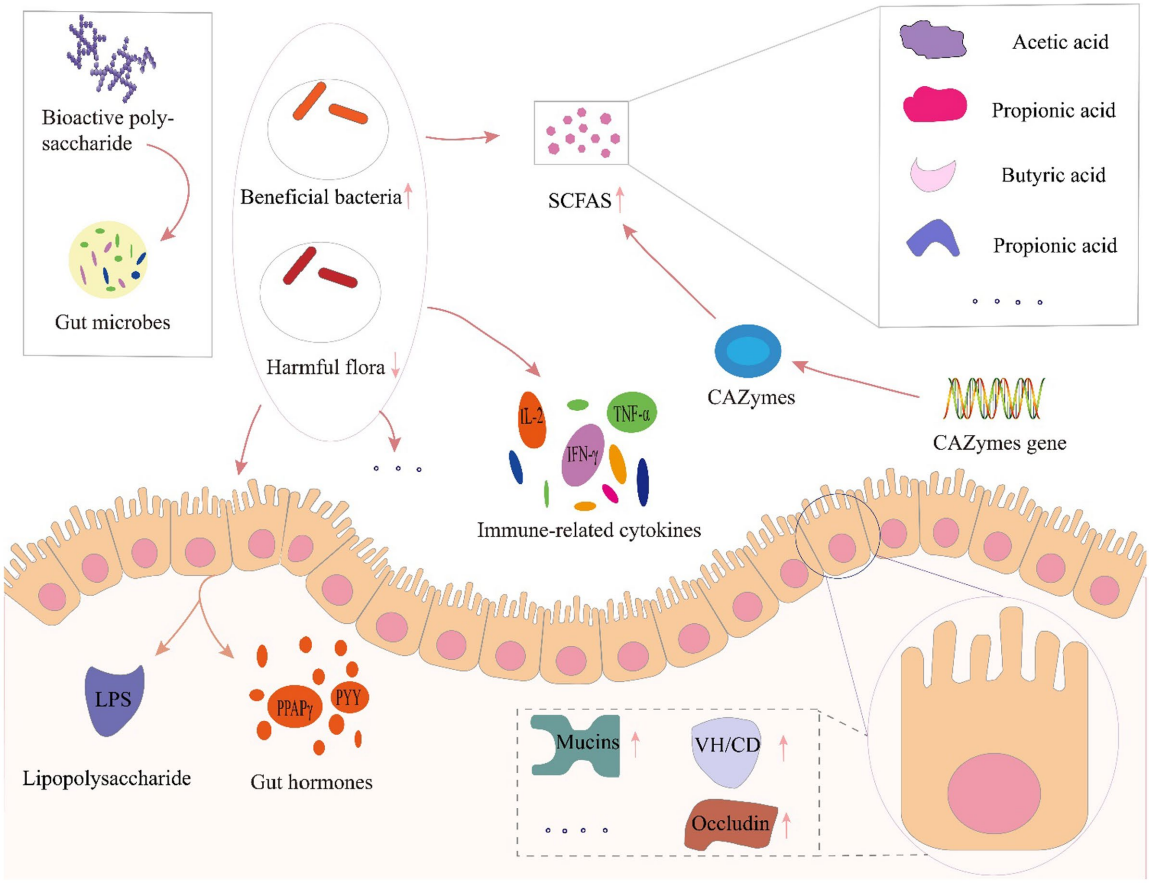

FIGURE 4
Prebiotic effects of polysaccharides toward gut microbial modulation. LPS, lipopolysaccharide; VH/CD, villus height/crypt depth; CAZymes, carbohydrate-active enzymes; SCFA, short-chain fatty acids; PPARγ, peroxisome proliferator-activated receptor-γ; PYY, peptide tyrosine; IL-2, interleukin-2; IFN-γ, interferon-γ; TNF-α, tumor necrosis factor-α.

Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Verrucomicrobia, and Fusobacteria (Gong et al., 2020), which collectively contribute to protecting the intestinal barrier, preventing pathogen invasion, participating in vitamin synthesis (Vitamin K, Vitamin B), host metabolism, and other functions related to nutrient uptake (Cresci and Bawden, 2015). In addition, mutual interactions between gut microbiota and their host were found to provide important functions in host health, and dysbiosis in gut microbiota has been linked to inflammatory bowel disease, obesity, allergies, and psychological disorders (Coyte et al., 2015). Patterson et al. (2016) proposed a possible association between gut microbiota and obesity and metabolic syndrome, which are both accompanied by an increased ratio of Firmicutes to Bacteroidetes in obese phenotypes patients, compared with that in non-obese individuals (Patterson et al., 2016). Other studies have indicated that gut microbiota can influence the development of neurological disorders as autism, depression, Alzheimer's disease, and Parkinson's disease (Dinan and Cryan, 2017).

Several factors, including infant delivery modes and feeding patterns, host, diet, antibiotic usage, and other factors have all been reported to affect gut microbiota composition and diversity (Cresci and Bawden, 2015). Among these factors, host and diet exert a particularly strong influence on gut microbiota. Our own previous study identified clear differences in gut microbiota driven by dietary variation among people living in close regional proximity (Liu et al., 2020). Furthermore, dietary intervention was recently recognized as a reliable strategy for altering gut microbiota and enhancing gut function, especially through prebiotics. Prebiotics are food components that may promote health in the host by exert activity in the gastrointestinal microbiota, such as fructose oligosaccharides, arabino-oligosaccharides, isomalt oligosaccharides, polyphenols and polyunsaturated fatty acids and etc. (Gibson et al., 2004, 2017). Among these prebiotics, dietary polysaccharides are relatively large macromolecules that are difficult to digest and absorbed, but can serve as carbon sources that potentially enrich beneficial gut microbes, or regulate microbial growth, activity, and metabolite production (Koropatkin et al., 2012; Chang et al., 2015; Wang et al., 2018). Furthermore, the polysaccharides affected gut microbiota modulation *via* enrichment for beneficial taxa and suppression of potential pathogens, leading to increased microbial expression of carbohydrate-active enzymes and enhanced short chain fatty acid production. In addition, polysaccharide mediated improvements in gut function such as influencing immune-related cytokines and hormone secretion in host intestinal epithelial cells and maintaining gut barrier function (Figure 4).

Plant polysaccharides have been frequently proposed to serve as regulators of gut microecology (Fang et al., 2019), and have been

shown to regulate the diversity and structure of gut microbiota, thus altering metabolic functions in the gastrointestinal tract (Zhao et al., 2021). Oral administration of polysaccharides from *Ziziphus Jujuba* cv. Pozao in cyclophosphamide-induced mice led to enrichment of Firmicutes and decreased Bacteroidetes, with genus-level increases in the relative abundance of *Bacteroidales-S24-7-group*, *Lachnospiraceae*, *Alloprevotella*, *Alistipes,* and *Bacteroides* (Han et al., 2020). Chen and colleagues showed that *Sarcodon aspratus* polysaccharides could serve as a prebiotic treatment to prevent obesity in mice fed with a high-fat diet, and led to increased relative abundance of *Lactobacillus*, *Bacteroides* and *Akkermansia*, decreased Firmicutes-to-Bacteroidetes ratio, inhibitory effects on immune cells activation and adipocyte differentiation in adipose tissues (Chen et al., 2020). *Poria cocos* polysaccharides were shown to alleviate intestinal mucosal injury, improve intestinal integrity, and restore the composition and structure of gut microbiota following dysbiosis by promoting the proliferation of beneficial bacteria in Ob/Ob Mice (Sun et al., 2019). Li et al. (2020) investigated the hypoglycemic and hypolipidemic effects of tea polysaccharides, potentially mediated by changes in gut microbiota and metabolism, in a rat model of type 2 diabetes. They stated that treatment with tea polysaccharides restored some specific bacterial taxa, such as *Lachnospira*, *Victivallis*, *Roseburia*, and *Fluviicola* in diabetic rats, whereas *Bacteroides* was decreased (Li et al., 2020). Taken together, polysaccharides play an important role in improving body health by increasing the enrichment of beneficial bacteria and reducing the proximity of harmful bacteria.

In addition, gut microbiota could produce short-chain fatty acids (SCFAs) and other metabolites, such as acetic acid, propionic acid, butyric acid, lactic acid and succinic acids, etc. These SCFAs can, in turn, affect gut functions to jointly regulate health (Wu Y et al., 2019). SCFAs are the final product of polysaccharide fermentation by gut microbiota (Mitsou et al., 2020), and are well-known to contribute to maintaining epithelial cell growth in the colon, modulate host metabolism, participate in immune regulation of the intestinal system, and play an indispensable role in maintaining the homeostasis of human gut microbiota (Morrison and Preston, 2016). In a study by Guo et al., treatment with polysaccharide from hawthorn (*Crataegus monogyna*) by gavage ameliorated inflammation symptoms in mice with dextran sulfate sodium (DSS)-induced colitis through significantly increased production of total SCFAs and acetic acid, attributable to greater relative abundance of *Alestipes* and *Odoribacter* (Guo et al., 2021b). Investigation of *Cyclocarya paliurus* polysaccharides in type 2 diabetic rat models. Suggested that these molecules could enrich for SCFA-producing bacteria, leading to elevated SCFA production and upregulation of associated sensory mediators that alleviate symptoms of type 2 diabetes (Yao et al., 2020). β-glucans, inulin, and oligofructose have all been found to significantly increase butyric acid production, which serve as a nutrient source for intestinal epithelial cells, reduces pH in the lumen, and provides energy for human activities (Song et al., 2014; Kim and Jazwinski, 2018), whereas polysaccharides such as α-glucan, fructan, and arabinoxylan can reportedly increase intestinal acetic acid levels (Shang et al., 2018). Insoluble polysaccharide isolated from the sclerotium of *Poria cocos*, as a prebiotic, increased the abundance of butyrate-producing bacteria such as *Lachnospiracea* and *Clostridium*, leading to an increase in the level of butyrate and improvement of gut mucosal integrity and activated the intestinal PPAR-γ pathway, significantly improving glucose and lipid metabolism and alleviating hepatic steatosis in ob/ob mice, suggesting its potential for the treatment of metabolic diseases (Sun et al., 2019). As one of the important natural polysaccharides source, marine polysaccharides were also well studied recently. Lentinan and sea anemone polysaccharides were recently reported to facilitate the production of total SCFAs, maintain gut homeostasis, and provide energy (Wang et al., 2018). In addition, polysaccharides from tea, *C. sinensis,* can also reduce blood glucose and lipid levels, promote SCFA production, attenuate insulin resistance, confer protective effects against pancreatic damage in type 2 diabetic rat model (Li et al., 2020).

In addition to regulating SCFA production, study showed that plant polysaccharides can up-regulate the expression of genes encoding carbohydrate-active enzymes (CAZymes), enhancing CAZyme activity and thus leads to higher SCFA production and increased tight junction protein expression with concurrent suppression of metabolic endotoxemia and decreased expression of inflammatory factors (Nguyen et al., 2016). Guo et al. (2021a) reported that *Ganoderma lucidum* polysaccharide could also upregulate CAZyme expression, especially glycoside hydrolases, polysaccharide lyases, glycosyltransferases, and carbohydrate esterases, leading to improved health.

Furthermore, polysaccharides can facilitate repair of damaged intestinal barrier to ensure the integrity of intestinal structures (Liang J. et al., 2021). Zhou et al. (2021) conducted co-treatment of American ginseng polysaccharide and ginsenoside altered uric acid, xanthurenic acid, acylcarnitine and restored the morphology of intestine. Specifically, the co-treatment resulted in an up-regulation of the villus height (VH)/crypt depth (CD) ratio, as well as an increase in the areas of mucins expression, quantity of goblet cells and expression of tight junction proteins (ZO-1, occludin) and then protecting the intestinal barrier (Zhou et al., 2021). Additionally, study of Yu et al. (2021) found that *Cyclocarya paliurus* polysaccharides dramatically increased the intestine antioxidant defense of CTX-induced mice, repaired the intestinal barrier by restoring the length of villi and depth of crypt, up-regulating the expression of tight junction proteins, shifting the composition and diversity of the gut microbiota, and regulating the relative abundances of specific taxa, then restoring intestinal mucosal barrier function (Yu et al., 2021).

Additionally, polysaccharide mediated improvements in gut function such as influencing immune-related cytokines and hormone secretion. Study of *Lycium barbarum* polysaccharide also suggested that polysaccharide treatment could protect immune organs, enhance the production of immune-related cytokines (IL-2, IL-6, IL-1β, TNF-α, and IFN-γ) and prevent the hepatotoxicity in cyclophosphamide (CTX)-induced mice (Ding et al., 2019). In immunosuppressed mice, *Cordyceps sinensis* polysaccharide was found to modulate gut microbiota, alleviate gut injury, and regulate the balance of T helper (Th)1/Th2 cells (Chen S. et al., 2021). Khan I. et al. (2019) reported that the treatment of *Ganoderma lucidum* polysaccharides markedly promoted SCFA produced bacteria and abridged sulfate-reducing bacteria in a time-dependent manner, altered expressions of histone deacetylases, anti-cancer gut hormone PYY, and PPAPγ in ApcMin/+ mice. Collectively, both *in vitro* and animal experiments applied for polysaccharide from different sources has been proved to have gut modulation capacity as prebiotic, however, clinical application of well recognized polysaccharides could help further understanding the actual response of human gut microbiota. These studies collectively indicate that plant

polysaccharides share a complex relationship with gut microbes, and further study is required to reveal the full scope of mechanisms. In addition, gut regulation by postbiotic as secreted polysaccharides and extracellular polysaccharides has aroused attention recently. In several studies, the lactic acid bacteria-exopolysaccharides showed anti-oxidative and immunomodulatory activities as well as gut microbiota regulation effect (Xu R. et al., 2019; Xu Y. et al., 2019; Nataraj et al., 2020; Wang et al., 2020a). Further research into the biological activities of these metabolites is expected to reveal novel uses for postbiotics in medicine and beyond.

In general, the influence of polysaccharides on gut microbes can be summarized in three main aspects. Firstly, polysaccharides can attenuate damage to intestinal mucosa, alleviate intestinal inflammation, facilitate repair of damaged intestinal barrier, and ensure the integrity of the intestinal structure. Second, polysaccharides can alter intestinal microbial community composition and function, enriching beneficial bacteria while suppressing the proliferation of potential pathogens, consequently improving the relative content of various intestinal metabolites, especially SCFAs, that positively impact overall health. Third, polysaccharides can directly regulate gut function, such as modulating the secretion of interleukins and hormones in intestinal epithelial cells, or activating microbial CAZyme expression, which can reduce the likelihood of gut disease. However, most of these interventional studies are based on *in vitro* experiments or model animals, and thus clinical trials examining the effects of polysaccharide-based treatments in gut dysbiosis could improve our understanding of the interactions between polysaccharides, gut microbiota, and gastrointestinal function in humans.

4. Conclusion

Dietary polysaccharides have been shown to exert a range of biological activities, including anticoagulation, anti-inflammatory, immunomodulatory, hypoglycemic, antitumor, and antioxidant effects that impact gut health, in addition to modulating gut function and microbiota composition. These diverse functions are largely dependent on the primary structure and functional groups of the saccharide monomers. Ongoing innovations in data mining and compound screening continually expand the range of available bioactive polysaccharides, and drive the development of their pharmacological applications. It is noteworthy that these bioactive polysaccharides are not strictly limited to plants, and have been screened from fungi and algal sources, suggesting that there are likely a multitude of such metabolites with potential health benefits that are yet to be discovered in nature.

Author contributions

BL and ZL: conceptualization. YN and WL: writing – original draft preparation. BL, ZL, XF, DWX, DW, and HW: revising entire manuscript draft. BL, ZL, and XF: supervising, reviewing and editing final version of article. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

Björkstén, B., Sepp, E., Julge, K., Voor, T., and Mikelsaar, M. (2001). Allergy development and the intestinal microflora during the first year of life. *J. Allergy Clin. Immunol.* 108, 516–520. doi: 10.1067/mai.2001.118130

Bunyatyan, N. D., Bukhtiyarova, I. P., Drogovoz, S. M., Kononenko, A. V., Olefir, Y. V., Prokof'ev, A. B., et al. (2017). Influence of human biorhythms on the blood glucose level and the efficacy of hypoglycemic drugs (review). *Pharm. Chem. J.* 51, 399–401. doi: 10.1007/s11094-017-1621-4

Cao, Y.-Y., Ji, Y.-H., Liao, A.-M., Huang, J.-H., Thakur, K., Li, X.-L., et al. (2020). Effects of sulfated, phosphorylated and carboxymethylated modifications on the antioxidant activities *in-vitro* of polysaccharides sequentially extracted from *Amana edulis*. *Int. J. Biol. Macromol.* 146, 887–896. doi: 10.1016/j.ijbiomac.2019.09.211

Chaisuwan, W., Phimolsiripol, Y., Chaiyaso, T., Techapun, C., Leksawasdi, N., Jantanasakulwong, K., et al. (2021). The antiviral activity of bacterial, fungal, and algal polysaccharides as bioactive ingredients: potential uses for enhancing immune systems and preventing viruses. *Front. Nutr.* 8:772033. doi: 10.3389/fnut.2021.772033

Chang, C.-J., Lin, C.-S., Lu, C.-C., Martel, J., Ko, Y.-F., Ojcius, D. M., et al. (2015). *Ganoderma lucidum* reduces obesity in mice by modulating the composition of the gut microbiota. *Nat. Commun.* 6:7489. doi: 10.1038/ncomms8489

Chen, G., Chen, R., Chen, D., Ye, H., Hu, B., Zeng, X., et al. (2019). Tea polysaccharides as potential therapeutic options for metabolic diseases. *J. Agric. Food Chem.* 67, 5350–5360. doi: 10.1021/acs.jafc.8b05338

Chen, F., Huang, G., Yang, Z., and Hou, Y. (2019). Antioxidant activity of *Momordica charantia* polysaccharide and its derivatives. *Int. J. Biol. Macromol.* 138, 673–680. doi: 10.1016/j.ijbiomac.2019.07.129

Chen, J., Liu, J., Yan, C., Zhang, C., Pan, W., Zhang, W., et al. (2020). *Sarcodon aspratus* polysaccharides ameliorated obesity-induced metabolic disorders and modulated gut microbiota dysbiosis in mice fed a high-fat diet. *Food Funct.* 11, 2588–2602. doi: 10.1039/C9FO00963A

Chen, X., Qian, L., Wang, B., Zhang, Z., Liu, H., Zhang, Y., et al. (2019). Synergistic Hypoglycemic effects of pumpkin polysaccharides and Puerarin on type II diabetes mellitus mice. *Molecules* 24:955. doi: 10.3390/molecules24050955

Chen, S., Wang, J., Fang, Q., Dong, N., Fang, Q., Cui, S. W., et al. (2021). A polysaccharide from natural *cordyceps sinensis* regulates the intestinal immunity and gut microbiota in mice with cyclophosphamide-induced intestinal injury. *Food Funct.* 12, 6271–6282. doi: 10.1039/d1fo00596k

Chen, Y.-C., Wu, Y.-J., and Hu, C.-Y. (2019). Monosaccharide composition influence and immunomodulatory effects of probiotic exopolysaccharides. *Int. J. Biol. Macromol.* 133, 575–582. doi: 10.1016/j.ijbiomac.2019.04.109

Chen, C., Xie, X., and Li, X. (2021). Immunomodulatory effects of four polysaccharides purified from *Erythronium sibiricum* bulb on macrophages. *Glycoconj. J.* 38, 517–525. doi: 10.1007/s10719-021-10005-z

Chiu, Y.-H., Lin, S.-L., Tsai, J.-J., and Lin, M.-Y. (2014). Probiotic actions on diseases: implications for therapeutic treatments. *Food Funct.* 5, 625–634. doi: 10.1039/c3fo60600g

Coyte, K. Z., Schluter, J., and Foster, K. R. (2015). The ecology of the microbiome: networks, competition, and stability. *Science* 350, 663–666. doi: 10.1126/science.aad2602

Cresci, G. A., and Bawden, E. (2015). Gut microbiome: what we do and don't know. *Nutr. Clin. Pract.* 30, 734–746. doi: 10.1177/0884533615609899

Cui, M., Zhou, R., Wang, Y., Zhang, M., Liu, K., and Ma, C. (2020). Beneficial effects of sulfated polysaccharides from the red seaweed *Gelidium pacificum* Okamura on mice with antibiotic-associated diarrhea. *Food Funct.* 11, 4625–4637. doi: 10.1039/D0FO00598C

D'Ayala, G., Malinconico, M., and Laurienzo, P. (2008). Marine derived polysaccharides for biomedical applications: chemical modification approaches. *Molecules* 13, 2069–2106. doi: 10.3390/molecules13092069

de Vrese, M., and Schrezenmeir, J. (2008). Probiotics, prebiotics, and synbiotics. *Adv. Biochem. Eng. Biotechnol.* 111, 1–66. doi: 10.1007/10_2008_097

Dinan, T. G., and Cryan, J. F. (2017). Gut instincts: microbiota as a key regulator of brain development, ageing and neurodegeneration. *J. Physiol.* 595, 489–503. doi: 10.1113/JP273106

Ding, Y., Yan, Y., Chen, D., Ran, L., Mi, J., Lu, L., et al. (2019). Modulating effects of polysaccharides from the fruits of *Lycium barbarum* on the immune response and gut microbiota in cyclophosphamide-treated mice. *Food Funct.* 10, 3671–3683. doi: 10.1039/C9FO00638A

Fang, Q., Hu, J., Nie, Q., and Nie, S. (2019). Effects of polysaccharides on glycometabolism based on gut microbiota alteration. *Trends Food Sci. Technol.* 92, 65–70. doi: 10.1016/j.tifs.2019.08.015

Fang, Y. Z., Yang, S., and Wu, G. (2002). Free radicals, antioxidants, and nutrition. *Nutrition* 18, 872–879. doi: 10.1016/S0899-9007(02)00916-4

Fridovich, I. (1999). Fundamental aspects of reactive oxygen species, or what's the matter with oxygen? *Ann. N. Y. Acad. Sci.* 893, 13–18. doi: 10.1111/j.1749-6632.1999.tb07814.x

Fu, Y.-P., Li, C.-Y., Peng, X., Zou, Y.-F., Rise, F., Paulsen, B. S., et al. (2022). Polysaccharides from *Aconitum carmichaelii* leaves: structure, immunomodulatory and anti-inflammatory activities. *Carbohydr. Polym.* 291:119655. doi: 10.1016/j.carbpol.2022.119655

Fuke, N., Nagata, N., Suganuma, H., and Ota, T. (2019). Regulation of gut microbiota and metabolic endotoxemia with dietary factors. *Nutrients* 11:2277. doi: 10.3390/nu11102277

Gabriela das Chagas Faustino Alves, M., Almeida-Lima, J., Paiva, A. A. O., Leite, E. L., and Rocha, H. A. O. (2016). Extraction process optimization of sulfated galactan-rich fractions from *Hypnea musciformis* in order to obtain antioxidant, anticoagulant, or immunomodulatory polysaccharides. *J. Appl. Phycol.* 28, 1931–1942. doi: 10.1007/s10811-015-0705-3

Ganesan, K., and Xu, B. (2019). Anti-diabetic effects and mechanisms of dietary polysaccharides. *Molecules* 24:E2556. doi: 10.3390/molecules24142556

Gao, Z., Zhang, C., Tian, W., Liu, K., Hou, R., Yue, C., et al. (2017). The antioxidative and hepatoprotective effects comparison of Chinese angelica polysaccharide (CAP) and selenizing CAP (sCAP) in CCl4 induced hepatic injury mice. *Int. J. Biol. Macromol.* 97, 46–54. doi: 10.1016/j.ijbiomac.2017.01.013

Gibson, G. R., Hutkins, R., Sanders, M. E., Prescott, S. L., Reimer, R. A., Salminen, S. J., et al. (2017). Expert consensus document: the International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. *Nat. Rev. Gastroenterol. Hepatol.* 14, 491–502. doi: 10.1038/nrgastro.2017.75

Gibson, G. R., Probert, H. M., Loo, J. V., Rastall, R. A., and Roberfroid, M. B. (2004). Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. *Nutr. Res. Rev.* 17, 259–275. doi: 10.1079/NRR200479

Gong, W.-P., Han, R., Li, H.-S., Song, J.-M., Yan, H.-F., Li, G.-Y., et al. (2017). Agronomic traits and molecular marker identification of wheat- *Aegilops caudata* addition lines. *Front. Plant Sci.* 8:1743. doi: 10.3389/fpls.2017.01743

Gong, X., Li, X., Bo, A., Shi, R.-Y., Li, Q.-Y., Lei, L.-J., et al. (2020). The interactions between gut microbiota and bioactive ingredients of traditional Chinese medicines: a review. *Pharmacol. Res.* 157:104824. doi: 10.1016/j.phrs.2020.104824

Govindan, S., Shanmugam, J., Rajendran, G., Ramani, P., Unni, D., Venkatachalam, B., et al. (2023). Antidiabetic activity of polysaccharide from *Hypsizygus ulmarius* in streptozotocin-nicotinamide induced diabetic rats. *Bioact. Carbohydr. Diet. Fibre* 29:100350. doi: 10.1016/j.bcdf.2023.100350

Guo, C., Guo, D., Fang, L., Sang, T., Wu, J., Guo, C., et al. (2021a). *Ganoderma lucidum* polysaccharide modulates gut microbiota and immune cell function to inhibit inflammation and tumorigenesis in colon. *Carbohydr. Polym.* 267:118231. doi: 10.1016/j.carbpol.2021.118231

Guo, L., Ma, R., Sun, H., Raza, A., Tang, J., and Li, Z. (2018). Anti-inflammatory activities and related mechanism of polysaccharides isolated from *Sargentodoxa cuneata*. *Chem. Biodivers.* 15:e1800343. doi: 10.1002/cbdv.201800343

Guo, C., Wang, Y., Zhang, S., Zhang, X., Du, Z., Li, M., et al. (2021b). *Crataegus pinnatifida* polysaccharide alleviates colitis via modulation of gut microbiota and SCFAs metabolism. *Int. J. Biol. Macromol.* 181, 357–368. doi: 10.1016/j.ijbiomac.2021.03.137

Han, X., Bai, B., Zhou, Q., Niu, J., Yuan, J., Zhang, H., et al. (2020). Dietary supplementation with polysaccharides from *Ziziphus Jujuba* cv. Pozao intervenes in immune response via regulating peripheral immunity and intestinal barrier function in cyclophosphamide-induced mice. *Food Funct.* 11, 5992–6006. doi: 10.1039/D0FO00008F

Han, Y., Nan, S., Fan, J., Chen, Q., and Zhang, Y. (2019). *Inonotus obliquus* polysaccharides protect against Alzheimer's disease by regulating Nrf2 signaling and exerting antioxidative and antiapoptotic effects. *Int. J. Biol. Macromol.* 131, 769–778. doi: 10.1016/j.ijbiomac.2019.03.033

Hasan, A., Waibhaw, G., Tiwari, S., Dharmalingam, K., Shukla, I., and Pandey, L. M. (2017). Fabrication and characterization of chitosan, polyvinylpyrrolidone, and cellulose nanowhiskers nanocomposite films for wound healing drug delivery application. *J. Biomed. Mater. Res. A* 105, 2391–2404. doi: 10.1002/jbm.a.36097

Hatcher, E., Sawen, E., Widmalm, G., and MacKerell, A. D. J. (2011). Conformational properties of methyl β-maltoside and methyl α- and β-cellobioside disaccharides. *J. Phys. Chem. B* 115, 597–608. doi: 10.1021/jp109475p

Jayachandran, M., Wu, Z., Ganesan, K., Khalid, S., Chung, S. M., and Xu, B. (2019). Isoquercetin upregulates antioxidant genes, suppresses inflammatory cytokines and regulates AMPK pathway in streptozotocin-induced diabetic rats. *Chem. Biol. Interact.* 303, 62–69. doi: 10.1016/j.cbi.2019.02.017

Kalinina, T. S., Zlenko, D. V., Kiselev, A. V., Litvin, A. A., and Stovbun, S. V. (2020). Antiviral activity of the high-molecular-weight plant polysaccharides (Panavir®). *Int. J. Biol. Macromol.* 161, 936–938. doi: 10.1016/j.ijbiomac.2020.06.031

Kemp, M. M., Kumar, A., Mousa, S., Dyskin, E., Yalcin, M., Ajayan, P., et al. (2009). Gold and silver nanoparticles conjugated with heparin derivative possess anti-angiogenesis properties. *Nanotechnology* 20:455104. doi: 10.1088/0957-4484/20/45/455104

Khan, T., Date, A., Chawda, H., and Patel, K. (2019). Polysaccharides as potential anticancer agents-a review of their progress. *Carbohydr. Polym.* 210, 412–428. doi: 10.1016/j.carbpol.2019.01.064

Khan, I., Huang, G., Li, X.-A., Liao, W., Leong, W. K., Xia, W., et al. (2019). Mushroom polysaccharides and jiaogulan saponins exert cancer preventive effects by shaping the gut microbiota and microenvironment in ApcMin/+ mice. *Pharmacol. Res.* 148:104448. doi: 10.1016/j.phrs.2019.104448

Kiddane, A. T., and Kim, G.-D. (2021). Anticancer and immunomodulatory effects of polysaccharides. *Nutr. Cancer* 73, 2219–2231. doi: 10.1080/01635581.2020.1861310

Kim, S., and Jazwinski, S. M. (2018). The gut microbiota and healthy aging: a mini-review. *Gerontology* 64, 513–520. doi: 10.1159/000490615

Koropatkin, N. M., Cameron, E. A., and Martens, E. C. (2012). How glycan metabolism shapes the human gut microbiota. *Nat. Rev. Microbiol.* 10, 323–335. doi: 10.1038/nrmicro2746

Kouakou, K., Schepetkin, I. A., Yapi, A., Kirpotina, L. N., Jutila, M. A., and Quinn, M. T. (2013). Immunomodulatory activity of polysaccharides isolated from *Alchornea cordifolia*. *J. Ethnopharmacol.* 146, 232–242. doi: 10.1016/j.jep.2012.12.037

Lafond, M., Sulzenbacher, G., Freyd, T., Henrissat, B., Berrin, J.-G., and Garron, M.-L. (2016). The quaternary structure of a glycoside hydrolase dictates specificity toward β-glucans. *J. Biol. Chem.* 291, 7183–7194. doi: 10.1074/jbc.M115.695999

Li, W., Deng, Y., Chu, Q., and Zhang, P. (2019). Gut microbiome and cancer immunotherapy. *Cancer Lett.* 447, 41–47. doi: 10.1016/j.canlet.2019.01.015

Li, H., Fang, Q., Nie, Q., Hu, J., Yang, C., Huang, T., et al. (2020). Hypoglycemic and hypolipidemic mechanism of tea polysaccharides on type 2 diabetic rats via gut microbiota and metabolism alteration. *J. Agric. Food Chem.* 68, 10015–10028. doi: 10.1021/acs.jafc.0c01968

Li, J.-P., and Kusche-Gullberg, M. (2016). Heparan Sulfate: biosynthesis, structure, and function. *Int. Rev. Cell Mol. Biol.* 325, 215–273. doi: 10.1016/bs.ircmb.2016.02.009

Li, W., Wu, D., Wei, B., Wang, S., Sun, H., Li, X., et al. (2014). Anti-tumor effect of cactus polysaccharides on lung squamous carcinoma cells (SK-MES-1). *Afr. J. Tradit. Complement. Altern. Med.* 11, 99–104. doi: 10.4314/ajtcam.v11i5.16

Li, S., Xiong, Q., Lai, X., Li, X., Wan, M., Zhang, J., et al. (2016). Molecular modification of polysaccharides and resulting bioactivities. *Compr. Rev. Food Sci. Food Saf.* 15, 237–250. doi: 10.1111/1541-4337.12161

Liang, Q., Dong, J., Wang, S., Shao, W., Ahmed, A. F., Zhang, Y., et al. (2021). Immunomodulatory effects of *Nigella sativa* seed polysaccharides by gut microbial and proteomic technologies. *Int. J. Biol. Macromol.* 184, 483–496. doi: 10.1016/j.ijbiomac.2021.06.118

Liang, J., Zhang, M., Wang, X., Ren, Y., Yue, T., Wang, Z., et al. (2021). Edible fungal polysaccharides, the gut microbiota, and host health. *Carbohydr. Polym.* 273:118558. doi: 10.1016/j.carbpol.2021.118558

Liao, W., Luo, Z., Liu, D., Ning, Z., Yang, J., and Ren, J. (2015). Structure characterization of a novel polysaccharide from *Dictyophora indusiata* and its macrophage immunomodulatory activities. *J. Agric. Food Chem.* 63, 535–544. doi: 10.1021/jf504677r

Lin, G., Luo, D., Liu, J., Wu, X., Chen, J., Huang, Q., et al. (2018). Hepatoprotective effect of polysaccharides isolated from *Dendrobium officinale* against acetaminophen-induced liver injury in mice via regulation of the Nrf2-Keap1 Signaling pathway. *Oxidative Med. Cell. Longev.* 2018:6962439. doi: 10.1155/2018/6962439

Lin, Y., Pi, J., Jin, P., Liu, Y., Mai, X., Li, P., et al. (2022). Enzyme and microwave co-assisted extraction, structural characterization and antioxidant activity of polysaccharides from *purple-heart radish*. *Food Chem.* 372:131274. doi: 10.1016/j.foodchem.2021.131274

Liu, Y., Chen, D., You, Y., Zeng, S., Hu, Y., Duan, X., et al. (2016a). Structural characterization and antidiabetic activity of a glucopyranose-rich heteropolysaccharide from *Catathelasma ventricosum*. *Carbohydr. Polym.* 149, 399–407. doi: 10.1016/j.carbpol.2016.04.106

Liu, Z., Jiao, Y., Wang, Y., Zhou, C., and Zhang, Z. (2008). Polysaccharides-based nanoparticles as drug delivery systems. *Adv. Drug Deliv. Rev.* 60, 1650–1662. doi: 10.1016/j.addr.2008.09.001

Liu, Y., Li, X., Xie, C., Luo, X., Bao, Y., Wu, B., et al. (2016b). Prevention effects and possible molecular mechanism of mulberry leaf extract and its formulation on rats with insulin-insensitivity. *PLoS One* 11:e0152728. doi: 10.1371/journal.pone.0152728

Liu, K., Zhang, Y., Li, Q., Li, H., Long, D., Yan, S., et al. (2020). Ethnic differences shape the alpha but not Beta diversity of gut microbiota from school children in the absence of environmental differences. *Microorganisms* 8:254. doi: 10.3390/microorganisms8020254

Lu, J., He, R., Sun, P., Zhang, F., Linhardt, R. J., and Zhang, A. (2020). Molecular mechanisms of bioactive polysaccharides from *Ganoderma lucidum* (Lingzhi), a review. *Int. J. Biol. Macromol.* 150, 765–774. doi: 10.1016/j.ijbiomac.2020.02.035

Luo, Y., Peng, B., Wei, W., Tian, X., and Wu, Z. (2019). Antioxidant and anti-diabetic activities of polysaccharides from guava leaves. *Molecules* 24:1343. doi: 10.3390/molecules24071343

Ma, L., Xu, G. B., Tang, X., Zhang, C., Zhao, W., Wang, J., et al. (2020). Anti-cancer potential of polysaccharide extracted from hawthorn (*Crataegus*.) on human colon cancer cell line HCT116 via cell cycle arrest and apoptosis. *J. Funct. Foods* 64:103677. doi: 10.1016/j.jff.2019.103677

Maji, B. (2019). Introduction to natural polysaccharides. *Funct. Polysacch. Biomed. Applic.* 1–31. doi: 10.1016/B978-0-08-102555-0.00001-7

Mata, Y. N., Torres, E., Blázquez, M. L., Ballester, A., González, F., and Muñoz, J. A. (2009). Gold(III) biosorption and bioreduction with the brown alga *Fucus vesiculosus*. *J. Hazard. Mater.* 166, 612–618. doi: 10.1016/j.jhazmat.2008.11.064

Matsubara, K., Matsuura, Y., Bacic, A., Liao, M., Hori, K., and Miyazawa, K. (2001). Anticoagulant properties of a sulfated galactan preparation from a marine green alga, *Codium cylindricum*. *Int. J. Biol. Macromol.* 28, 395–399. doi: 10.1016/S0141-8130(01)00137-4

Min, Z., Xiaona, H., Aziz, T., Jian, Z., and Zhennai, Y. (2020). Exopolysaccharides from *Lactobacillus plantarum* YW11 improve immune response and ameliorate inflammatory bowel disease symptoms. *Acta Biochim. Pol.* 67, 485–493. doi: 10.18388/abp.2020_5371

Mirzadeh, M., Keshavarz Lelekami, A., and Khedmat, L. (2021). Plant/algal polysaccharides extracted by microwave: a review on hypoglycemic, hypolipidemic, prebiotic, and immune-stimulatory effect. *Carbohydr. Polym.* 266:118134. doi: 10.1016/j.carbpol.2021.118134

Mitsou, E. K., Saxami, G., Stamoulou, E., Kerezoudi, E., Terzi, E., Koutrotsios, G., et al. (2020). Effects of rich in B-glucans edible mushrooms on aging gut microbiota characteristics: an *in vitro* study. *Molecules* 25:E2806. doi: 10.3390/molecules25122806

Morrison, D. J., and Preston, T. (2016). Formation of short chain fatty acids by the gut microbiota and their impact on human metabolism. *Gut Microbes* 7, 189–200. doi: 10.1080/19490976.2015.1134082

Mousavi, S., Moradi, M., Khorshidahmad, T., and Motamedi, M. (2015). Anti-inflammatory effects of heparin and its derivatives: a systematic review. *Adv. Pharmacol. Sci.* 2015:507151, 1–14. doi: 10.1155/2015/507151

Nadkarni, V. D., Pervin, A., and Linhardt, R. J. (1994). Directional immobilization of heparin onto beaded supports. *Anal. Biochem.* 222, 59–67. doi: 10.1006/abio.1994.1454

Nataraj, B. H., Ali, S. A., Behare, P. V., and Yadav, H. (2020). Postbiotics-parabiotics: the new horizons in microbial biotherapy and functional foods. *Microb. Cell Factories* 19:168. doi: 10.1186/s12934-020-01426-w

Nguyen, S. G., Kim, J., Guevarra, R. B., Lee, J.-H., Kim, E., Kim, S.-I., et al. (2016). Laminarin favorably modulates gut microbiota in mice fed a high-fat diet. *Food Funct.* 7, 4193–4201. doi: 10.1039/C6FO00929H

Oh, W. K., Lee, C. H., Lee, M. S., Bae, E. Y., Sohn, C. B., Oh, H., et al. (2005). Antidiabetic effects of extracts from *Psidium guajava*. *J. Ethnopharmacol.* 96, 411–415. doi: 10.1016/j.jep.2004.09.041

Paik, W., Alonzo, F., and Knight, K. L. (2019). Probiotic exopolysaccharide protects against *systemic Staphylococcus aureus* infection, inducing dual-functioning macrophages that restrict bacterial growth and limit inflammation. *Infect. Immun.* 87, e00791–e00718. doi: 10.1128/IAI.00791-18

Park, Y., Hong, Y. N., Weyers, A., Kim, Y. S., and Linhardt, R. J. (2011). Polysaccharides and phytochemicals: a natural reservoir for the green synthesis of gold and silver nanoparticles. *IET Nanobiotechnol.* 5, 69–78. doi: 10.1049/iet-nbt.2010.0033

Patterson, E., Ryan, P. M., Cryan, J. F., Dinan, T. G., Ross, R. P., Fitzgerald, G. F., et al. (2016). Gut microbiota, obesity and diabetes. *Postgrad. Med. J.* 92, 286–300. doi: 10.1136/postgradmedj-2015-133285

Pawlaczyk, I., Czerchawski, L., Kuliczkowski, W., Karolko, B., Pilecki, W., Witkiewicz, W., et al. (2011). Anticoagulant and anti-platelet activity of polyphenolic-polysaccharide preparation isolated from the medicinal plant *Erigeron canadensis* L. *Thromb. Res.* 127, 328–340. doi: 10.1016/j.thromres.2010.11.031

Rajakovich, L. J., and Balskus, E. P. (2019). Metabolic functions of the human gut microbiota: the role of metalloenzymes. *Nat. Prod. Rep.* 36, 593–625. doi: 10.1039/C8NP00074C

Ru, Y., Liu, K., Kong, X., Li, X., Shi, X., and Chen, H. (2020). Synthesis of selenylated polysaccharides from *Momordica charantia* L. and its hypoglycemic activity in streptozotocin-induced diabetic mice. *Int. J. Biol. Macromol.* 152, 295–304. doi: 10.1016/j.ijbiomac.2020.02.288

Schepetkin, I. A., and Quinn, M. T. (2006). Botanical polysaccharides: macrophage immunomodulation and therapeutic potential. *Int. Immunopharmacol.* 6, 317–333. doi: 10.1016/j.intimp.2005.10.005

Shan, X., Zhou, J., Ma, T., and Chai, Q. (2011). *Lycium barbarum* polysaccharides reduce exercise-induced oxidative stress. *Int. J. Mol. Sci.* 12, 1081–1088. doi: 10.3390/ijms12021081

Shang, Q., Jiang, H., Cai, C., Hao, J., Li, G., and Yu, G. (2018). Gut microbiota fermentation of marine polysaccharides and its effects on intestinal ecology: an overview. *Carbohydr. Polym.* 179, 173–185. doi: 10.1016/j.carbpol.2017.09.059

Shen, X., Wang, Z., Song, X., Xu, J., Jiang, C., Zhao, Y., et al. (2014). Transcriptomic profiling revealed an important role of cell wall remodeling and ethylene signaling pathway during salt acclimation in *Arabidopsis*. *Plant Mol. Biol.* 86, 303–317. doi: 10.1007/s11103-014-0230-9

Sinha, V. R., and Kumria, R. (2001). Polysaccharides in colon-specific drug delivery. *Int. J. Pharm.* 224, 19–38. doi: 10.1016/S0378-5173(01)00720-7

Song, S. K., Beck, B. R., Kim, D., Park, J., Kim, J., Kim, H. D., et al. (2014). Prebiotics as immunostimulants in aquaculture: a review. *Fish Shellfish Immunol.* 40, 40–48. doi: 10.1016/j.fsi.2014.06.016

Song, J., Chen, M., Li, Z., Zhang, J., Hu, H., Tong, X., et al. (2019). Astragalus polysaccharide extends lifespan via mitigating endoplasmic reticulum stress in the silkworm, *Bombyx mori*. *Aging Dis.* 10, 1187–1198. doi: 10.14336/AD.2019.0515

Souza, R. O. S., Assreuy, A. M. S., Madeira, J. C., Chagas, F. D. S., Parreiras, L. A., Santos, G. R. C., et al. (2015). Purified polysaccharides of *Geoffroea spinosa* barks have anticoagulant and antithrombotic activities devoid of hemorrhagic risks. *Carbohydr. Polym.* 124, 208–215. doi: 10.1016/j.carbpol.2015.01.069

Sun, S.-S., Wang, K., Ma, K., Bao, L., and Liu, H.-W. (2019). An insoluble polysaccharide from the sclerotium of *Poria cocos* improves hyperglycemia, hyperlipidemia and hepatic steatosis in *Ob/Ob* mice via modulation of gut microbiota. *Chin. J. Nat. Med.* 17, 3–14. doi: 10.1016/S1875-5364(19)30003-2

Surayot, U., Wangtueai, S., You, S., Palanisamy, S., Krusong, W., Brennan, C. S., et al. (2021). Extraction, structural characterisation, and immunomodulatory properties of edible *Amanita hemibapha* subspecies *Javanica* (corner and bas) mucilage polysaccharide as a potential of functional food. *J Fungi (Basel)* 7:683. doi: 10.3390/jof7090683

Tan, X., Zhou, X., and Chen, H.-G. (2017). Structure-activity relationship of plant polysaccharides. *Zhongguo Zhong Yao Za Zhi* 42, 4104–4109. doi: 10.19540/j.cnki.cjcmm.20170928.016

Tomé, L. C., Fernandes, S. C. M., Perez, D. S., Sadocco, P., Silvestre, A. J. D., Neto, C. P., et al. (2013). The role of nanocellulose fibers, starch and chitosan on multipolysaccharide based films. *Cellulose* 20, 1807–1818. doi: 10.1007/s10570-013-9959-6

Wang, K., Niu, M., Song, D., Song, X., Zhao, J., Wu, Y., et al. (2020a). Preparation, partial characterization and biological activity of exopolysaccharides produced from *Lactobacillus fermentum* S1. *J. Biosci. Bioeng.* 129, 206–214. doi: 10.1016/j.jbiosc.2019.07.009

Wang, L.-Y., Wang, Y., Xu, D.-S., Ruan, K.-F., Feng, Y., and Wang, S. (2012). MDG-1, a polysaccharide from *Ophiopogon japonicus* exerts hypoglycemic effects through the PI3K/Akt pathway in a diabetic KKAy mouse model. *J. Ethnopharmacol.* 143, 347–354. doi: 10.1016/j.jep.2012.06.050

Wang, Y., Wang, X., Zhang, K., Zhang, X., Li, S., Li, Y., et al. (2020). Extraction kinetics, thermodynamics, rheological properties and anti-BVDV activity of the hot

water assisted extraction of Glycyrrhiza polysaccharide. *Food Funct.* 11, 4067–4080. doi: 10.1039/d0fo00608d

Wang, M., Xie, Z., Li, L., Chen, Y., Li, Y., Wang, Y., et al. (2019). Supplementation with compound polysaccharides contributes to the development and metabolic activity of young rat intestinal microbiota. *Food Funct.* 10, 2658–2675. doi: 10.1039/c8fo02565g

Wang, L., Xie, Y., Yang, W., Yang, Z., Jiang, S., Zhang, C., et al. (2019a). Alfalfa polysaccharide prevents H2O2-induced oxidative damage in MEFs by activating MAPK/Nrf2 signaling pathways and suppressing NF-κB signaling pathways. *Sci. Rep.* 9:1782. doi: 10.1038/s41598-018-38466-7

Wang, X., Wang, X., Jiang,, H., Cai, C., Li, G., Hao, J., et al. (2018). Marine polysaccharides attenuate metabolic syndrome by fermentation products and altering gut microbiota: An overview. *Carbohydr. Polym.* 195, 601–612. doi: 10.1016/j.carbpol.2018.05.003

Wang, G., Yang, X., Wang, J., Zhong, D., Zhang, R., Zhang, Y., et al. (2021). Walnut green husk polysaccharides prevent obesity, chronic inflammatory responses, nonalcoholic fatty liver disease and colonic tissue damage in high-fat diet fed rats. *Int. J. Biol. Macromol.* 182, 879–898. doi: 10.1016/j.ijbiomac.2021.04.047

Wang, K., Yang, X., Wu, Z., Wang, H., Li, Q., Mei, H., et al. (2020b). *Dendrobium officinale* polysaccharide protected CCl4-induced liver fibrosis through intestinal homeostasis and the LPS-TLR4-NF-κB signaling pathway. *Front. Pharmacol.* 11:240. doi: 10.3389/fphar.2020.00240

Wang, L., Zhang, X., Niu, Y., Ahmed, A. F., Wang, J., and Kang, W. (2019b). Anticoagulant activity of two novel polysaccharides from flowers of *Apocynum venetum* L. *Int. J. Biol. Macromol.* 124, 1230–1237. doi: 10.1016/j.ijbiomac.2018.12.015

Wei, H., Shi, Y., Yuan, Z., Huang, Z., Cai, F., Zhu, J., et al. (2021). Isolation, identification, and anti-inflammatory activity of polysaccharides of *Typha angustifolia*. *Biomacromolecules* 22, 2451–2459. doi: 10.1021/acs.biomac.1c00235

Witvrouw, M., Este, J. A., Mateu, M. Q., Reymen, D., Andrei, G., Snoeck, R., et al. (1994). Activity of a Sulfated polysaccharide extracted from the red seaweed *Aghardhiella Tenera* against human immunodeficiency virus and other enveloped viruses. *Antivir. Chem. Chemother.* 5, 297–303. doi: 10.1177/095632029400500503

Wu, J., Shi, S., Wang, H., and Wang, S. (2016). Mechanisms underlying the effect of polysaccharides in the treatment of type 2 diabetes: a review. *Carbohydr. Polym.* 144, 474–494. doi: 10.1016/j.carbpol.2016.02.040

Wu, Y., Wan, J., Choe, U., Pham, Q., Schoene, N. W., He, Q., et al. (2019). Interactions between food and gut microbiota: impact on human health. *Annu. Rev. Food Sci. Technol.* 10, 389–408. doi: 10.1146/annurev-food-032818-121303

Wu, J., Xu, Y., Liu, X., Chen, M., Zhu, B., Wang, H., et al. (2020). Isolation and structural characterization of a non-competitive α-glucosidase inhibitory polysaccharide from the seeds of *Litchi chinensis Sonn*. *Int. J. Biol. Macromol.* 154, 1105–1115. doi: 10.1016/j.ijbiomac.2019.11.170

Wu, S., Zhang, X., Liu, J., Song, J., Yu, P., Chen, P., et al. (2019). Physicochemical characterization of *Sargassum fusiforme* fucoidan fractions and their antagonistic effect against P-selectin-mediated cell adhesion. *Int. J. Biol. Macromol.* 133, 656–662. doi: 10.1016/j.ijbiomac.2019.03.218

Xie, J.-H., Jin, M.-L., Morris, G. A., Zha, X.-Q., Chen, H.-Q., Yi, Y., et al. (2016). Advances on bioactive polysaccharides from medicinal plants. *Crit. Rev. Food Sci. Nutr.* 56, S60–S84. doi: 10.1080/10408398.2015.1069255

Xu, R., Aruhan, , Xiu, L., Sheng, S., Liang, Y., Zhang, H., et al. (2019). Exopolysaccharides from *Lactobacillus buchneri* TCP016 attenuate LPS- and d-GalN-induced liver injury by modulating the gut microbiota. *J. Agric. Food Chem.* 67, 11627–11637. doi: 10.1021/acs.jafc.9b04323

Xu, X., Shan, B., Liao, C.-H., Xie, J.-H., Wen, P.-W., and Shi, J.-Y. (2015). Anti-diabetic properties of *Momordica charantia* L. polysaccharide in alloxan-induced diabetic mice. *Int. J. Biol. Macromol.* 81, 538–543. doi: 10.1016/j.ijbiomac.2015.08.049

Xu, Y., Wu, Y.-J., Sun, P.-L., Zhang, F.-M., Linhardt, R. J., and Zhang, A.-Q. (2019). Chemically modified polysaccharides: synthesis, characterization, structure activity relationships of action. *Int. J. Biol. Macromol.* 132, 970–977. doi: 10.1016/j.ijbiomac.2019.03.213

Xu, R., Ye, H., Sun, Y., Tu, Y., and Zeng, X. (2012). Preparation, preliminary characterization, antioxidant, hepatoprotective and antitumor activities of polysaccharides from the flower of tea plant (*Camellia sinensis*). *Food Chem. Toxicol.* 50, 2473–2480. doi: 10.1016/j.fct.2011.10.047

Xue, Y.-T., Li, S., Liu, W.-J., Xin, M., Li, H.-H., Yu, G.-L., et al. (2018). The mechanisms of sulfated polysaccharide drug of propylene glycol alginate sodium sulfate (PSS) on bleeding side effect. *Carbohydr. Polym.* 194, 365–374. doi: 10.1016/j.carbpol.2018.04.048

Yang, Y., Ji, J., di, L., Li, J., Hu, L., Qiao, H., et al. (2020). Resource, chemical structure and activity of natural polysaccharides against alcoholic liver damages. *Carbohydr. Polym.* 241:116355. doi: 10.1016/j.carbpol.2020.116355

Yao, Y., Yan, L., Chen, H., Wu, N., Wang, W., and Wang, D. (2020). *Cyclocarya paliurus* polysaccharides alleviate type 2 diabetic symptoms by modulating gut microbiota and short-chain fatty acids. *Phytomedicine* 77:153268. doi: 10.1016/j.phymed.2020.153268

Yoon, S.-J., Yu, M.-A., Pyun, Y.-R., Hwang, J.-K., Chu, D.-C., Juneja, L. R., et al. (2003). The nontoxic mushroom *Auricularia auricula* contains a polysaccharide with anticoagulant activity mediated by antithrombin. *Thromb. Res.* 112, 151–158. doi: 10.1016/j.thromres.2003.10.022

You, L., Gao, Q., Feng, M., Yang, B., Ren, J., Gu, L., et al. (2013). Structural characterisation of polysaccharides from *Tricholoma matsutake* and their antioxidant and antitumour activities. *Food Chem.* 138, 2242–2249. doi: 10.1016/j.foodchem.2012.11.140

Yu, Y., Zhu, H., Shen, M., Yu, Q., Chen, Y., and Xie, J. (2021). Sulfation modification enhances the intestinal regulation of *Cyclocarya paliurus* polysaccharides in cyclophosphamide-treated mice via restoring intestinal mucosal barrier function and modulating gut microbiota. *Food Funct.* 12, 12278–12290. doi: 10.1039/D1FO03042F

Zeng, P., Li, J., Chen, Y., and Zhang, L. (2019). The structures and biological functions of polysaccharides from traditional Chinese herbs. *Prog. Mol. Biol. Transl. Sci.* 163, 423–444. doi: 10.1016/bs.pmbts.2019.03.003

Zhang, L., Hao, J., Zheng, Y., Su, R., Liao, Y., Gong, X., et al. (2018). Fucoidan protects dopaminergic neurons by enhancing the mitochondrial function in a rotenone-induced rat model of Parkinson's disease. *Aging Dis.* 9, 590–604. doi: 10.14336/AD.2017.0831

Zhang, R., Yuan, S., Ye, J., Wang, X., Zhang, X., Shen, J., et al. (2020). Polysaccharide from *Flammuliana velutipes* improves colitis via regulation of colonic microbial dysbiosis and inflammatory responses. *Int. J. Biol. Macromol.* 149, 1252–1261. doi: 10.1016/j.ijbiomac.2020.02.044

Zhang, Z., Zhang, L., and Xu, H. (2019). Effect of Astragalus polysaccharide in treatment of diabetes mellitus: a narrative review. *J. Tradit. Chin. Med.* 39, 133–138. doi: 10.19852/j.cnki.jtcm.2019.01.017

Zhang, X., Zhao, S., Song, X., Jia, J., Zhang, Z., Zhou, H., et al. (2018). Inhibition effect of glycyrrhiza polysaccharide (GCP) on tumor growth through regulation of the gut microbiota composition. *J. Pharmacol. Sci.* 137, 324–332. doi: 10.1016/j.jphs.2018.03.006

Zhao, R., Ji, Y., Chen, X., Hu, Q., and Zhao, L. (2021). Polysaccharide from *Flammulina velutipes* attenuates markers of metabolic syndrome by modulating the gut microbiota and lipid metabolism in high fat diet-fed mice. *Food Funct.* 12, 6964–6980. doi: 10.1039/D1FO00534K

Zhao, R., Ji, Y., Chen, X., Su, A., Ma, G., Chen, G., et al. (2020). Effects of a β-type glycosidic polysaccharide from *Flammulina velutipes* on anti-inflammation and gut microbiota modulation in colitis mice. *Food Funct.* 11, 4259–4274. doi: 10.1039/C9FO03017D

Zhou, R., He, D., Xie, J., Zhou, Q., Zeng, H., Li, H., et al. (2021). The synergistic effects of polysaccharides and ginsenosides from American ginseng (*Panax quinquefolius* L.) ameliorating cyclophosphamide-induced intestinal immune disorders and gut barrier dysfunctions based on microbiome-metabolomics analysis. *Front. Immunol.* 12:665901. doi: 10.3389/fimmu.2021.665901

Zhou, N., Long, H., Wang, C., Yu, L., Zhao, M., and Liu, X. (2020). Research progress on the biological activities of selenium polysaccharides. *Food Funct.* 11, 4834–4852. doi: 10.1039/C9FO02026H

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Microbiomes in the context of developing sustainable intensified aquaculture

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With an ever-growing human population, the need for sustainable production of nutritional food sources has never been greater. Aquaculture is a key industry engaged in active development to increase production in line with this need while remaining sustainable in terms of environmental impact and promoting good welfare and health in farmed species. Microbiomes fundamentally underpin animal health, being a key part of their digestive, metabolic and defense systems, in the latter case protecting against opportunistic pathogens in the environment. The potential to manipulate the microbiome to the advantage of enhancing health, welfare and production is an intriguing prospect that has gained considerable traction in recent years. In this review we first set out what is known about the role of the microbiome in aquaculture production systems across the phylogenetic spectrum of cultured animals, from invertebrates to finfish. With a view to reducing environmental footprint and tightening biological and physical control, investment in "closed" aquaculture systems is on the rise, but little is known about how the microbial systems of these closed systems affect the health of cultured organisms. Through comparisons of the microbiomes and their dynamics across phylogenetically distinct animals and different aquaculture systems, we focus on microbial communities in terms of their functionality in order to identify what features within these microbiomes need to be harnessed for optimizing healthy intensified production in support of a sustainable future for aquaculture.

KEYWORDS

functionality, health, immune system, microbiota, sustainability

1. Introduction

As the global population continues to expand rapidly, so too does the need for sustainably produced food. The world's water bodies cover over 70% of the planet and are home to a bounty of protein-rich seafood (edible fish, invertebrates and algae from marine, brackish and freshwater environments). Global consumption of seafood (from capture and culture production in inland and marine waters) is growing at an annual rate of 3.1% (1961–2017), which is faster than for any livestock and animal production sector (FAO, 2020). Concurrent with stagnation of wild fisheries, aquaculture has progressively increased its contribution to fish and shellfish production with a mean annual growth of 5.3% between 2001 and 2018 (FAO, 2020), accounting for 52%

of all seafood produced for human consumption in 2018 (FAO, 2020). The continued expansion of aquaculture has however been hampered by outbreaks of infectious disease, concerns over environmental footprint, and impacts of climate change.

Health management in aquaculture has historically relied upon antibiotics and other chemotherapeutics (de Bruijn et al., 2018), but continued development of targeted vaccines (Ma et al., 2019) and selective breeding to generate disease resistant lines (Houston et al., 2020) are now coming to the forefront as environmentally sustainable alternatives. A more holistic approach to health management in aquaculture is to consider the cultured animal and its surrounding environment together. Emerging evidence indicates that microbial communities both within and surrounding an animal in aquaculture can contribute directly to productivity in terms of growth, disease resistance and animal welfare (Perry et al., 2020; Rajeev et al., 2021; Dai et al., 2022; Murphy et al., 2022). Microbial communities play dynamic functional roles with both commonalities and contrasts across cultured animal groups from invertebrates such as mollusks and crustaceans to vertebrates—predominantly finfish—and should be viewed as central components in the development of a sustainable aquaculture industry.

The associated microbial communities both within and surrounding farmed aquatic animals are commonly referred to as the microbiome (Figure 1). Bacterial communities are the focal point of the vast majority of microbiome studies and a paucity of information exists relating to the communities of other microbes including viruses, fungi, archaea and protozoa. The fungal community has been characterized in telelosts including zebrafish, the gut of which contained fungal taxa belonging to more than 15 classes across the phyla *Ascomycota*, *Basidiomycota*, and *Zygomycota*, and distinct

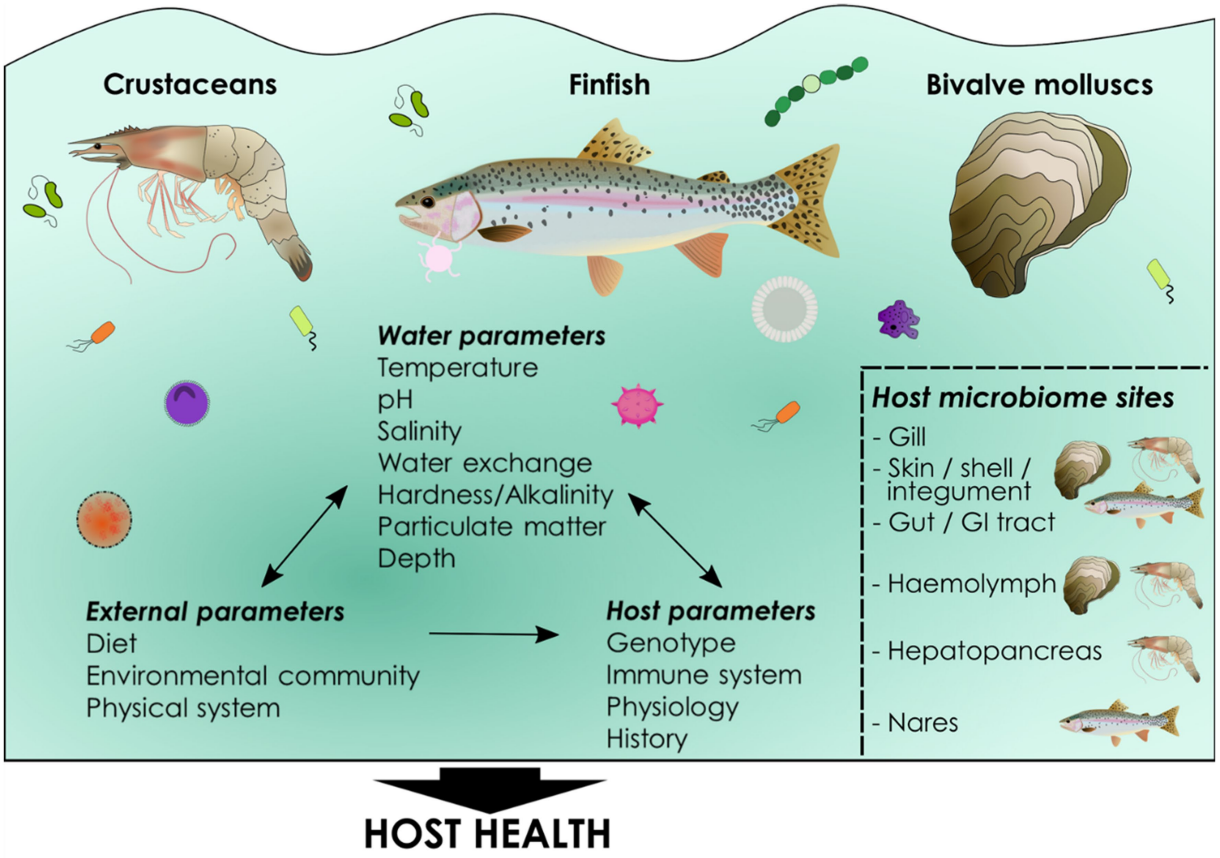

FIGURE 1
What is a microbiome? A microbiome can be defined simply as "a community of commensal, symbiotic, and pathogenic microorganisms within a body space or other environment" (Lederberg and McCray, 2001), although many varying definitions exist (Berg et al., 2020). A microbiome comprises a community of microbes including bacteria, viruses, fungi, microeukaryotic and metazoan parasites and Archaea (collectively known as the microbiota) as well as the downstream products and functionality of the microbiota. Cultured aquatic species harbor communities of microbes in the gut or gastrointestinal tract, as well as in the skin, nasal and oral cavities, gills, hemolymph, shells, integument and hepatopancreas, some of which are common across all cultured animal phyla (e.g., gills). The established microbiome composition is complex and involves not only host factors such as genotype and the host immune system, but also external environmental influences such as diet, physical parameters of culture systems, microbes in the aquatic environment and an array of water physiochemical parameters, including temperature, salinity, and dissolved oxygen. Together these parameters determine microbiome composition in host niches and these communities of commensals influence host health. Key determinants of host microbiome composition in cultured aquatic animals. Host microbiomes are determined by both environmental and host-associated factors. Environmental parameters can also reciprocally impact each other, as well as the host, particularly the microbial community within the water. Environmental parameters can be measured and, in some cases, controlled in aquaculture as can host factors such as genetics. Microbiomes occur in all cultured taxa including finfish, bivalve mollusks and crustaceans. Common microbial niches are found in a number of mucosal tissues including gut, gill and skin while others are specific to particular phyla, for example invertebrate hemolymph. Host microbiome composition itself has important implications for host health, but the functional and mechanistic pathways underlying these associations are poorly understood in aquaculture.

profiles in wild versus laboratory reared fish (Siriyappagouder et al., 2018). In the wood-eating Amazonian catfish (*Panaque nigrolineatus*), the mycobiome varied in different gut regions, and as a function of diet (Marden et al., 2017). Using a metatranscriptomic approach in the intestine of gilthead sea bream (*Sparus aurata*), fungal transcripts were found to be as abundant as bacterial, and while bacteria were important in vitamin and amino acid metabolism as well as rhythmic and symbiotic pathways, fungi were determined to play a role in host immunity, digestion and endocrine processes (Naya-Català et al., 2022). In green-lipped mussels (*Perna canaliculus*), the fungal community was determined in gills, hemolymph, digestive gland and stomach, and the majority of amplicon sequence variants (ASVs) belonged to the phylum *Ascomycota* in line with other studies (Li et al., 2022). However, more than 50% of ASVs for fungal taxa were only classified at the phylum level, highlighting the lack of fungal studies and thus lack of reference sequences, a hurdle which will need to be overcome to fully characterize complete microbial communities.

Across cultured phyla there are common tissues which host microbial communities including gut and gill, but microbiomes also occur in fish on their skin, and in their nasal and oral cavities, and in crustaceans and mollusks in their hemolymph (blood), digestive glands, shells (mollusks) and integument (crustaceans). Differences in microbiome composition in different tissues is driven by niche specialization and host selective pressures (Kelly and Salinas, 2017). A common feature across phylogenetically distinct animals in aquaculture is the primary role of internal mucosal microbiomes in digestion and nutrient absorption while external mucosal communities assume a primarily defensive role from the perspective of outcomes for the host. Yet microbiomes can also contain opportunistic pathogens, and to some extent are determined by the surrounding environment and not regulated by the host. In this perspective we explore how microbiomes contribute to productivity and health in aquaculture, with a particular focus on the commonalities and differences between phylogenetically diverse cultured animals and culture systems. We also present future prospects on how we may harness the power of the microbiome to promote sustainable aquaculture as production seeks to be intensified.

2. Establishing the host microbiome—where do commensal microbes come from in aquaculture?

Distinct from their terrestrial counterparts, aquatic organisms are in constant contact with (potentially) microbially rich water and this constant exposure may offer adaptive advantages, in addition to some inherent threats. In aquaculture systems, the aquatic environment is highly dynamic and can change both frequently and rapidly in response to many factors, including weather, temperature, and husbandry practices. The majority of aquaculture species, both invertebrate and vertebrate, reproduce by external fertilization, producing an independent egg exposed to the environment. External mucosal surfaces are first colonized by environmental microbes shortly after hatching (Giatsis et al., 2014; Stephens et al., 2016). As initial colonizers originate from the environment, hatchery conditions play a strong role in determining the diversity and richness of the initial colonizing community (Minich et al., 2021). In contrast, the internal gut mucosa is not colonized until the time of mouth opening and first feeding, exposing this internal niche to a potentially different pool of colonizers from the environment as well as diet-associated microbes. Critically, a sterile or partially sanitized environment restricts the pool of microbes from which to establish resident communities.

2.1. Selectivity in colonization—how to tell friend from foe?

Interactions between mucosal microbiota and the host immune system is particularly intense during initial colonization by commensals. Commonalities exist between invertebrates and vertebrates during the first phase of colonization in that acceptance or rejection of microbial taxa is controlled by a triad of the environmental conditions, the microbes themselves, and the host innate immune system. Germline-encoded cell receptors, where they exist, can play an early role in community selection and the innate immune system must have the ability for tolerance and allow settlement of commensals while selectively excluding opportunists or undesirable taxa (Kelly and Salinas, 2017). These cell receptors are likely to differ between mucosal surfaces, which raises interesting mechanistic questions. However, it is not solely the case that the immune system "selects" symbionts, but also that microbes from the environment may successfully colonize as they are best adapted to survive in the nutrient rich biochemical environment of the mucosal surface, independent of host influences.

In contrast with crustaceans and mollusks, the immune systems of which are devoid of lymphocytes and immunoglobulins (Igs), the acquired immune system or immunological memory capability of fish develops at first-feeding (Castro et al., 2015), while initial colonization of the gut occurs between mouth opening and first-feeding. As such, in addition to being exposed to a temporally distinct group of potential colonizers, colonization of the gut may be regulated by different immune pathways in invertebrates and vertebrates. In fish, as in other vertebrates, Igs are a key feature in acquired mucosal immunity, coating resident microbes to aid in selectivity (Woof and Mestecky, 2005; Kelly and Salinas, 2017). Dominant Ig isotypes have been identified at different mucosal sites, suggesting a role in determining site specific microbiota (Salinas et al., 2021). Although mollusks and crustaceans lack immunological memory in terms of Ig-mediated mechanisms, they harbor complex repertoires of lectins and other binding molecules which enable recognition of diverse microbes, resulting in control of undesirable species for successful innate immune protection (Vasta et al., 2007, 2012; Gerdol et al., 2018; Coates et al., 2022). Thus, successful colonizers are microbes in the environment which are suited to a mucosal niche and evade exclusion by the host immune system. This has been well studied in bobtail squid, *Euprymna scolopes* which acquire a specific strain of *Vibrio fischeri* from the surrounding environment into a specialized light organ, excluding all other bacterial taxa (Kostic et al., 2013). An additional facet of the immune system to consider when trying to understand microbial colonization are antimicrobial peptides (AMPs). AMPs refer to small peptides also known as bacteriocins including hepcidins, beta-defensins, cathelicidins and fish-specific piscidins (Masso-Silva and Diamond, 2014; Katzenback, 2015). AMPs are generally toxic to closely-related strains, although wider target ranges also occur (Rodney et al., 2014) and thus have the potential to impact colonization and succession by making an inhospitable environment

for certain taxa. Less well understood in aquaculture is the interaction between environmental microbes and host genetics in microbiome colonization.

2.2. How do host genetics impact microbial colonization?

Host genetics may play a role in the establishment of microbial associations through microbial recognition, immune selection, and determination of the biochemical niche (Spor et al., 2011). Fish of different species reared in the same or closely related environments can differ in their microbiome composition (Nikouli et al., 2021). Additionally, a genetic component may persist within species. In genetically divergent three-spined stickleback (*Gasterosteus aculeatus*) populations, divergence in the intestinal microbiota is greater than could be accounted for by environment and ecology, supporting a role for host genetics in the selection of bacterial species (Smith et al., 2015; Steury et al., 2019). Even within populations, an interaction between major histocompatibility complex class IIb (MHC class IIb) polymorphisms and genotype could impact microbiome diversity (Bolnick et al., 2014). However, other studies in Atlantic salmon (*Salmo salar*) identified a relatively minor role of host genetics (Dvergedal et al., 2020), particularly when compared to environmental effects (Uren Webster et al., 2018). Yet using hybrid populations of Chinook salmon, compositional differences in microbiota were attributed to quantitative genetic architecture (Ziab et al., 2023). It may be the case that, in common with mammalian systems, genotype-dependent selection may influence specific microbes, as opposed to the whole microbial community (Tabrett and Horton, 2020). Some animals in aquaculture, particularly salmonids, generally originate from brood-stock under genetic selection for production traits, notably related to growth, which may influence the contribution of environmental relative to genetic factors. In the case of shrimp farming, traits selected for are almost entirely focused on disease resistance. Importantly, genetic processes select microbes after the host has come into contact with bacterial communities in the environment, an important consideration in trait selection for different environments.

Little is known about the interactions between host genetics, microbiome stability, and health outcomes in relation to animals in aquaculture. Pacific oyster [*Crassostrea* (*Magallana*) *gigas*] families susceptible to Ostreid herpesvirus-1 have been shown to have differences in bacterial community structure and evenness compared to resistant families (King et al., 2019; Clerissi et al., 2020) and there are similar observations for the gill and gut of susceptible and resistant rainbow trout (*Oncorhynchus mykiss*) in response to *Flavobacterium psychrophilum* infection (Brown et al., 2019). In addition to genetic influences, epigenetic processes provide a link between host and microbiota. Metabolites resulting from cellular metabolic processes can impact the activity of enzymes involved in histone and DNA methylation and demethylation (Narne et al., 2017; Reid et al., 2017). The environmental microbiome is important in host immune system maturation, including the development of pathogen recognition systems. In the Pacific oyster, early life exposure to microbes improved survival during a challenge with Pacific oyster mortality syndrome (POMS) not just in the exposed generation, but in the following generation also. A combination of microbiota, transcriptomics, genetics and epigenetic analyses determined a distinct change in epigenetic methylation marks during microbial exposure leading to an altered immune gene expression and long-lasting, intergenerational immune protection against POMS (Fallet et al., 2022).

Overall, host genetics appear to play a central role in microbiome establishment, but little is known about the genomics *x* environment interaction for establishment and maintenance of microbial communities. The interactive nature of genetics and environment raises the potential for targeted environmental conditioning in aquaculture. To consider such approaches, it is important to first understand the role of physical aquaculture systems in dictating parameters which influence both host and environmental microbes.

3. Aquaculture system dynamics and host microbiome determination

Water has many variable properties including temperature, salinity, pH, ionic composition and chemistry related to dissolved gasses. Additionally, physical aquaculture systems dictate water exchange rate, retention time, and a level of biological sterility, particularly in hatcheries, a key stage for community establishment. Water harbors an array of microbes which provide ecosystem services within wild, and aquaculture systems and system parameters are important determinants of microbial dynamics. In low diversity microbial environments with input of organic material, fast-growing opportunistic *r*-strategists characteristic of instability can outcompete slow-growing *K*-strategists which are markers of stability. However, if water retention time in the system is short, opportunists are less able to replicate and stability of the system is more likely maintained by virtue of this management practice (Attramadal et al., 2012; Vadstein et al., 2018). A key end-goal of system optimization in aquaculture is to define environmental parameters which are conducive to desirable microbiomes in terms of sustainability and productivity, and to then have the ability to monitor and control these parameters to ensure optimum performance. However, due to the wide range of aquaculture system types (categorized as flow-through, open, or recirculating) and their associated temporal dynamics, a "one-size fits all" approach to determining optimum parameters is unlikely to succeed.

3.1. Aquaculture systems and evolving microbial stability

The nature of the aquaculture system can have major impacts on microbiomes of finfish (Minich et al., 2020, 2021; Uren Webster et al., 2020) and invertebrates, as illustrated for oyster (Arfken et al., 2021) and shrimp (Tepaamorndech et al., 2020). Such impacts are determined by physical and environmental parameters within systems and can influence production outcomes including growth and survival (Table 1). Culture systems come in an array of shapes and sizes and can be broadly described on a scale from open to closed (Figure 2). Larval stages are generally cultivated in highly controlled flow-through or recirculating aquaculture system (RAS) hatcheries with many species requiring planktonic organisms as feed before being transferred to on-growing facilities that vary between species. On-growing occurs in tanks, ponds and cages, or on other structures such longlines and rafts for bivalves (Tidwell, 2012). Most commonly

TABLE 1 Key published literature describing system parameter effects on animal microbiomes and production outcomes.

Species	Microbiomes (s)	System (s)	Factor	Description	References
Atlantic salmon (*Salmo salar*)	Gut and water	RAS	Membrane filtration	Salmon parr in RAS with membrane ultrafiltration (mRAS) compared against conventional RAS (cRAS) in periods of high and low organic loading. With high organic loading in cRAS, opportunistic bacteria colonized the gut microbiome. Ultrafiltration in mRAS stabilized the water microbiota, preventing growth of and colonization by opportunists.	Bugten et al. (2022)
Nile tilapia (*Oreochromis niloticus*)	Gut	RAS and FTS	Hatchery type	Tilapia embryos reared to fry in FTS, RAS or RAS with probiotic feed (RASB). Lower survival in fish reared in FTS compared to RAS and RASB. Gut microbiota composition differed in fish from different hatchery types and showed correlation with survival rate and size at harvest.	Deng et al. (2022)
Tilapia (*Coptodon rendalli* and *Oreochromis shiranus*)	Skin and water	Pond	Location	Tilapia reared in seven earthen ponds in two pond systems in distinct geographic regions. 92% of taxa shared by skin and water, but enriched and core taxa differed. Strong site-specific clustering of water samples, but not skin, highlighting some independence of skin microbiome from that of the environment.	McMurtrie et al. (2022)
Common carp (*Cyprinus carpio*)	Gut, sediment and water	Pond	Water quality	Carp reared in pond and sampled on 10 occasions across five months. The impact of water quality on water microbiota was stronger than the influence of gut or sediment microbiota. Gut microbiota dynamics were most closely associated with sediment microbiota.	Jing et al. (2021)
European lobster (*Homarus Gammarus*)	Larvae, biofilm and water	RAS and FTS	UV disinfection	Lobster larvae reared in RAS, RAS with UV disinfection or FTS in two separate experiments. Significantly different larval and water microbiomes were identified in each system. Survival was consistently highest in RAS without disinfection in replicate tanks and experiments.	Attramadal et al. (2021)
Eastern oyster (*Crassostrea virginica*)	Larvae and water	RAS and FTS	Hatchery type	Compared microbiomes of larvae originating from 4 different hatcheries for two consecutive spawning events. Larval microbiota were distinct from water and between hatcheries and spawning events. Hatchery had the strongest effect. Core OTUs (n = 25) identified across larval microbiomes.	Arfken et al. (2021)
Nile tilapia (*Oreochromis niloticus*)	Gut, biofloc, water and feed	RAS	Biofloc	Tilapia reared in RAS, RAS with *in situ* biofloc or fed a diet containing live or dead *ex situ* biofloc. *In situ* biofloc increased microbiome diversity in the gut with an increase in abundance of potentially beneficial taxa. Growth was also increased in fish from the *in situ* biofloc treatment.	Deng et al. (2021)
Pacific whiteleg shrimp (*Litopenaeus vannamei*)	Gut and biofloc	Static tank	Biofloc	Shrimp reared in indoor tanks with no water exchange, with or without biofloc. Microbiota composition similar with or without biofloc, but individual taxa enriched. Expression of immune-related genes and immune status enhanced in shrimp reared with biofloc.	Tepaamorndech et al. (2020)

RAS, recirculating aquaculture system; FTS, flow-through system.

these on-growing stages are exposed to the natural environment and generally employ the use of processed diets for finfish and crustaceans, or natural feed for filter feeding animals. Most open systems such as sea cages are exposed to natural fluctuations in the environment and may experience temperature extremes, exposure to harmful algal blooms, jellyfish (Clinton et al., 2021), and pollutants (Trabal Fernández et al., 2014). Such challenges have direct impacts on microbial communities and exposure to pathogens. The desire to have greater control over environmental parameters with a view to optimizing water quality to promote growth and minimize pathogen exposure, has led to an expansion of closed or semi-closed systems such as RAS. However, although it is more feasible in closed environments to maintain a high degree of stability, water chemistry can change over time in closed systems, depending on how they are maintained, and this can affect the biological load (Fossmark et al., 2020) and in turn the environmental microbiome. How the

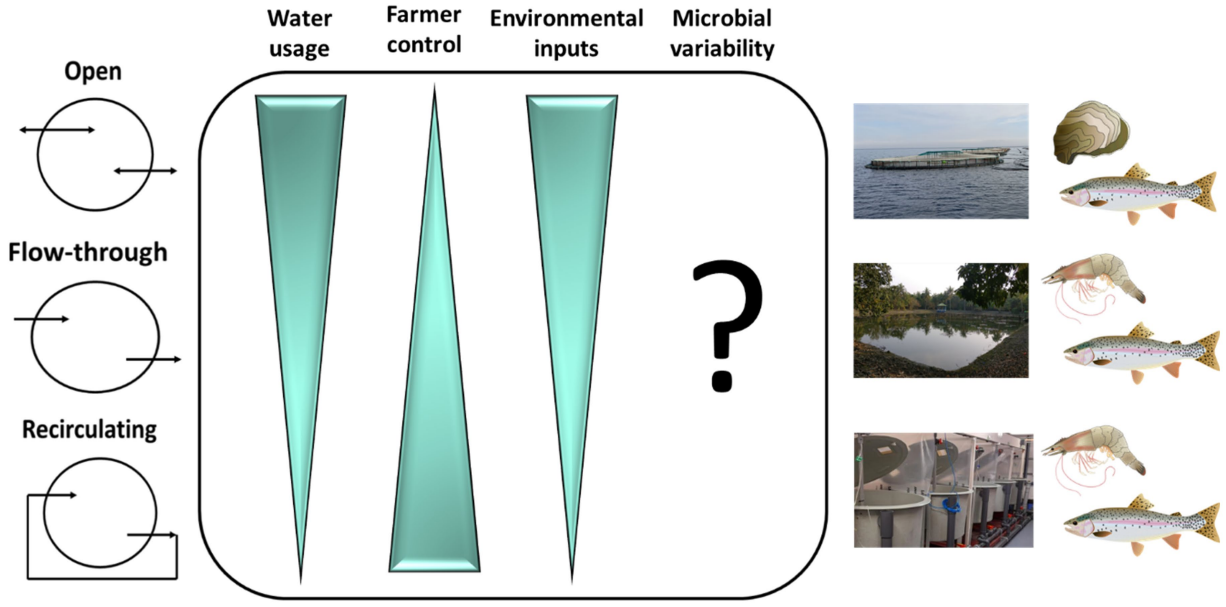

FIGURE 2
Microbiome dynamics in different aquaculture systems. Water usage, farmer control, environmental inputs and microbial variability in aquaculture systems. An important consideration in microbial maturation within aquaculture systems is water usage, particularly in terms of water flow and exchange. Systems vary from open (e.g., Atlantic salmon pens in lochs or fjords) to closed (e.g., land-based recirculating aquaculture systems; RAS) with systems between the two extremes referred to as flow-through (e.g., tilapia or shrimp ponds). Farmer control over environmental parameters including temperature and photoperiod are greater moving from open to recirculating systems and environmental factors such as natural seasonality and exposure to pollutants decrease. These dynamics may suggest a more stable microbial environment in closed systems, but in reality microbial variability is unlikely to follow a gradient from open to closed systems. Open systems are exposed to a rich pool of natural microbes which vary with season and geographic location. Contrastingly, strict husbandry practices to ensure pathogen exclusion in RAS, may result in periods of near sterility in the system, effectively re-setting the microbial community and introducing variability. In both cases, the result is potential instability in the system, but it is not known if stability is essential to a functioning microbiome.

physiochemistry of the different aquaculture systems may affect microbial stability in aquaculture is now a significant area of research interest. Although the physical systems in which invertebrates and vertebrates are reared in and on-grown differ, commonalities can be defined in terms of the nature of the systems on the scale from open to closed.

3.2. Open versus closed—what do aquaculture system structures mean for the microbiome?

In open aquaculture systems with constant water exchange—such as those used in shellfish cultivation and finfish cage culture—water quality, chemistry and microbial composition vary with geographical location, time, and season, with temperature having a strong impact on the microbiota (Sunagawa et al., 2015). For example, in response to increased water temperature, a surge in opportunistic pathogenic taxa in the Pacific oyster, *C. gigas* with dominance of *Vibrionaceae* occurs (Green et al., 2019). Similarly in marine Atlantic salmon, gut microbiomes are dominated by *Leuconostoc* and *Weissella* in cold water temperatures, but by *Vibrio*, *Allivibrio*, and *Photobacterium* in warmer water (Zarkasi et al., 2014). Circadian rhythmicity is naturally determined by latitude and influences microbiome composition in rainbow trout skin (Ellison et al., 2021), but photoperiod is also often manipulated in aquaculture to promote growth and enable year-round production (Mero et al., 2019; Wang et al., 2020; Pino Martinez et al., 2021), and is likely to impact on immune function with consequences in mucosal immune activity (West et al., 2021).

In RAS, microbial processes are vital in maintaining water quality (Attramadal et al., 2012; Blancheton et al., 2013). Biofilters in the RAS loop host essential communities of bacteria which regulate conversion of waste nutrients to prevent build-up of toxic metabolites such as ammonia and hydrogen sulfide resulting from uneaten feed and fecal matter. Build-up of organic matter or introduction of pathogens result in mass mortalities, and therefore various forms and combinations of filtration and disinfection are often carried out in a RAS loop (Summerfelt et al., 2009). Filtration in the RAS loop assists in stabilizing carrying capacity (Fossmark et al., 2020), while disinfection is an effective means of removing pathogens, but may effectively "re-set" the community. Such dynamics raise questions about how, where, and when RAS water should be subject to disinfection. Simply put, how clean is too clean? At the other end of the sterility scale, the use of biofloc technology, a microbial feed source added to culture water to maintain microbial communities in culture water, has associated benefits for immune reactivity in shrimp (Tepaamorndech et al., 2020), and faster growth in tilapia (Nguyen et al., 2021). Importantly, despite the relative stability of RAS in comparison to open systems, microbiomes in Atlantic salmon gut in RAS are still temporally dynamic, possibly related to biological load in the system (Lorgen-Ritchie et al., 2021).

Intermediate between open and closed states, pond systems are the dominant forms of global aquaculture, particularly prominent in

low-middle income countries of Asia, responsible for farming carp, tilapia and shrimp (FAO, 2020). Similar to open systems, ponds are exposed to fluctuating external environmental parameters, but the physiochemical and biotic condition of ponds can shift rapidly due to their shallow depth and limited water exchange, especially when compared to the large water body buffer of open cage culture. During the rainy season, rapid salinity fluxes occur due to rainwater diluting ion concentrations. In intensive culture, algal blooms can form due to excessive food and fertilizer (e.g., manure) inputs providing the key phytoplankton nutrients phosphorous and nitrogen (Huang et al., 2016). In summer months overgrowth of photoautotrophic bacteria can lead to highly turbid pond water, limiting oxygen production (Sriyasak et al., 2015; Dien et al., 2019). In the oriental river prawn (*Macrobrachium nipponense*) hypoxia induced changes in the intestinal microbiome composition with a deviation toward higher levels of pathogenic bacteria (*Aeromonas*) (Sun et al., 2020). Mitigation strategies for anoxic conditions include pond aeration (Boyd, 1998), and multi-trophic farming with mollusks that filter feed on phytoplankton resulting in a net increase of dissolved oxygen (Cunha et al., 2019). As aquaculture continues to expand, it would be beneficial to employ an ecosystem-based approach when designing and optimizing aquaculture systems, attempting to integrate interactions between physical environment, host, and microbiome to promote establishment of desirable microbiomes for productivity and host health.

4. Which microbiomes promote animal health in aquaculture?

Productivity and welfare in all areas of the aquaculture industry are under constant threat from opportunistic pathogens, infectious disease and syndromes which result in poor health. A growing body of evidence challenges the idea of a single pathogen causing a single disease; the concept of a "pathobiome" implies that a triad of interactions between environmental microbes, host-associated commensals and the host itself steer overall host health (Bass et al., 2019). For example, in skin mucus of brook charr (*Salvelinus fontinalis*), host stress was associated with a decreased abundance of beneficial commensals including *Methylobacterium* and *Propionibacterium*, known to produce pathogen growth inhibitors poly-β-hydroxybutyrate and bacteriocin, coincident with a rise in abundance of opportunistic pathogenic genera including *Aeromonas*, *Psychrobacter* and *Acientobacter* (Boutin et al., 2013). Additionally, cryptic infection is possible whereby pathogens with dormant, or non-pathogenic life stages may infect a host asymptomatically, but be activated as a pathogen in response to a change in the environment, for example, seasonal changes in temperature (Overstreet and Lotz, 2016; Bass et al., 2019). Mucosal surfaces and their associated microbiota are the first line of defense against pathogens and are equipped with a range of protective molecules such as antimicrobial peptides and immune-related cell components (Reverter et al., 2018) which may induce competitive advantage directly or indirectly. It is becoming increasingly clear that disease impacts host microbiomes, but also that host micorbiomes can mediate disease severity or progression (Table 2). For example, in Atlantic salmon infected with amoebic gill disease (AGD), a significant reduction in bacterial diversity was apparent when compared with healthy individuals and a particular taxon (*Tenacibaculum dicentrarchi*) was particularly prevalent in lesions of infected gill tissue (Slinger et al., 2020). In red abalone suffering from Withering syndrome (WS), *Mycoplasma* was replaced as the dominant taxon in the digestive gland by *Candidatus Xenohaliotis californiensis*, but *C. X. californiensis* was also a member of the microbiome of healthy individuals, suggesting that in this case, the ratio between the two taxa may be key in determining pathogenicity of the disease (Villasante et al., 2020). The array of life history strategies, species and systems across which aquatic animals are farmed represent a challenge in identifying desirable microbiome signatures at the taxonomic level, yet at a functional level, commonalities can be identified. In this line of thinking, it is the ability of the microbiome to maintain functionality in the face of environmental change that promotes host health, rather than consistency of the taxonomic composition of microbial communities.

4.1. Can a "healthy" microbiome be defined for aquaculture practices?

Attempts have been made in the past to define a "healthy" microbiome in terms of aquaculture but realistically a single consistent "healthy" microbiome is unlikely to exist, even within a single species in a single culture system (Infante-Villamil et al., 2021; Rajeev et al., 2021). Thus, perhaps a desirable microbiome for aquaculture should instead be defined as one that has the capacity to adjust to its environment, and in doing so maintain a beneficial symbiotic relationship with the host. Maintenance of community homeostasis historically has been considered important, but this should be expanded to considering community homeostasis in terms of functionality rather than taxonomic identity. Microbial communities can be remarkably dynamic in response to changes in their environment and a highly plastic, dynamic microbiome may also contribute to host phenotypic plasticity, and thus host ability to tolerate environmental stressors. The "Anna Karenina principle" (Zaneveld et al., 2017) infers that stressors create more stochastic microbiomes as a consequence of a dampening of the host's ability to regulate its microbial communities, resulting in increased inter-individual variation in microbiomes of hosts experiencing stress (Boutin et al., 2013). However, the contrasting pattern has also been observed, with remarkably similar microbiome responses to environmental disturbance which have been attributed to reduced competition allowing increased dominance of certain taxa (Webster et al., 2019). Such differences may well reflect differences in external environment. In open systems with more variation in environmental microbes, we might expect greater stochasticity, but in closed systems with less environmental microbial variation, we might expect to see the domination of certain taxa.

Although maintaining low levels of stress in aquaculture is a central tenet of stock management, even routine husbandry practices can induce stress, which may increase disease susceptibility (Eissa and Wang, 2016). A microbiome with a high adaptive plasticity is less likely to be disturbed by environmental stressors and therefore there are less likely to be knock-on adverse effects of microbiome disturbance on host health. A plastic microbiome may also confer specific fitness benefits to the host (e.g., an ability to metabolize or sequester dietary toxins, adapt to novel nutrient or feed sources, or to cope with changing water temperatures) (Alberdi et al., 2016;

TABLE 2 Key published literature exploring the interaction between disease and host microbiome composition.

Species	Tissue (s)	Disease	Description	References
Crayfish (*Procambarus clakii*)	Gut and hepatopancreas	White spot syndrome virus (WSSV)	Crayfish infected with WSSV displayed decreased intestinal microbiota diversity and richness and relative abundance of an opportunistic pathogen (*Aeromonas*) increased. The potential pathogenicity in the gut microbiota of WSSV-infected crayfish was increased compared to healthy controls.	Xue et al. (2022)
Black tiger shrimp (*Penaeus monodon*)	Hepatopancreas	Vibriosis	Infected and non-infected shrimp obtained from six hatcheries. Alpha diversity was reduced in the hepatopancreas of infected shrimp. Eight bacterial genera were associated with a shift in the microbiome in infected shrimp.	Foysal et al. (2021)
Pacific oyster (*Crassostrea gigas*)	Whole oyster	Pacific oyster mortality syndrome (POMS)	Oysters from resistant or susceptible families infected with POMS. All individuals from susceptible families died, but during early infection, microbiota of the whole oyster showed a reduction in evenness compared to resistant families.	Clerissi et al. (2020)
Atlantic salmon (*Salmo salar*)	Gill	Amoebic gill disease (AGD)	The microbiota of AGD-affected and non-affected gill tissue biopsies was compared. Bacterial diversity was significantly reduced in biopsies from both AGD-affected and un-affected gill tissue from infected fish compared to uninfected fish. Lesions in AGD-affected tissue contained higher abundance of *Tenacibaculum dicentrarchi*.	Slinger et al. (2020)
Atlantic salmon (*Salmo salar*)	Mouth	Tenacibaculosis (Yellow Mouth, YM)	Comparing YM infected fish with uninfected control, a reduction in microbiota diversity and distinct dysbiosis were identified. High levels of the primary causative agent *Tenacibaculum maritimum* were seen in infected and healthy fish indicating additional unknown factors responsible for pathology of YM. An association between *T. maritimum* and *Vibrio* abudance was identified.	Wynne et al. (2020)
Red abalone (*Haliotis rufescens*)	Digestive gland	Withering syndrome (WS)	In red abalone affected by WS, *Candidatus Xenohaliotis californiensis* replaced *Mycoplasma* as the dominant taxon in the digestive gland microbiota. *C. X. californiensis* was also sequenced in healthy specimens, suggesting that the ratio between the two taxa may be more important in determining pathogenicity of WS.	Villasante et al. (2020)
Yellowtail Kingfish (*Seriola lalandi*)	Gut and skin	Gut enteritis	Microbiota and gene expression in gut and skin analyzed in fish affected by gut inflammation. The gut microbiota of affected fish was dominated by a *Mycoplasmataceae* sp., while a reduction in microbial diversity in the skin was identified at the early stages of disease. Gene expression analysis revealed little differentiation in the gut between healthy and affected fish while extensive differences were identified in the skin, related to pathways indicative of a weakened host.	Legrand et al. (2020a)
Pacific whiteleg shrimp (*Litopenaeus vannamei*)	Gut	White spot syndrome virus (WSSV)	In shrimp infected with WSSV, there was no significant impact on bacterial richness or diversity. However, community composition was different between infected and non-infected individuals with increased *Proteobacteria* and *Fusobacteria*, but reduced *Bacteroidetes* and *Tenericutes* in infected shrimp.	Wang et al. (2019)

Macke et al., 2017). It is important to consider that the nature and outcomes of stress responses will depend on the nature of the existing microbial community, with different mucosal sites displaying differing responses to stress in terms of both cortisol levels and microbiome dynamics (Webster et al., 2019). Despite variable responses, identification of markers of stress or dysbiosis across datasets could enable these to be leveraged in real-time microbiome monitoring in aquaculture through non-invasive sampling of water, feces, or external mucus to detect microbial signatures of potential ill-health. Going a step further, the future ideal would be to actively intervene and manipulate the microbiome during these periods of dysbiosis to help re-balance disruptions and maintain optimal host health.

4.2. Dysbiosis and rebiosis—an opportunity to condition microbiomes for sustainable aquaculture?

As explained above, the first microbial colonizers are key in determining succession via competitive exclusion or "colonization resistance," competing for nutrients and physical space (Vonaesch

et al., 2018). Post-colonization, development of the fish microbiome is characterized by periods of establishment and proliferation which are especially dynamic and strongly influenced by environment, and periods of stabilization which are strongly influenced by the host. Husbandry practices can introduce instability to the environment and these transitional periods are often accompanied by high levels of mortality or increased disease susceptibility (Paul-Pont et al., 2013; Johansson et al., 2016). As an example, on-growing of Atlantic salmon requires transition from a freshwater to a saltwater environment, exposing commensal microbes to a distinct shift in salinity and ultimately creating a new niche for salt-tolerant taxa to the detriment of freshwater commensals. Such distinct shifts in environmental parameters are accompanied by rapid remodeling of host microbiomes (Dehler et al., 2017; Lorgen-Ritchie et al., 2021) characterized by an initial loss of community structure, a process known as dysbiosis, followed by re-establishment of a new community—rebiosis. Periods of rapid change in the microbiome are especially sensitive to external influences of incoming microbes that may later be more inhibited by colonization resistance, and this may have a lasting effect on microbiome composition and function via priority effects (Debray et al., 2021). This could have important implications for aquaculture as these periods may be especially sensitive to disruption but may also represent an opportunity for increased sensitivity to conditioning.

Dysbiosis in the naturally occurring microbiome of the host often coincides with infection or poor health, from invertebrates to humans. Dysbiotic states may manifest as (i) loss of beneficial organisms, (ii) expansion of pathobionts and/or (iii) loss of microbial diversity (Petersen and Round, 2014). It is important to consider community interactions when trying to understand these processes. Ecological theory suggests diverse communities are more resistant to dysbiosis and invasion by opportunistic pathogens (Levine and D'Antonio, 1999) as they have more diverse functionality and more effective competitive exclusion. Salmon experimentally infected with the copepod "louse" ectoparasite, *Lepeophtherius salmonis,* have been shown to display a reduced bacterial richness in their skin microbiome (Llewellyn et al., 2017). A similar pattern has been observed in the gut of shrimp suffering white feces syndrome (Hou et al., 2018). However, in shrimp suffering from white spot syndrome virus (WSSV), no significant difference in diversity was observed although community composition was distinct in control vs. diseased animals (Wang et al., 2019). Moving forward, defining dysbiosis in terms of loss of functional integrity and knock-on effects on host health may be more useful when it comes to potential development of therapeutics.

It remains unclear whether dysbiosis occurs in response to infection or if opportunists exploit an existing or incipient dysbiotic state, perhaps induced by change in environmental conditions or host physiology or immune capabilities, in order to infect. A combination of both is probably most likely—infection may well exacerbate an already dysbiotic state. To illustrate this scenario, production of the Pacific oyster (*C. gigas*) is hampered by disease, especially Pacific oyster mortality syndrome (POMS) that has caused major economic losses (Segarra et al., 2010). POMS is a polymicrobial disease where oysters become infected by osterid herpes virus 1 µvar (OSHV-1 µvar) and this leads to immune suppression facilitating dysbiotic shifts in the oyster's microbiome. This altered microbiome, the pathobiome, is characterized by dominance in taxa including *Vibrio*, *Aliivibrio*, *Arcobacter*, *Marinobacterium*, *Marinomonas*, *Photobacterium*, *Psychrobium*, and *Pschromonas* (de Lorgeril et al., 2018; Clerissi et al., 2020; Petton et al., 2021; Richard et al., 2021). Affected oysters show bacteremia where opportunistic vibrios including *V. aestuarianus* cause significant mortality. Oyster families displaying greater microbial diversity within individuals tend to be less likely to suffer from POMS (Clerissi et al., 2020) and selective breeding programs may improve disease resistance and hence reduce the impact of this condition (Dégremont et al., 2020). Similarly in Atlantic salmon, amoebic gill disease (AGD) is a increasing gill health issue, the etiological agent of which is the free-living amoeba *Neoparamoeba perurans* (Crosbie et al., 2012). AGD infection is initiated by adherence of *N. perurans* to the gill mucosa where commensal bacteria persist (Embar-Gopinath et al., 2005). Microbial profiling revealed lower bacterial diversity and a moderate positive correlation between *N. perurans* and *Tenacibaculum dicentarchi* in infected tissues (Slinger et al., 2020). Altering bacterial load on the gills prior to *N. perurans* infection using antimicrobial treatments resulted in differing progression of AGD with significantly higher severity observed in chloramine-trihydrate (CI-T) treated fish vs. control fish. High levels of *Tenacibaculum* were observed in the CI-T group again suggesting a protagonistic role for this taxon in AGD infection highlighting the potential for dysbiosis to allow expansion of potentially pathogenic communities which may affect subsequent disease progression (Slinger et al., 2021). Future research is needed to identify useful commonalities in these intertwined relationships between dysbiosis, disease progression, and host health.

5. Moving from microbiome characterization to beneficial manipulation in aquaculture

Identifying commensal microbes and their dynamics is the first step in understanding the value of microbiomes for aquaculture productivity and sustainability. If "*the microbiome*" (accepting there are many), is to be used as a tool to improve health and performance of the animals, we need to know not just which microbes are present, but how these microbial communities function (Gibbons, 2017). In recent years there has been a distinct shift in microbiome research to "function over phylogeny." Functionality has historically been inferred from high-throughput amplicon sequencing, but predictions are limited in their use in aquaculture studies due to poor taxonomic resolution and low numbers of characterized bacterial genomes across aquaculture environments (Infante-Villamil et al., 2021). Additionally, such predictions relate to a specific set of conditions, and functional analysis tools assume that all genes are transcriptionally active in all microbial taxa at that moment in time, which more often than not is simply not the case. More holistic 'omics strategies such as metagenomics (Tepaamorndech et al., 2020), metatranscriptomics (Nam et al., 2018), metaproteomics (Jose et al., 2020), and metabolomics (Roques et al., 2020), as well as integration of these approaches (multi-omics) (Uengwetwanit et al., 2020) are increasingly being adopted. A step further is to consider the "hologenome," that is the collection of genes in the microbiomes along with those of the host (Rosenberg et al., 2007; Rosenberg and Zilber-Rosenberg, 2018). The strength of these approaches is the ability to examine microbiomes in terms of community ecology, considering interactions among microbiota, with the host and with the environment, but integration requires well designed and executed sampling strategies, sufficient levels of sampling (generally producing very large datasets), and

complex bioinformatics analyses. Omics approaches are key to uncovering contrasts and commonalities between microbiome function across aquaculture but must be applied in combination with carefully designed mechanistic studies.

5.1. Modeling a desirable microbiome function for aquaculture

One approach to understanding functionality is to use germ-free (GF) or gnotobiotic models. Gnotobiotic refers to animals hatched and reared in a sterile environment which provide a "blank canvas" on which to test the impact of introducing a single microbe or a community of microbes. Although gnotobiotic research has been applied to aquaculture species since the 1940s (Zhang et al., 2020), full use of this approach as a tool to understand microbiome functionality is only now being realized. Nile tilapia (*Oreochromis niloticus*) models have been used to test the efficacy of therapeutics and to study colonization by free-living microbes (Situmorang et al., 2014; Giatsis et al., 2016) while in gnotobiotic rainbow trout larvae commensal taxa which offered protection against pathogenic infection have been identified (Pérez-Pascual et al., 2021). Furthermore, cod (*Gadus morhua*) models have recently been used to study the role of microbiota in innate immune development and gut morphology (Vestrum et al., 2022). Gnotobiotic oyster (*Crassostrea gigas*) models have also been utilized to detect effects of pathogens (Arzul et al., 2001) while in crustacea, germ-free *Artemia* brine-shrimp (Baruah et al., 2017) and *Daphnia* (Macke et al., 2020) models are widely used to study host-microbiome-environment interactions.

Pioneering work in zebrafish (reviewed in Murdoch and Rawls, 2019) established a germ-free model, but also a conventionalized model by harvesting bacteria from tank water of conventionally reared zebrafish and adding this community to gnotobiotic zebrafish medium to colonize germ-free animals (Rawls et al., 2004). Using these models and transcriptomic analysis, 212 genes regulated by the microbiota were identified in the intestine, and 59 responses were conserved in the mouse intestine indicative of an evolutionary conserved role of the vertebrate gut microbiome. Similarly, exposure of germ free zebrafish to yeast in the form of *Pseudozyma* sp. elucidated differential expression of 59 genes compared to naive controls, many involved in metabolism and immune response, indicating that commensal fungi also have the potential to influence the early development of fish larvae (Siriyappagouder et al., 2020). This work was extended using reciprocal gut microbiome transplants from zebrafish and mice to germ-free recipients and identified the importance of host habitat selection (Rawls et al., 2006). Transplanted communities resembled the community of origin in terms of taxonomic lineages present, but relative abundances were modified to resemble the normal microbial community composition of the recipient host. These transplantation studies are important in understanding microbiome function and selection pressures during colonization, but also have the potential to be adapted for therapeutic means similar to fecal microbiota transplantation (FMT) (Petersen and Round, 2014; Vargas-Albores et al., 2021). Such approaches may well be used in aquaculture as understanding of functionality of microbiomes improves.

Limitations exist in terms of long-term germ-free husbandry and untangling complexities of microbe-microbe interactions. Genetic manipulations including knock-out, knock-in and clonal approaches are additional avenues by which to uncover mechanistic microbiome pathways and potential genetic influences on microbiome composition. IgT, a teleost mucosal immunoglobulin believed to be an important adaptive immune determinant of microbial homeostasis, was depleted in a rainbow trout model. This depletion resulted in dysbiosis characterized by an increased presence of pathobionts, tissue damage and inflammation as well as increased susceptibility to mucosal parasites, highlighting the importance of this immunoglobulin in microbiome homeostasis in finfish (Xu et al., 2020). Clonal lines of aquaculture species are an additional tool that can be utilized to uncover genetic contributions to microbiome composition under varying environmental conditions. Clonal lines have advantages over inbred lines by avoiding artificially low levels of heterozygosity and inbreeding depression. The first clonal line of Atlantic salmon has recently been verified (Hansen et al., 2020) adding to previous lines in aquaculture species, and future work could potentially see the extension of aquaculture breeding programs to select for resistant microbiome types if these can be determined. These tools take time to develop, but with the increasing availability of fully sequenced genomes of aquaculture species, genetic manipulations to enhance functional understanding are becoming more accessible. Despite the evolution of 'omic tools, the practice of culturing microbes is also making a resurgence as a tool for understanding functionality by enabling mechanistic experiments to determine physiological processes of microbes, or how they interact with their host. Culturing also allows for more in depth genomic analyses of taxa and discovery and description of novel taxa which can then feed into sequencing databases (Hitch et al., 2021).

5.2. Applying microbial manipulation effectively in aquaculture systems

Potential therapeutics for the aquaculture industry must be biologically- and cost-effective to manufacture and apply. Evidence-based procedures should be adopted with a view to identifying pivotal taxa, whether that is a single species which plays a role in a specific disease or resistance to infection provided by controlled communities of bacteria and other microbes.

Gut microbiomes of many organisms play an important role in aiding digestion and supporting gut (and organismal) health. Perhaps the most well-known or widely-applied example of microbial therapeutics in aquaculture (and human) health are the inclusion of pre- pro- or synbiotics in the diet. Probiotics refer to live microbial feed supplements which promote host health (Hill et al., 2014) whereas pre-biotics are non-digestible feed ingredients which selectively stimulate the growth of one or a limited number of particular microbes (Yadav et al., 2022). Synbiotics refers to products containing both pre- and pro-biotics working in synergy (Schrezenmeir and de Vrese, 2001). Recent reviews on prebiotic and probiotic use have shown favorable outcomes including increased growth or survival, improved immune responses, increased digestive efficiency, and improved water quality (López Nadal et al., 2020; El-Saadony et al., 2021; Knipe et al., 2021), but caution that more knowledge is needed in fish to promote efficacy (Brugman et al., 2018).

Probiotics can exert their function via the principles of competitive exclusion, be that exploitation or interference competition (Knipe et al., 2021). In exploitation, competition is indirect and characterized by consumption of resources while in interference, direct harm is caused, for example via the production of antimicrobial compounds. For example, the lactic acid bacteria (LAB) family of probiotics produce

antimicrobial substances including lactic acid, acetic acid, hydrogen peroxide and bacteriocins interfering with the growth of other bacteria (Ringø, 2020; Ringø et al., 2020). Feeding shrimp (*Litopenaeus stylirostris*) with a diet containing the probiotic *Pediococcus acidilactici* resulted in the purging of potentially pathogenic vibrios from the gut (Castex et al., 2008). Similarly, in freshwater marron crayfish (*Cherax cainii*), feeding with *Clostridium butyricum* resulted in a reduction of *Vibrio* and *Aeromonas* counts in the hindgut (Foysal et al., 2019). In Atlantic salmon, feeding with *Peiococcus acidilactici* resulted in distinct reduction of *Fusobacteriia*, *Clostridiales*, *Actinomycetales*, *Pasteurellales*, and *Streptococcacea*, and an increase in *Bradyrhizobiaceae* in gut mucosa, but also identified that this probiotic effect is dependent on the water habitat (fresh vs. seawater) (Jaramillo-Torres et al., 2019). Contrastingly, in Nile tilapia, probiotic feeding with a mix of three *Bacillus* species did not modify gut microbiota to any large extent (Adeoye et al., 2016). Despite differences in efficacy, intervention with probiotics, prebiotics or synbiotics in an aquaculture setting more often than not illicit changes in the host microbiome (Table 3).The complex and dynamic nature of

TABLE 3 Key published literature analyzing the impact of probiotic, prebiotic, and synbiotic interventions on microbiome composition.

Species	Intervention (s)	Supplement (s)	Description	References
Rainbow trout (*Oncorhynchus mykiss*)	Probiotic and synbiotic	Probiotic – BACTOCELL (*Pediococcus acidilactici*) Synbiotic – BACTOCELL + galacto-oligosaccharides	Multiomics approach identified changes in the microbiome with a reduced relative abundance of *Candidatus Mycoplasma salmoninae* directly associated with changes in microbial arginine biosynthesis and terpenoid backbone synthesis pathways. Additionally, differences in microbiota composition were associated with alterations in metabolomic profiles.	Rasmussen et al. (2022)
Nile tilapia (*Oreochromis niloticus*)	Prebiotic	Honey	Oligosaccharides of honey can act as a prebiotic. The impact of four dietary treatments (honey inclusion of 0, 0.25, 0.5 and 1%) were tested. Honey inclusion increased growth performance, reduced feed conversion ratio, increased microvilli length, and improved microbiota diversity in the gut of tilapia with the best dietary dose determined to be 1%.	Aryati et al. (2021)
Pacific whiteleg shrimp (*Litopenaeus vannamei*)	Probiotic, prebiotic and synbiotic	Probiotic – *Bacillus* sp. NP_5 Rf^R Prebiotic – honey Synbiotic – honey + *Bacillus* sp. NP_5 Rf^R	Shrimp were fed with control, pre-, pro- or symbiotic treatments. All experimental treatments resulted in increased growth rate, feed conversion ratios, and digestive enzyme activities compared to controls with the prebiotic treatment being the most effective. The prebiotic also increased the presence of a number of known probiotic candidates.	Hasyimi et al. (2020)
Pacific whiteleg shrimp (*Litopenaeus vannamei*)	Prebiotic	Mannan oligosaccharides	Shrimp in intensive pond culture were fed with mannan oligosaccharide (MOS) supplementation. Shrimp survival was increased by 30%. Changes were identified in the gut microbiota with the prevalence of potential opportunistic pathogens (*Vibrio*, *Aeromonas*, *Shewanella*) negligible in MOS fed shrimp.	Gainza and Romero (2020)
Atlantic salmon (*Salmo salar*)	Probiotic	*Pediococcus acidilactici* MA18/5 M	The impact of seawater transfer (SWT) on gut microbiome in fish with or without dietary inclusion of probiotic was assessed. Both probiotic supplementation and SWT impacted gut microbiome composition. A higher antiviral response of fish fed the probiotic diet was indicative of a causal link between microbiome composition and activation of the antiviral response.	Jaramillo-Torres et al. (2019)
Marron crayfish (*Cherax cainii*)	Probiotic	*Clostridium butyricum*	Feeding with probiotic supplementation resulted in increased growth, attributed to an increase in molt occurrence. Probiotic inclusion also increased bacterial diversity and abundance of pathogenic taxa (*Vibrio* and *Aeromonas*) were significantly reduced. Additionally, the expression of immune-responsive genes was modulated in probiotic fed individuals when challenged with *Vibrio mimicus*.	Foysal et al. (2019)
Eastern oyster (*Crassostrea virginica*)	Probiotic	*Bacillus pumilus* RI06-95	Daily addition of a probiotic to culture water had no impact on the diversity of bacterial communities in oyster larvae and water. However, abundances of *Oceanospirillales* and *Bacillus* were higher in probiotic treated water and oyster larvae, and co-occurrence network analysis indicated a role for probiotic treatment in decreasing potentially pathogenic taxa.	Stevick et al. (2019)

changing environments in aquaculture systems has confounded wide usage of probiotics to date. Variability in efficacy may be a consequence of therapeutics often being derived from non-aquatic hosts or systems, resulting in poor tolerance of biophysical conditions within the host and subsequently poor survival and establishment. In reality, to ensure efficacy, any potential pro- or pre-biotic therapeutic needs to be evaluated under different environmental circumstances to target optimum windows of application for maximum impact, particularly in aquaculture where the environment itself can often be manipulated. Although pre- and pro-biotics are generally applied via feed, the opportunity also exists in aquaculture systems to apply such therapeutics directly to the water with a view to directly influencing external microbial communities (Rowley, 2022). Utilizing specific microbial probiotics targeted at specific pathogens carries a degree of risk whether it be the invasion of secondary pathogens or evolution of target pathogen to evade the mechanism of exclusion (Knipe et al., 2021). AMPs are an intriguing route toward developing therapeutic compounds in aquaculture as they have the power to modulate the immune system while maintaining a low probability of the development of bacterial resistance (Chaturvedi et al., 2020). The gut microbiome represents an environment which may harbor AMPs with therapeutic and microbiome sequencing datasets can be mined to uncover candidate AMPs (Dong et al., 2017).

6. Future prospects

The importance of the microbiome in the future development of sustainable aquaculture systems is clearly evident considering the vast outpouring of research in this area over the past 5–10 years, including a raft of recent reviews (de Bruijn et al., 2018; Perry et al., 2020; Legrand et al., 2020b; Infante-Villamil et al., 2021; Parata et al., 2021; Rajeev et al., 2021; Vargas-Albores et al., 2021; Diwan et al., 2022; Paillard et al., 2022). Despite this great interest, few focused studies have been conducted relating to the impact of microbiomes on host health and subsequent productivity, and the vast majority of studies have focused only on bacteria, mainly due to technical difficulties or cost associated with characterizing eukaryotes and viruses. Understanding more about these other communities and their interactions is imperative and also holds many opportunities for microbiome engineering, for example using phage to control opportunistic pathogens (Donati et al., 2021). There is now a critical need for better understanding of microbiome function in the context of aquaculture for enabling application to enhancing improved production systems. Poor animal welfare and loss of revenue due to disease remains one of the most restrictive issues in aquaculture development. One future focus should be pinpointing optimum periods for microbiome conditioning or manipulation, both during immune system development at larval stages and within periods of dysbiosis caused by disease or husbandry practices. Directing studies also toward functionality over identity will also be key in unlocking the full therapeutic potential of the microbiome in the aquaculture sector and employing next generation sequencing technologies in tandem with culture-based approaches will be essential in making this transition.

Author contributions

SM, CT, AR, and ML-R conceived the original concept. TW, JM, and DB expanded on scientific areas. JM and ML-R created the figures. ML-R wrote the initial draft and managed. All authors edited and contributed equally to the final manuscript.

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Conflict of interest

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References

Adeoye, A. A., Yomla, R., Jaramillo-Torres, A., Rodiles, A., Merrifield, D. L., and Davies, S. J. (2016). Combined effects of exogenous enzymes and probiotic on Nile tilapia (*Oreochromis niloticus*) growth, intestinal morphology and microbiome. *Aquaculture* 463, 61–70. doi: 10.1016/j.aquaculture.2016.05.028

Alberdi, A., Aizpurua, O., Bohmann, K., Zepeda-Mendoza, M. L., and Gilbert, M. T. P. (2016). Do vertebrate gut metagenomes confer rapid ecological adaptation? *Trends Ecol. Evol.* 31, 689–699. doi: 10.1016/j.tree.2016.06.008

Arfken, A., Song, B., Allen, S. K., and Carnegie, R. B. (2021). Comparing larval microbiomes of the eastern oyster (*Crassostrea virginica*) raised in different hatcheries. *Aquaculture* 531:735955. doi: 10.1016/j.aquaculture.2020.735955

Aryati, Y., Widanarni, W., Wahjuningrum, D., Rusmana, I., and Lusiastuti, A. M. (2021). The effect of dietary honey prebiotic on microbiota diversity in the digestive tract of Nile tilapia (*Oreochromis niloticus*) and its growth performance. *Aquac. Res.* 52, 1215–1226. doi: 10.1111/are.14980

Arzul, I., Renault, T., and Lipart, C. (2001). Experimental herpes-like viral infections in marine bivalves: demonstration of interspecies transmission. *Dis. Aquat. Org.* 46, 1–6. doi: 10.3354/dao046001

Attramadal, K. J. K., Øien, J. V., Kristensen, E., Evjemo, J. O., Kjørsvik, E., Vadstein, O., et al. (2021). UV treatment in RAS influences the rearing water microbiota and reduces the survival of european lobster larvae (*Homarus gammarus*). *Aquac. Eng.* 94:102176. doi: 10.1016/j.aquaeng.2021.102176

Attramadal, K. J. K., Salvesen, I., Xue, R., Øie, G., Størseth, T. R., Vadstein, O., et al. (2012). Recirculation as a possible microbial control strategy in the production of marine larvae. *Aquac. Eng.* 46, 27–39. doi: 10.1016/j.aquaeng.2011.10.003

Baruah, K., Norouzitallab, P., Phong, H. P. P. D., Smagghe, G., and Bossier, P. (2017). Enhanced resistance against *Vibrio harveyi* infection by carvacrol and its association with the induction of heat shock protein 72 in gnotobiotic *Artemia franciscana*. *Cell Stress Chaperones* 22, 377–387. doi: 10.1007/s12192-017-0775-z

Bass, D., Stentiford, G. D., Wang, H., Koskella, B., and Tyler, C. R. (2019). The pathobiome in animal and plant diseases. *Trends Ecol. Evol.* 34, 996–1008. doi: 10.1016/j.tree.2019.07.012

Berg, G., Rybakova, D., Fischer, D., Cernava, T., Vergès, M. C., Charles, T., et al. (2020). Microbiome definition re-visited: old concepts and new challenges. *Microbiome* 8:103. doi: 10.1186/s40168-020-00875-0

Blancheton, J. P., Attramadal, K. J. K., Michaud, L., d'Orbcastel, E. R., and Vadstein, O. (2013). Insight into bacterial population in aquaculture systems and its implication. *Aquac. Eng.* 53, 30–39. doi: 10.1016/j.aquaeng.2012.11.009

Bolnick, D. I., Snowberg, L. K., Caporaso, J. G., Lauber, C., Knight, R., and Stutz, W. E. (2014). Major histocompatibility complex class IIb polymorphism influences gut microbiota composition and diversity. *Mol. Ecol.* 23, 4831–4845. doi: 10.1111/mec.12846

Boutin, S., Bernatchez, L., Audet, C., and Derôme, N. (2013). Network analysis highlights complex interactions between pathogen, host and commensal microbiota. *PLoS One* 8:e84772. doi: 10.1371/journal.pone.0084772

Boyd, C. E. (1998). Pond water aeration systems. *Aquac. Eng.* 18, 9–40. doi: 10.1016/S0144-8609(98)00019-3

Brown, R. M., Wiens, G. D., and Salinas, I. (2019). Analysis of the gut and gill microbiome of resistant and susceptible lines of rainbow trout (*Oncorhynchus mykiss*). *Fish Shellfish Immunol.* 86, 497–506. doi: 10.1016/j.fsi.2018.11.079

Brugman, S., Ikeda-Ohtsubo, W., Braber, S., Folkerts, G., Pieterse, C. M., and Bakker, P. A. (2018). A comparative review on microbiota manipulation: lessons from fish, plants, livestock, and human research. *Front. Nutr.* 5:80. doi: 10.3389/fnut.2018.00080

Bugten, A. V., Attramadal, K. J. K., Fossmark, R. O., Rosten, T. W., Vadstein, O., and Bakke, I. (2022). Changes in rearing water microbiomes in RAS induced by membrane filtration alters the hindgut microbiomes of Atlantic salmon (*Salmo salar*) parr. *Aquaculture* 548:737661. doi: 10.1016/j.aquaculture.2021.737661

Castex, M., Chim, L., Pham, D., Lemaire, P., Wabete, N., Nicolas, J., et al. (2008). Probiotic *P. acidilactici* application in shrimp *Litopenaeus stylirostris* culture subject to vibriosis in New Caledonia. *Aquaculture* 275, 182–193. doi: 10.1016/j.aquaculture.2008.01.011

Castro, R., Jouneau, L., Tacchi, L., Macqueen, D. J., Alzaid, A., Secombes, C. J., et al. (2015). Disparate developmental patterns of immune responses to bacterial and viral infections in fish. *Sci. Rep.* 5:15458. doi: 10.1038/srep15458

Chaturvedi, P., Bhat, R. A. H., and Pande, A. (2020). Antimicrobial peptides of fish: innocuous alternatives to antibiotics. *Rev. Aquacult.* 12, 85–106. doi: 10.1111/raq.12306

Clerissi, C., de Lorgeril, J., Petton, B., Lucasson, A., Escoubas, J., Gueguen, Y., et al. (2020). Microbiota composition and evenness predict survival rate of oysters confronted to Pacific oyster mortality syndrome. *Front. Microbiol.* 11:311. doi: 10.3389/fmicb.2020.00311

Clinton, M., Ferrier, D. E. K., Martin, S. A. M., and Brierley, A. S. (2021). Impacts of jellyfish on marine cage aquaculture: an overview of existing knowledge and the challenges to finfish health. *ICES J. Mar. Sci.* 78, 1557–1573. doi: 10.1093/icesjms/fsaa254

Coates, C., Rowley, A., Smith, L., and Whitten, M. (2022). "Host defences of invertebrates to pathogens and parasites" in *Invertebrate pathology*. eds. A. F. Rowley, C. J. Coates and M. W. Whitten (Oxford: Oxford University Press), 3–40.

Crosbie, P. B. B., Bridle, A. R., Cadoret, K., and Nowak, B. F. (2012). *In vitro* cultured *Neoparamoeba perurans* causes amoebic gill disease in Atlantic salmon and fulfils Koch's postulates. *Int. J. Parasitol.* 42, 511–515. doi: 10.1016/j.ijpara.2012.04.002

Cunha, M. E., Quental-Ferreira, H., Parejo, A., Gamito, S., Ribeiro, L., Moreira, M., et al. (2019). Understanding the individual role of fish, oyster, phytoplankton and macroalgae in the ecology of integrated production in earthen ponds. *Aquaculture* 512:734297. doi: 10.1016/j.aquaculture.2019.734297

Dai, W., Dong, Y., Ye, J., Xue, Q., and Lin, Z. (2022). Gut microbiome composition likely affects the growth of razor clam *Sinonovacula constricta*. *Aquaculture* 550:737847. doi: 10.1016/j.aquaculture.2021.737847

de Bruijn, I., Liu, Y., Wiegertjes, G. F., and Raaijmakers, J. M. (2018). Exploring fish microbial communities to mitigate emerging diseases in aquaculture. *FEMS Microbiol. Ecol.* 94:fix161. doi: 10.1093/femsec/fix161

de Lorgeril, J., Lucasson, A., Petton, B., Toulza, E., Montagnani, C., Clerissi, C., et al. (2018). Immune-suppression by OsHV-1 viral infection causes fatal bacteraemia in Pacific oysters. *Nat. Commun.* 9:4215. doi: 10.1038/s41467-018-06659-3

Debray, R., Herbert, R. A., Jaffe, A. L., Crits-Christoph, A., Power, M. E., and Koskella, B. (2021). Priority effects in microbiome assembly. *Nat. Rev. Microbiol.* 20, 109–121. doi: 10.1038/s41579-021-00604-w

Dégremont, L., Azéma, P., Maurouard, E., and Travers, M. (2020). Enhancing resistance to *Vibrio aestuarianus* in *Crassostrea gigas* by selection. *Aquaculture* 526:735429. doi: 10.1016/j.aquaculture.2020.735429

Dehler, C. E., Secombes, C. J., and Martin, S. A. M. (2017). Seawater transfer alters the intestinal microbiota profiles of Atlantic salmon (*Salmo salar* L.). *Sci. Rep.* 7:13877. doi: 10.1038/s41598-017-13249-8

Deng, Y., Borewicz, K., van Loo, J., Olabarrieta, M. Z., Kokou, F., Sipkema, D., et al. (2021). *In-situ* biofloc affects the core prokaryotes community composition in gut and enhances growth of Nile tilapia (*Oreochromis niloticus*). *Microb. Ecol.* 84, 879–892. doi: 10.1007/s00248-021-01880-y

Deng, Y., Verdegem, M. C. J., Eding, E., and Kokou, F. (2022). Effect of rearing systems and dietary probiotic supplementation on the growth and gut microbiota of Nile tilapia (*Oreochromis niloticus*) larvae. *Aquaculture* 546:737297. doi: 10.1016/j.aquaculture.2021.737297

Dien, L. D., Hiep, L. H., Faggotter, S. J., Chen, C., Sammut, J., and Burford, M. A. (2019). Factors driving low oxygen conditions in integrated rice-shrimp ponds. *Aquaculture* 512:734315. doi: 10.1016/j.aquaculture.2019.734315

Diwan, A. D., Harke, S. N., Gopalkrishna, , and Panche, A. N. (2022). Aquaculture industry prospective from gut microbiome of fish and shellfish: an overview. *J. Anim. Physiol. Anim. Nutr.* 106, 441–469. doi: 10.1111/jpn.13619

Donati, V. L., Dalsgaard, I., Sundell, K., Castillo, D., Er-Rafik, M., Clark, J., et al. (2021). Phage-mediated control of *Flavobacterium psychrophilum* in aquaculture: *in vivo* experiments to compare delivery methods. *Front. Microbiol.* 12:628309. doi: 10.3389/fmicb.2021.628309

Dong, B., Yi, Y., Liang, L., and Shi, Q. (2017). High throughput identification of antimicrobial peptides from fish gastrointestinal microbiota. *Toxins* 9:266. doi: 10.3390/toxins9090266

Dvergedal, H., Sandve, S. R., Angell, I. L., Klemetsdal, G., and Rudi, K. (2020). Association of gut microbiota with metabolism in juvenile Atlantic salmon. *Microbiome* 8:160. doi: 10.1186/s40168-020-00938-2

Eissa, N., and Wang, H. (2016). Transcriptional stress responses to environmental and husbandry stressors in aquaculture species. *Rev. Aquac.* 8, 61–88. doi: 10.1111/raq.12081

Ellison, A. R., Wilcockson, D., and Cable, J. (2021). Circadian dynamics of the teleost skin immune-microbiome interface. *Microbiome* 9:222. doi: 10.1186/s40168-021-01160-4

El-Saadony, M., Alagawany, M., Patra, A. K., Kar, I., Tiwari, R., Dawood, M. A. O., et al. (2021). The functionality of probiotics in aquaculture: an overview. *Fish Shellfish Immunol.* 117, 36–52. doi: 10.1016/j.fsi.2021.07.007

Embar-Gopinath, S., Butler, R., and Nowak, B. (2005). Influence of salmonid gill bacteria on development and severity of amoebic gill disease. *Dis. Aquat. Org.* 67, 55–60. doi: 10.3354/dao067055

Fallet, M., Montagnani, C., Petton, B., Dantan, L., de Lorgeril, J., Comarmond, S., et al. (2022). Early life microbial exposures shape the *Crassostrea gigas* immune system for lifelong and intergenerational disease protection. *Microbiome* 10:85. doi: 10.1186/s40168-022-01280-5

FAO. (2020). *The state of world fisheries and aquaculture: sustainability in action*. Food and Agriculture Organization of the United Nations, Rome.

Fossmark, R. O., Vadstein, O., Rosten, T. W., Bakke, I., Košeto, D., Bugten, A. V., et al. (2020). Effects of reduced organic matter loading through membrane filtration on the microbial community dynamics in recirculating aquaculture systems (RAS) with Atlantic salmon parr (*Salmo salar*). *Aquaculture* 524:735268. doi: 10.1016/j.aquaculture.2020.735268

Foysal, M. J., Momtaz, F., Kawser, A. Q. M. R., Ali, M. H., Raihan, T., Siddik, M. A. B., et al. (2021). Amplicon sequencing reveals significantly increased vibrio abundance and associated gene functions in vibriosis-infected black tiger shrimp (*Penaeus monodon*). *J. Fish Dis.* 44, 591–599. doi: 10.1111/jfd.13304

Foysal, M. J., Nguyen, T. T. T., Chaklader, M. R., Siddik, M. A. B., Tay, C., Fotedar, R., et al. (2019). Marked variations in gut microbiota and some innate immune responses of fresh water crayfish, marron (*Cherax cainii*, Austin 2002) fed dietary supplementation of *Clostridium butyricum*. *PeerJ* 7:e7553. doi: 10.7717/peerj.7553

Gainza, O., and Romero, J. (2020). Effect of mannan oligosaccharides on the microbiota and productivity parameters of *Litopenaeus vannamei* shrimp under intensive cultivation in Ecuador. *Sci. Rep.* 10:2719. doi: 10.1038/s41598-020-59587-y

Gerdol, M., Gomez-Chiarri, M., Castillo, M. G., Figueras, A., Fiorito, G., Moreira, R., et al. (2018). "Immunity in molluscs: recognition and effector mechanisms, with a focus on Bivalvia" in *Advances in comparative immunology*. ed. E. L. Cooper (Cham: Springer International Publishing), 225–341.

Giatsis, C., Sipkema, D., Ramiro-Garcia, J., Bacanu, G. M., Abernathy, J., Verreth, J., et al. (2016). Probiotic legacy effects on gut microbial assembly in tilapia larvae. *Sci. Rep.* 6:33965. doi: 10.1038/srep33965

Giatsis, C., Sipkema, D., Smidt, H., Verreth, J., and Verdegem, M. (2014). The colonization dynamics of the gut microbiota in tilapia larvae. *PLoS One* 9:e103641. doi: 10.1371/journal.pone.0103641

Gibbons, S. M. (2017). Microbial community ecology: function over phylogeny. *Nat. Ecol. Evol.* 1:32. doi: 10.1038/s41559-016-0032

Green, T. J., Siboni, N., King, W. L., Labbate, M., Seymour, J. R., and Raftos, D. (2019). Simulated marine heat wave alters abundance and structure of *Vibrio* populations associated with the Pacific oyster resulting in a mass mortality event. *Microb. Ecol.* 77, 736–747. doi: 10.1007/s00248-018-1242-9

Hansen, T. J., Penman, D., Glover, K. A., Fraser, T. W. K., Vågseth, T., Thorsen, A., et al. (2020). Production and verification of the first Atlantic salmon (*Salmo salar* L.) clonal lines. *BMC Genet.* 21:71. doi: 10.1186/s12863-020-00878-8

Hasyimi, W., Widanarni, W., and Yuhana, M. (2020). Growth performance and intestinal microbiota diversity in pacific white shrimp *Litopenaeus vannamei* fed with a probiotic bacterium, honey prebiotic, and synbiotic. *Curr. Microbiol.* 77, 2982–2990. doi: 10.1007/s00284-020-02117-w

Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., et al. (2014). The international scientific association for probiotics and prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nat. Rev. Gastroenterol. Hepatol.* 11, 506–514. doi: 10.1038/nrgastro.2014.66

Hitch, T. C. A., Afrizal, A., Riedel, T., Kioukis, A., Haller, D., Lagkouvardos, I., et al. (2021). Recent advances in culture-based gut microbiome research. *Int. J. Med. Microbiol.* 311:151485. doi: 10.1016/j.ijmm.2021.151485

Hou, D., Huang, Z., Zeng, S., Liu, J., Wei, D., Deng, X., et al. (2018). Intestinal bacterial signatures of white feces syndrome in shrimp. *Appl. Microbiol. Biotechnol.* 102, 3701–3709. doi: 10.1007/s00253-018-8855-2

Houston, R. D., Bean, T. P., Macqueen, D. J., Gundappa, M. K., Jin, Y. H., Jenkins, T. L., et al. (2020). Harnessing genomics to fast-track genetic improvement in aquaculture. *Nat. Rev. Genet.* 21, 389–409. doi: 10.1038/s41576-020-0227-y

Huang, S., Wu, M., Zang, C., Du, S., Domagalski, J., Gajewska, M., et al. (2016). Dynamics of algae growth and nutrients in experimental enclosures culturing bighead carp and common carp: phosphorus dynamics. *Int. J. Sediment Res.* 31, 173–180. doi: 10.1016/j.ijsrc.2016.01.003

Infante-Villamil, S., Huerlimann, R., and Jerry, D. R. (2021). Microbiome diversity and dysbiosis in aquaculture. *Rev. Aquac.* 13, 1077–1096. doi: 10.1111/raq.12513

Jaramillo-Torres, A., Rawling, M. D., Rodiles, A., Mikalsen, H. E., Johansen, L., Tinsley, J., et al. (2019). Influence of dietary supplementation of probiotic *Pediococcus acidilactici* MA18/5M during the transition from freshwater to seawater on intestinal health and microbiota of Atlantic salmon (*Salmo salar* L.). *Front. Microbiol.* 10:2243. doi: 10.3389/fmicb.2019.02243

Jing, X., Su, S., Zhang, C., Zhu, J., Hou, Y., Li, Z., et al. (2021). Dynamic changes in microbial community structure in farming pond water and their effect on the intestinal microbial community profile in juvenile common carp (*Cyprinus carpio* L.). *Genomics* 113, 2547–2560. doi: 10.1016/j.ygeno.2021.05.024

Johansson, L., Timmerhaus, G., Afanasyev, S., Jørgensen, S. M., and Krasnov, A. (2016). Smoltification and seawater transfer of Atlantic salmon (*Salmo salar* L.) is associated with systemic repression of the immune transcriptome. *Fish Shellfish Immunol.* 58, 33–41. doi: 10.1016/j.fsi.2016.09.026

Jose, D., Preena, P. G., Kumar, V. J. R., Philip, R., and Singh, I. S. B. (2020). Metaproteomic insights into ammonia oxidising bacterial consortium developed for bioaugmenting nitrification in aquaculture systems. *Biologia* 75, 1751–1757. doi: 10.2478/s11756-020-00481-3

Katzenback, B. A. (2015). Antimicrobial peptides as mediators of innate immunity in teleosts. *Biology* 4, 607–639. doi: 10.3390/biology/4040607

Kelly, C., and Salinas, I. (2017). Under pressure: interactions between commensal microbiota and the teleost immune system. *Front. Immunol.* 8:559. doi: 10.3389/fimmu.2017.00559

King, W. L., Siboni, N., Williams, N. L. R., Kahlke, T., Nguyen, K. V., Jenkins, C., et al. (2019). Variability in the composition of Pacific oyster microbiomes across oyster families exhibiting different levels of susceptibility to OsHV-1 µvar disease. *Front. Microbiol.* 10:473. doi: 10.3389/fmicb.2019.00473

Knipe, H., Temperton, B., Lange, A., Bass, D., and Tyler, C. R. (2021). Probiotics and competitive exclusion of pathogens in shrimp aquaculture. *Rev. Aquac.* 13, 324–352. doi: 10.1111/raq.12477

Kostic, A. D., Howitt, M. R., and Garrett, W. S. (2013). Exploring host–microbiota interactions in animal models and humans. *Genes Dev.* 27, 701–718. doi: 10.1101/gad.212522.112

Lederberg, J., and McCray, A. T. (2001). 'Ome sweet 'omics - a genealogical treasury of words. *Scientist* 15:8.

Legrand, T. P. R. A., Wynne, J. W., Weyrich, L. S., and Oxley, A. P. A. (2020a). Investigating both mucosal immunity and microbiota in response to gut enteritis in yellowtail kingfish. *Microorganisms* 8:1267. doi: 10.3390/microorganisms8091267

Legrand, T. P. R. A., Wynne, J. W., Weyrich, L. S., and Oxley, A. P. A. (2020b). A microbial sea of possibilities: current knowledge and prospects for an improved understanding of the fish microbiome. *Rev. Aquac.* 12, 1101–1134. doi: 10.1111/raq.12375

Levine, J. M., and D'Antonio, C. M. (1999). Elton revisited: a review of evidence linking diversity and invasibility. *Oikos* 87, 15–26. doi: 10.2307/3546992

Li, S., Young, T., Archer, S., Lee, K., Sharma, S., and Alfaro, A. C. (2022). Mapping the Green-lipped mussel (*Perna canaliculus*) microbiome: a multi-tissue analysis of bacterial and fungal diversity. *Curr. Microbiol.* 79:76. doi: 10.1007/s00284-021-02758-5

Llewellyn, M. S., Leadbeater, S., Garcia, C., Sylvain, F. -E., Custodio, M., Ang, K. P., et al. (2017). Parasitism perturbs the mucosal microbiome of Atlantic salmon. *Sci. Rep.* 7:43465. doi: 10.1038/srep43465

López Nadal, A., Ikeda-Ohtsubo, W., Sipkema, D., Peggs, D., McGurk, C., Forlenza, M., et al. (2020). Feed, microbiota, and gut immunity: using the zebrafish model to understand fish health. *Front. Immunol.* 11:114. doi: 10.3389/fimmu.2020.00114

Lorgen-Ritchie, M., Clarkson, M., Chalmers, L., Taylor, J. F., Migaud, H., and Martin, S. A. M. (2021). A temporally dynamic gut microbiome in Atlantic salmon during freshwater recirculating aquaculture system (RAS) production and post-seawater transfer. *Front. Mar. Sci.* 8:869. doi: 10.3389/fmars.2021.711797

Ma, J., Bruce, T. J., Jones, E. M., and Cain, K. D. (2019). A review of fish vaccine development strategies: conventional methods and modern biotechnological approaches. *Microorganisms* 7:569. doi: 10.3390/microorganisms7110569

Macke, E., Callens, M., De Meester, L., and Decaestecker, E. (2017). Host-genotype dependent gut microbiota drives zooplankton tolerance to toxic cyanobacteria. *Nat. Commun.* 8:1608. doi: 10.1038/s41467-017-01714-x

Macke, E., Callens, M., Massol, F., Vanoverberghe, I., De Meester, L., and Decaestecker, E. (2020). Diet and genotype of an aquatic invertebrate affect the composition of free-living microbial communities. *Front. Microbiol.* 11:380. doi: 10.3389/fmicb.2020.00380

Marden, C. L., McDonald, R., Schreier, H. J., and Watts, J. E. M. (2017). Investigation into the fungal diversity within different regions of the gastrointestinal tract of *Panaque nigrolineatus*, a wood-eating fish. *AIMS Microbiol.* 3, 749–761. doi: 10.3934/microbiol.2017.4.749

Masso-Silva, J. A., and Diamond, G. (2014). Antimicrobial peptides from fish. *Pharmaceuticals* 7, 265–310. doi: 10.3390/ph7030265

McMurtrie, J., Alathari, S., Chaput, D. L., Bass, D., Ghambi, C., Nagoli, J., et al. (2022). Relationships between pond water and tilapia skin microbiomes in aquaculture ponds in Malawi. *Aquaculture* 558:738367. doi: 10.1016/j.aquaculture.2022.738367

Mero, F. F. C., Pedroso, F. L., Apines-Amar, M., Cadangin, J. F., Rendaje, D. C., Verde, C. S., et al. (2019). Influence of water management, photoperiod and aeration on growth, survival, and early spat settlement of the hatchery-reared green mussel, *Perna viridis*. *Int. Aquat. Res.* 11, 159–172. doi: 10.1007/s40071-019-0226-9

Minich, J. J., Nowak, B., Elizur, A., Knight, R., Fielder, S., and Allen, E. E. (2021). Impacts of the marine hatchery built environment, water and feed on mucosal microbiome colonization across ontogeny in yellowtail kingfish, *Seriola lalandi*. *Front. Mar. Sci.* 8:516. doi: 10.3389/fmars.2021.676731

Minich, J. J., Poore, G. D., Jantawongsri, K., Johnston, C., Bowie, K., Bowman, J., et al. (2020). Microbial ecology of Atlantic salmon (*Salmo salar*) hatcheries: impacts of the built environment on fish mucosal microbiota. *Appl. Environ. Microbiol.* 86, e00411–e00420. doi: 10.1101/828749

Murdoch, C. C., and Rawls, J. F. (2019). Commensal microbiota regulate vertebrate innate immunity-insights from the zebrafish. *Front. Immunol.* 10:2100. doi: 10.3389/fimmu.2019.02100

Murphy, T. R., Xiao, R., Brooks, M. L., Rader, B. A., and Hamilton-Brehm, S. (2022). Aquaculture production of hatchling Hawaiian bobtail squid (*Euprymna scolopes*) is negatively impacted by decreasing environmental microbiome diversity. *J. Appli. Microbiol.* 132, 1724–1737. doi: 10.1111/jam.15350

Nam, B., Jang, J., Caetano-Anolles, K., Kim, Y., Park, J. Y., Sohn, H., et al. (2018). Microbial community and functions associated with digestion of algal polysaccharides in the visceral tract of *Haliotis discus hannai*: insights from metagenome and metatranscriptome analysis. *PLoS One* 13:e0205594. doi: 10.1371/journal.pone.0205594

Narne, P., Pandey, V., and Phanithi, P. B. (2017). Interplay between mitochondrial metabolism and oxidative stress in ischemic stroke: an epigenetic connection. *Mol. Cell. Neurosci.* 82, 176–194. doi: 10.1016/j.mcn.2017.05.008

Naya-Català, F., Piazzon, M. C., Calduch-Giner, J. A., Sitjà-Bobadilla, A., and Pérez-Sánchez, J. (2022). Diet and host genetics drive the bacterial and fungal intestinal metatranscriptome of Gilthead Sea bream. *Front. Microbiol.* 13:883738. doi: 10.3389/fmicb.2022.883738

Nguyen, H. Y. N., Trinh, T. L., Baruah, K., Lundh, T., and Kiessling, A. (2021). Growth and feed utilisation of Nile tilapia (*Oreochromis niloticus*) fed different protein levels in a clear-water or biofloc-RAS system. *Aquaculture* 536:736404. doi: 10.1016/j.aquaculture.2021.736404

Nikouli, E., Meziti, A., Smeti, E., Antonopoulou, E., Mente, E., and Kormas, K. A. (2021). Gut microbiota of five sympatrically farmed marine fish species in the Aegean Sea. *Microb. Ecol.* 81, 460–470. doi: 10.1007/s00248-020-01580-z

Overstreet, R. M., and Lotz, J. M. (2016). "Host-symbiont relationships: understanding the change from guest to pest" in *The Rasputin effect; when commensals and symbionts become parasitic. Advances in environmental microbiology*. ed. C. Hurst, vol. *3* (Cham: Springer), 27–64.

Paillard, C., Gueguen, Y., Wegner, K. M., Bass, D., Pallavicini, A., Vezzulli, L., et al. (2022). Recent advances in bivalve-microbiota interactions for disease prevention in aquaculture. *Curr. Opin. Biotechnol.* 73, 225–232. doi: 10.1016/j.copbio.2021.07.026

Parata, L., Sammut, J., and Egan, S. (2021). Opportunities for microbiome research to enhance farmed freshwater fish quality and production. *Rev. Aquac.* 13, 2027–2037. doi: 10.1111/raq.12556

Paul-Pont, I., Dhand, N. K., and Whittington, R. J. (2013). Influence of husbandry practices on OsHV-1 associated mortality of Pacific oysters *Crassostrea gigas*. *Aquaculture* 412-413, 202–214. doi: 10.1016/j.aquaculture.2013.07.038

Pérez-Pascual, D., Vendrell-Fernández, S., Audrain, B., Bernal-Bayard, J., Patiño-Navarrete, R., Petit, V., et al. (2021). Gnotobiotic rainbow trout (*Oncorhynchus mykiss*) model reveals endogenous bacteria that protect against *Flavobacterium columnare* infection. *PLoS Pathog.* 17:e1009302. doi: 10.1371/journal.ppat.1009302

Perry, W. B., Lindsay, E., Payne, C. J., Brodie, C., and Kazlauskaite, R. (2020). The role of the gut microbiome in sustainable teleost aquaculture. *Proc. Royal Soc. B* 287:20200184. doi: 10.1098/rspb.2020.0184

Petersen, C., and Round, J. L. (2014). Defining dysbiosis and its influence on host immunity and disease. *Cell. Microbiol.* 16, 1024–1033. doi: 10.1111/cmi.12308

Petton, B., Destoumieux-Garzón, D., Pernet, F., Toulza, E., de Lorgeril, J., Degremont, L., et al. (2021). The Pacific oyster mortality syndrome, a polymicrobial and multifactorial disease: state of knowledge and future directions. *Front. Immunol.* 12:630343. doi: 10.3389/fimmu.2021.630343

Pino Martinez, E., Balseiro, P., Pedrosa, C., Haugen, T. S., Fleming, M. S., and Handeland, S. O. (2021). The effect of photoperiod manipulation on Atlantic salmon growth, smoltification and sexual maturation: a case study of a commercial RAS. *Aquac. Res.* 52, 2593–2608. doi: 10.1111/are.15107

Rajeev, R., Adithya, K. K., Kiran, G. S., and Selvin, J. (2021). Healthy microbiome: a key to successful and sustainable shrimp aquaculture. *Rev. Aquac.* 13, 238–258. doi: 10.1111/raq.12471

Rasmussen, J. A., Villumsen, K. R., Ernst, M., Hansen, M., Forberg, T., Gopalakrishnan, S., et al. (2022). A multi-omics approach unravels metagenomic and metabolic alterations of a probiotic and synbiotic additive in rainbow trout (*Oncorhynchus mykiss*). *Microbiome* 10:21. doi: 10.1186/s40168-021-01221-8

Rawls, J. F., Mahowald, M. A., Ley, R. E., and Gordon, J. I. (2006). Reciprocal gut microbiota transplants from zebrafish and mice to germ-free recipients reveal host habitat selection. *Cells* 127, 423–433. doi: 10.1016/j.cell.2006.08.043

Rawls, J. F., Samuel, B. S., and Gordon, J. I. (2004). Gnotobiotic zebrafish reveal evolutionarily conserved responses to the gut microbiota. *Proc. Natl. Acad. Sci. U. S. A.* 101:4596. doi: 10.1073/pnas.0400706101

Reid, M. A., Dai, Z., and Locasale, J. W. (2017). The impact of cellular metabolism on chromatin dynamics and epigenetics. *Nat. Cell Biol.* 19, 1298–1306. doi: 10.1038/ncb3629

Reverter, M., Tapissier-Bontemps, N., Lecchini, D., Banaigs, B., and Sasal, P. (2018). Biological and ecological roles of external fish mucus: a review. *Aust. Fish.* 3:41. doi: 10.3390/fishes3040041

Richard, M., Rolland, J. L., Gueguen, Y., de Lorgeril, J., Pouzadoux, J., Mostajir, B., et al. (2021). *In situ* characterisation of pathogen dynamics during a Pacific oyster mortality syndrome episode. *Mar. Environ. Res.* 165:105251. doi: 10.1016/j.marenvres.2020.105251

Ringø, E. (2020). Probiotics in shellfish aquaculture. Aquac. *Fisheries* 5, 1–27. doi: 10.1016/j.aaf.2019.12.001

Ringø, E., Doan, H. V., Lee, S., and Song, S. K. (2020). Lactic acid bacteria in shellfish: possibilities and challenges. *Rev. Fisheries Sci. Aquac.* 28, 139–169. doi: 10.1080/23308249.2019.1683151

Rodney, H. P., Takeshi, Z., and Kenji, S. (2014). Novel bacteriocins from lactic acid bacteria (LAB): various structures and applications. *Microb. Cell Factories* 13:S3. doi: 10.1186/1475-2859-13-S1-S3

Roques, S., Deborde, C., Richard, N., Skiba-Cassy, S., Moing, A., and Fauconneau, B. (2020). Metabolomics and fish nutrition: a review in the context of sustainable feed development. *Rev. Aquac.* 12, 261–282. doi: 10.1111/raq.12316

Rosenberg, E., Koren, O., Reshef, L., Efrony, R., and Zilber-Rosenberg, I. (2007). The role of microorganisms in coral health, disease and evolution. *Nat. Rev. Microbiol.* 5, 355–362. doi: 10.1038/nrmicro1635

Rosenberg, E., and Zilber-Rosenberg, I. (2018). The hologenome concept of evolution after 10 years. *Microbiome* 6:78. doi: 10.1186/s40168-018-0457-9

Rowley, A. F. (2022). "Bacterial diseases of crustaceans" in *Invertebrate pathology*. eds. A. F. Rowley, C. J. Coates and M. W. Whitten (Oxford: Oxford University Press), 400–435.

Salinas, I., Fernández-Montero, Á., Ding, Y., and Sunyer, J. O. (2021). Mucosal immunoglobulins of teleost fish: a decade of advances. *Dev. Comp. Immunol.* 121:104079. doi: 10.1016/j.dci.2021.104079

Schrezenmeir, J., and de Vrese, M. (2001). Probiotics, prebiotics, and synbiotics—approaching a definition. *Am. J. Clin. Nutr.* 73, 361s–364s. doi: 10.1093/ajcn/73.2.361s

Segarra, A., Pépin, J. F., Arzul, I., Morga, B., Faury, N., and Renault, T. (2010). Detection and description of a particular ostreid herpesvirus 1 genotype associated with massive mortality outbreaks of Pacific oysters, *Crassostrea gigas*, in France in 2008. *Virus Res.* 153, 92–99. doi: 10.1016/j.virusres.2010.07.011

Siriyappagouder, P., Galindo-Villegas, J., Dhanasiri, A. K. S., Zhang, Q., Mulero, V., Kiron, V., et al. (2020). *Pseudozyma* priming influences expression of genes involved in metabolic pathways and immunity in zebrafish larvae. *Front. Immunol.* 11:987. doi: 10.3389/fimmu.200.00978

Siriyappagouder, P., Kiron, V., Lokesh, J., Rajeish, M., Kopp, M., and Fernandes, J. (2018). The intestinal mycobiota in wild zebrafish comprises mainly Dothideomycetes while Saccharomycetes predominate in their laboratory-reared counterparts. *Front. Microbiol.* 9:387. doi: 10.3389/fmicb.2018.00387

Situmorang, M. L., Dierckens, K., Mlingi, F. T., Van Delsen, B., and Bossier, P. (2014). Development of a bacterial challenge test for gnotobiotic Nile tilapia *Oreochromis niloticus* larvae. *Dis. Aquat. Org.* 109, 23–33. doi: 10.3354/dao02721

Slinger, J., Adams, M. B., Stratford, C. N., Rigby, M., and Wynne, J. W. (2021). The effect of antimicrobial treatment upon the gill bacteriome of Atlantic salmon (*Salmo salar* L.) and progression of amoebic gill disease (AGD) *in vivo*. *Microorganisms* 9:987. doi: 10.3390/microorganisms9050987

Slinger, J., Adams, M. B., and Wynne, J. W. (2020). Bacteriomic profiling of branchial lesions induced by *Neoparamoeba perurans* challenge reveals commensal dysbiosis and an association with *Tenacibaculum dicentrarchi* in agd-affected Atlantic salmon (*Salmo salar* L.). *Microorganisms* 8, 1–17. doi: 10.3390/microorganisms8081189

Smith, C. C. R., Snowberg, L. K., Gregory Caporaso, J., Knight, R., and Bolnick, D. I. (2015). Dietary input of microbes and host genetic variation shape among-population differences in stickleback gut microbiota. *ISME J.* 9, 2515–2526. doi: 10.1038/ismej.2015.64

Spor, A., Koren, O., and Ley, R. (2011). Unravelling the effects of the environment and host genotype on the gut microbiome. *Nat. Rev. Microbiol.* 9, 279–290. doi: 10.1038/nrmicro2540

Sriyasak, P., Chitmanat, C., Whangchai, N., Promya, J., and Lebel, L. (2015). Effect of water de-stratification on dissolved oxygen and ammonia in tilapia ponds in northern Thailand. *Int. Aquat. Res.* 7, 287–299. doi: 10.1007/s40071-015-0113-y

Stephens, W. Z., Burns, A. R., Stagaman, K., Wong, S., Rawls, J. F., Guillemin, K., et al. (2016). The composition of the zebrafish intestinal microbial community varies across development. *ISME J.* 10, 644–654. doi: 10.1038/ismej.2015.140

Steury, R. A., Currey, M. C., Cresko, W. A., and Bohannan, B. J. M. (2019). Population genetic divergence and environment influence the gut microbiome in Oregon threespine stickleback. *Genes* 10:484. doi: 10.3390/genes10070484

Stevick, R. J., Sohn, S., Modak, T. H., Nelson, D. R., Rowley, D. C., Tammi, K., et al. (2019). Bacterial community dynamics in an oyster hatchery in response to probiotic treatment. *Front. Microbiol.* 10:1060. doi: 10.3389/fmicb.2019.01060

Summerfelt, S. T., Sharrer, M. J., Tsukuda, S. M., and Gearheart, M. (2009). Process requirements for achieving full-flow disinfection of recirculating water using ozonation and UV irradiation. *Aquac. Eng.* 40, 17–27. doi: 10.1016/j.aquaeng.2008.10.002

Sun, S., Yang, M., Fu, H., Ge, X., and Zou, J. (2020). Altered intestinal microbiota induced by chronic hypoxia drives the effects on lipid metabolism and the immune response of oriental river prawn *Macrobrachium nipponense*. *Aquaculture* 526:735431. doi: 10.1016/j.aquaculture.2020.735431

Sunagawa, S., Coelho, L. P., Chaffron, S., Kultima, J. R., Labadie, K., Salazar, G., et al. (2015). Ocean plankton. Structure and function of the global ocean microbiome. *Science* 348:1261359. doi: 10.1126/science.1261359

Tabrett, A., and Horton, M. W. (2020). The influence of host genetics on the microbiome. *F1000Res* 9:84. doi: 10.12688/f1000research.20835.1

Tepaamorndech, S., Nookaew, I., Higdon, S. M., Santiyanont, P., Phromson, M., Chantarasakha, K., et al. (2020). Metagenomics in bioflocs and their effects on gut microbiome and immune responses in Pacific white shrimp. *Fish Shellfish Immunol.* 106, 733–741. doi: 10.1016/j.fsi.2020.08.042

Tidwell, J. H. (2012). *Aquaculture production systems*. Oxford: John Wiley and Sons.

Trabal Fernández, N., Mazón-Suástegui, J. M., Vázquez-Juárez, R., Ascencio-Valle, F., and Romero, J. (2014). Changes in the composition and diversity of the bacterial microbiota associated with oysters (*Crassostrea corteziensis*, *Crassostrea gigas* and *Crassostrea sikamea*) during commercial production. *FEMS Microbiol. Ecol.* 88, 69–83. doi: 10.1111/1574-6941.12270

Uengwetwanit, T., Uawisetwathana, U., Arayamethakorn, S., Khudet, J., Chaiyapechara, S., Karoonuthaisiri, N., et al. (2020). Multi-omics analysis to examine microbiota, host gene expression and metabolites in the intestine of black tiger shrimp (*Penaeus monodon*) with different growth performance. *PeerJ* 8:e9646. doi: 10.7717/peerj.9646

Uren Webster, T. M., Consuegra, S., Hitchings, M., and Garcia de Leaniz, C. (2018). Interpopulation variation in the Atlantic salmon microbiome reflects environmental and genetic diversity. *Appl. Environ. Microbiol.* 84, e00691–e00618. doi: 10.1128/AEM.00691-18

Uren Webster, T. M., Rodriguez-Barreto, D., Castaldo, G., Gough, P., Consuegra, S., and Garcia de Leaniz, C. (2020). Environmental plasticity and colonisation history in the Atlantic salmon microbiome: a translocation experiment. *Mol. Ecol.* 29, 886–898. doi: 10.1111/mec.15369

Vadstein, O., Attramadal, K. J. K., Bakke, I., and Olsen, Y. (2018). K-selection as microbial community management strategy: a method for improved viability of larvae in aquaculture. *Front. Microbiol.* 9:2730. doi: 10.3389/fmicb.2018.02730

Vargas-Albores, F., Martínez-Córdova, L. R., Hernández-Mendoza, A., Cicala, F., Lago-Lestón, A., and Martínez-Porchas, M. (2021). Therapeutic modulation of fish gut microbiota, a feasible strategy for aquaculture? *Aquaculture* 544:737050. doi: 10.1016/j.aquaculture.2021.737050

Vasta, G. R., Ahmed, H., Bianchet, M. A., Fernández-Robledo, J. A., and Amzel, L. M. (2012). Diversity in recognition of glycans by F-type lectins and galectins: molecular, structural, and biophysical aspects. *Ann. N. Y. Acad. Sci.* 1253, E14–E26. doi: 10.1111/j.1749-6632.2012.06698.x

Vasta, G. R., Ahmed, H., Tasumi, S., Odom, E. W., and Saito, K. (2007). Biological roles of lectins in innate immunity: molecular and structural basis for diversity in self/non-self recognition. *Adv. Exp. Med. Biol.* 598, 389–406. doi: 10.1007/978-0-387-71767-8_27

Vestrum, R. I., Forberg, T., Luef, B., Bakke, I., Winge, P., Olsen, Y., et al. (2022). Commensal and opportunistic bacteria present in the microbiota in Atlantic cod (*Gadus*

morhua) larvae differentially alter the hosts' innate immune responses. *Microorganisms* 10:24. doi: 10.3390/microorganisms10010024

Villasante, A., Catalán, N., Rojas, R., Lohrmann, K. B., and Romero, J. (2020). Microbiota of the digestive gland of red abalone (*Haliotis rufescens*) is affected by withering syndrome. *Microorganisms* 8:1411. doi: 10.3390/microorganisms8091411

Vonaesch, P., Anderson, M., and Sansonetti, P. J. (2018). Pathogens, microbiome and the host: emergence of the ecological Koch's postulates. *FEMS Microbiol. Rev.* 42, 273–292. doi: 10.1093/femsre/fuy003

Wang, J., Huang, Y., Xu, K., Zhang, X., Sun, H., Fan, L., et al. (2019). White spot syndrome virus (WSSV) infection impacts intestinal microbiota composition and function in *Litopenaeus vannamei*. *Fish Shellfish Immunol.* 84, 130–137. doi: 10.1016/j.fsi.2018.09.076

Wang, K., Li, K., Liu, L., Tanase, C., Mols, R., and van der Meer, M. (2020). Effects of light intensity and photoperiod on the growth and stress response of juvenile Nile tilapia (*Oreochromis niloticus*) in a recirculating aquaculture system. *Aquac. Fish.* 8, 85–90. doi: 10.1016/j.aaf.2020.03.001

Webster, T. M. U., Rodriguez-Barreto, D., Consuegra, S., and Garcia de Leaniz, C. (2019). Cortisol-induced signatures of stress in the fish microbiome. *bioRxiv*:826503. doi: 10.1101/826503

West, A. C., Mizoro, Y., Wood, S. H., Ince, L. M., Iversen, M., Jørgensen, E. H., et al. (2021). Immunologic profiling of the Atlantic salmon gill by single nuclei transcriptomics. *Front. Immunol.* 12:669889. doi: 10.3389/fimmu.2021.669889

Woof, J. M., and Mestecky, J. (2005). Mucosal immunoglobulins. *Immunol. Rev.* 206, 64–82. doi: 10.1111/j.0105-2896.2005.00290.x

Wynne, J. W., Thakur, K. K., Slinger, J., Samsing, F., Milligan, B., Powell, J. F. F., et al. (2020). Microbiome profiling reveals a microbial dysbiosis during a natural outbreak of Tenacibaculosis (yellow mouth) in Atlantic salmon. *Front. Microbiol.* 11:586387. doi: 10.3389/fmicb.2020.586387

Xu, Z., Takizawa, F., Casadei, E., Shibasaki, Y., Ding, Y., Sauters, T. J. C., et al. (2020). Specialization of mucosal immunoglobulins in pathogen control and microbiota homeostasis occurred early in vertebrate evolution. *Sci. Immunol.* 5:eaay3254. doi: 10.1126/sciimmunol.aay3254

Xue, M., Jiang, N., Fan, Y., Yang, T., Li, M., Liu, W., et al. (2022). White spot syndrome virus (WSSV) infection alters gut histopathology and microbiota composition in crayfish (*Procambarus clarkii*). *Aquac. Rep.* 22:101006. doi: 10.1016/j.aqrep.2022.101006

Yadav, M. K., Kumari, I., Singh, B., Sharma, K. K., and Tiwari, S. K. (2022). Probiotics, prebiotics and synbiotics: safe options for next-generation therapeutics. *Appl. Microbiol. Biotechnol.* 106, 505–521. doi: 10.1007/s00253-021-11646-8

Zaneveld, J. R., McMinds, R., and Vega Thurber, R. (2017). Stress and stability: applying the Anna Karenina principle to animal microbiomes. *Nat. Microbiol.* 2:17121. doi: 10.1038/nmicrobiol.2017.121

Zarkasi, K. Z., Abell, G. C. J., Taylor, R. S., Neuman, C., Hatje, E., Tamplin, M. L., et al. (2014). Pyrosequencing-based characterization of gastrointestinal bacteria of Atlantic salmon (*Salmo salar* L.) within a commercial mariculture system. *J. Appl. Microbiol.* 117, 18–27. doi: 10.1111/jam.12514

Zhang, M., Shan, C., Tan, F., Limbu, S. M., Chen, L., and Du, Z. (2020). Gnotobiotic models: powerful tools for deeply understanding intestinal microbiota-host interactions in aquaculture. *Aquaculture* 517:734800. doi: 10.1016/j.aquaculture.2019.734800

Ziab, M., Chaganti, S. R., and Heath, D. D. (2023). The effects of host quantitative genetic architecture on the gut microbiota composition of Chinook salmon (*Oncorhynchus tshawytscha*). *Heredity*. doi: 10.1038/s41437-023-00620-x

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Ensiling hybrid *Pennisetum* with lactic acid bacteria or organic acids improved the fermentation quality and bacterial community

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The aim of this study was to compare the effect of different additives on nutritional quality, fermentation variables and microbial diversity of hybrid *Pennisetum* silages. A control (CK – no additives) and seven treatments were tested, namely, *Lactiplantibacillus plantarum* (LP), *Lentilactobacillus buchneri* (LB), propionic acid (PA), calcium propionate (CAP), LP+LB; LP+PA and LP+CAP. In comparison with CK, all treatments increased the contents of crude protein and lactic acid, decreased the content of butyric acid, and altered the bacterial communities of the silage. Except for the CAP and LP+CAP treatments, the additives decreased pH and the ammonia nitrogen:total nitrogen (NH_3-N:TN) ratio. The results of principal component analysis revealed that the PA, LP+PA and LP+LB treatments ranked as the top three silages. The PA and LP+PA treatments exhibited higher water-soluble carbohydrate content, but lower pH, and NH_3-N:TN ratio than the other treatments. With the PA and LP+PA treatments, the relative abundances of *Lactobacillus* and *Enterobacter* decreased, and of *Proteobacteria* and *Delftia* increased, while the carbohydrate metabolism of the microorganisms improved. The LP and LB treatments reduced the Shannon and Simpson diversities. In the beta diversity, PA and LP+PA separated from the other treatments, indicating that there were differences in the composition of bacterial species. The relative abundance of *Lactobacillus* increased in the LP and LB treatments and of *Leucanostoc* and *Weissella* increased in the CAP and LP+CAP treatments. In summary, the addition of *L. plantarum*, *L. buchneri*, propionic acid, calcium propionate, and their combinations improved fermentation quality, inhibited harmful bacteria and conserved the nutrients of hybrid *Pennisetum*.

KEYWORDS

calcium propionate, hybrid *Pennisetum*, silage, *Lactiplantibacillus plantarum*, microbial diversity, propionic acid

1. Introduction

With the rapid development of the livestock industry and the increased demand for animal products, more animal feed is needed. This has increased the cost of animal feed and the competition for land between food for humans and feed for livestock (Sandström et al., 2022; Zhao et al., 2022), which has led to utilization of different forage resources for ruminants

(Li D. et al., 2019; Wang N. et al., 2022). Hybrid *Pennisetum* (*Pennisetum americanum* × *Pennisetum purpureum*) has been incorporated into animal feed and has great potential to be used for ruminants. It is distributed widely in southern China, and is characterized by low cultivation input, high biomass, and strong stress tolerance (Song et al., 2019; Cai et al., 2020; Wu et al., 2020).

Ensiling is a traditional method of preserving forage (Ren et al., 2018; Drouin et al., 2021). However, the hybrid *Pennisetum* is difficult to ensile because of its low lactic acid bacteria (LAB) count (Shah et al., 2020). Bacterial inoculants and chemicals are often added to improve the fermentation and nutrient qualities of the silage, and to inhibit the activity of harmful microbiota (Queiroz et al., 2018; He Q. et al., 2021). The LAB inoculants are known for their ability to alter fermentation patterns, and are added widely to improve fermentation in the production of silage (Ogunade et al., 2017). *Lactiplantibacillus plantarum*, the most common additive, is a homofermentative LAB that produces lactic acid efficiently and reduces the pH rapidly (Xu et al., 2021; Xian et al., 2022). *Lentilactobacillus buchneri*, also a heterofermentative LAB, produces acetic acid during fermentation, inhibits yeast and mold, improves aerobic stability and reduces feed loss (Romero et al., 2017; Zhang et al., 2020). Propionic acid is an aerobic microbial inhibitor that affects nitrogen conversion and reduces the degradation of protein by acidizing the silage or limiting the activity of undesirable bacteria at the early stage of fermentation (Carvalho et al., 2012; He et al., 2020; Ren et al., 2021). Due to the volatility of propionic acid and its relatively short residual time, calcium propionate (CAP), which has the same antibacterial effect as propionic acid after ionization in water, was developed (Xiong et al., 2017). The above-mentioned additives have advantages, but little information is available on their effect on silage quality and the bacterial community of hybrid *Pennisetum*. To fill this knowledge gap, we compared the effects of *L. plantarum*, *L. buchneri*, propionic acid, calcium propionate, and their combinations on the chemical composition, fermentation quality, and microbial community of hybrid *Pennisetum*.

2. Materials and methods

2.1. Silage preparation

Hybrid *Pennisetum* was harvested in Fujian Province (117.93 °E, 26.79 °N, subtropical monsoon climate) in May 2021 by manually mowing at 8–10 cm above ground level, and was transported to the laboratory immediately. The *Pennisetum* was spread out evenly, and air-dried for 6 h, resulting in a dry matter (DM) content of 181.3 g/kg fresh weight (FW), and was chopped into 1–2 cm lengths using a paper cutter. The following were added to the *Pennisetum*: (1) distilled water (CK); (2) *L. plantarum* (LP, provided by Fujian Academy of Agricultural Sciences, viable counts $\geq 1 \times 10^6$ cfu/g FW); (3) *L. buchneri* (LB, BNCC187961, Beijing Beina Chuanglian Biotechnology Institute, Beijing, China, viable counts $\geq 1 \times 10^6$ cfu/g FW); (4) propionic acid (PA, 0.5% FW, analytical pure, Fuzhou Mili Biotechnology Co., Ltd., Fuzhou, China); (5) calcium propionate (CAP, 0.5% FW, Fuzhou Mili Biotechnology Co., Ltd., Fuzhou, China); (6) LP + LB; (7) LP + PA; and (8) LP + CAP. Each additive was dissolved in 10 mL sterile water and sprayed evenly onto the surface of the Pennisetum (CK was sprayed with an equal volume of distilled water). Subsequently, 400 g of the sprayed hybrid *Pennisetum* samples were placed in a polyethylene bag (248 mm × 344 mm), with 3 replicates for each treatment. The bags were vacuum sealed, and ensiled at room temperature of 26°C for 30 or 60 days.

2.2. Nutritional composition and fermentation variables of hybrid *Pennisetum* silage

After ensiling, DM content of the silage was determined by oven drying at 65°C for 48 h, and the oven-dried samples were sieved through a 0.425 mm screen. The content of water-soluble carbohydrates (WSC) was determined by anthrone sulfuric acid colorimetry (Fu and Diao, 2007); total nitrogen (TN) was determined using an automatic nitrogen analyzer (K9840 Kjeldahl, Hanon, Jinan, China), the crude protein (CP) content was calculated as TN × 6.25; and the neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents were measured following Van Soest et al. (1991).

Ten g of sample were added to 90 mL of distilled water for 24 h at 4°C, and filtered through 4 layers of gauze for pH determination (pHS-3D, Shandong, China). Ammonia nitrogen (NH_3-N) was determined by phenol sodium hypochlorite colorimetry (Arthur Thomas, 1977), and lactic, acetic, propionic, and butyric acids were determined using high-performance liquid chromatography (Wang et al., 2020).

2.3. The pH and microbial count during aerobic exposure

The pH and microbial counts of the treatments after 60 days of ensiling were determined at 0, 3, 6, and 9 days after aerobic exposure. The pH was determined as described previously. The methods of Dong et al. (2017) were used to measure the counts of LAB, yeast, and aerobic bacteria during aerobic exposure with the de Man Rogosa Sharpe medium, Potato Dextrose Agar medium, and Plate Count Agar medium, respectively (Fuzhou Mili Biotechnology Co., Ltd., Fuzhou, China). No antibiotics were added to the culture media.

2.4. Microbial diversity analysis

After 60 days of ensiling, a sample of each treatment was stored at −80°C for microbial diversity determination. High throughput sequencing was performed in triplicate, and total DNA was extracted using the CTAB/SDS method to check DNA concentration and purity with a 1% agarose gel. The 16S rDNA gene of the bacterial V3 ~ V4 hypervariable region was obtained by primer sequences 338F (ACTCCTACGGGAGGCAGCAG) and 806R (GGACTAC HVGGGTWTCTAAT) (Ling et al., 2022). PCR amplicons were identified by agarose gel electrophoresis using NEXTFLEX® Rapid DNA-Seq Kit for Miseq library construction and sequencing. After the library qualified, it was sent to Majorbio BioPharm Technology Co. Ltd. (Shanghai, China) for sequencing. The library was paired-end sequenced based on the Illumina Novaseq sequencing platform resulting in a complete microbial community.

2.5. 16S rRNA gene sequence analysis

Raw fastq files were demultiplexed and quality filtered using Trimomatic, and further merged by FLASH software. Uparse software (Uparse v7.0.1001)[1] was used to cluster the entire high-quality sequences of all samples, and, by default, the sequences were clustered to operational taxonomic units (OTUs) with 97% similarity (He L. et al., 2021). Alpha diversity was determined using species richness indices (Ace and Chao 1) and species diversity indices (Shannon and Simpson) (Zheng et al., 2020). Beta diversity was determined using principal coordinates analysis (PCoA), and was further analyzed using the ANOSIM test (Dong et al., 2017). The metabolic function of bacteria was predicted by comparing the Kyoto Encyclopedia of Genes and Genomes (KEGG) database with the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 (PICRUSt2) (Langille et al., 2013). Spearman's correlation tested the relationships between silage quality and the relative abundances of bacteria at the genus levels in each treatment. Raw sequencing files and associated metadata have been deposited in NCBI's Sequence Read Archive (PRJNA946341).

2.6. Statistical analyses

The data of silage quality were analyzed by the GLM program in SPSS software (version 26.0, Chicago, IL, United States) with ensiling time, additive treatment and their interaction. The principal component analysis used SPSS software. The eigenvalues of the variance matrix, the variance contribution rate and the weight coefficient of each factor were calculated to generate the principal component equation. The principal component comprehensive score was calculated by standardizing the original data into the principal component equation (Tharangani et al., 2021).

3. Results and discussion

3.1. Nutritional quality of hybrid *Pennisetum* silage

The DM and WSC contents of hybrid *Pennisetum* silage were affected by ensiling time, additives and their interactions (Table 1). When compared to CK, the LP treatment had a lesser ($p < 0.05$) DM content, while the LP + LB, PA, LP + PA, CAP and LP + CAP treatments had greater ($p < 0.05$) DM contents. The loss of DM from silage was due to the breakdown of nutrients, as aerobic microbes converted carbohydrates to water, carbon dioxide, and heat (Haas et al., 2011). In the present study, the DM content in the PA treatment was greater than in the other treatments after 60 days of ensiling. It is likely that the addition of propionic acid inhibited the growth of undesirable microorganisms and reduced their consumption of nutrients (Li et al., 2022). Propionic acid has antifungal properties by maintaining its activity on the surface of microorganisms and competing with amino acids for enzyme activity sites or by altering the cell permeability of the organisms (Gheller et al., 2021).

With the prolongation of ensiling, the CP content in each treatment decreased. The degradation of protein during ensiling involves a series of plant and microbial enzymes (Xiong et al., 2017). Proteins are converted into free amino acids and peptides through the catalytic hydrolysis of plant enzymes, and then are degraded further (Zheng et al., 2022). In the present study, the CP content was greater ($p < 0.05$) with each additive than in CK. Previous studies reported that propionic acid inhibited *Clostridia* and *Enterobacteria* effectively, as these bacteria were poor acid resistant bacteria, and reduced protein breakdown (Ali and Tahir, 2021). Adding calcium propionate also reduced CP loss, but less so than propionate, which might be due to the lesser concentration of dissociated propionate ions with CAP. The increase ($p < 0.05$) of CP content with the LB treatment most likely involved propionic acid. *L. buchneri* produces 1,2-propanediol from sugars and then propionic acid in the metabolic process, resulting in a bacteriostatic effect (Ling et al., 2022).

The WSC content of all silages was lower ($p < 0.05$) at 60 days than at 30 days of ensiling. The WSC serves as an energy source for microorganisms and its consumption implies microbial activity (Gheller et al., 2021). The WSC is converted into organic acid to reduce the pH of the silage (Zhang et al., 2019; Zhu et al., 2022). The NDF content of silage was reduced ($p < 0.05$) with the LP + LB treatments. Similarly, Du et al. (2022) reported that the content of NDF in ryegrass silage inoculated with *L. plantarum*, *L. buchneri* and *L. casei* was reduced after 60 days of ensiling. When ensiled for 30 days, the PA and LP + PA treatments had a lesser ($p < 0.05$) NDF content than CK. This could be due to the increase in total organic acids after the addition of PA, which could hydrolyze digestible cell walls (Jiang et al., 2020; Ren et al., 2020).

3.2. Fermentation quality of hybrid *Pennisetum* silage

Table 2 presents the effect of different additives on the silage fermentation parameters of hybrid *Pennisetum*. A pH in the range of 3.6–4.2 for silage is considered optimal, as it effectively reduces undesirable microorganisms (Lv et al., 2020; Bao et al., 2023). In the current study, the pH was below 4.2 at 30 days of ensiling in all treatments except for CAP, LP + CAP and LB, in which the pHs were greater ($p < 0.05$) than for CK. Li M. et al. (2019) concluded that CAP led to an increase in buffering energy and, thus, a rise in pH in the silage, which could explain the results in the present study. *L. plantarum*, which is regarded as the most commonly used homofermentative LAB, has the ability to reduce pH rapidly (Muck et al., 2018; Bai et al., 2022). *L. buchneri* could improve the aerobic stability of silage (Magnusson and Schnürer, 2001), and when combined with *L. plantarum*, reduced the pH at the initial stage of fermentation. Consequently, the pH of the LP + LB treatment was lesser than the LP and LB treatments.

After 30 days of ensiling, the LB and LP + LB treatments increased acetic acid content, and after 60 days of ensiling all treatments had greater ($p < 0.05$) lactic acid content than CK. The LP treatment had the lowest pH and highest lactic acid content of all treatments. It is likely that lactic acid has a lower pH than other organic acids and plays a vital role during fermentation (Jaipolsaen et al., 2021). In this study,

1 http://www.drive5.com/uparse/

TABLE 1 Nutritional quality of hybrid *Pennisetum* silage after 30 and 60 days of ensiling.

Ensiling days	Treatments								SEM	*P*		
	CK	LP	LB	LP+LB	PA	LP+PA	CAP	LP+CAP		D	T	D×T
Dry matter (g/kg FW)												
30	201.0^{b}	196.5^{a}	202.5^{Ab}	206.5^{Ac}	220.5^{Ad}	265.0^{Bg}	224.5^{e}	248.5^{Bf}	3.404	<0.001	<0.001	<0.001
60	207.0^{b}	193.5^{a}	206.1^{Bb}	219.0^{Bd}	239.5^{Bf}	211.0^{Ac}	224.5^{e}	221.5^{Ade}				
Crude protein (g/kg DM)												
30	94.6^{Ba}	113.0^{b}	110.1^{b}	119.6^{Bbc}	126.6^{cd}	134.6^{Bd}	123.8^{Bc}	123.5^{Bc}	2.008	<0.001	<0.001	0.009
60	82.5^{Aa}	103.2^{b}	109.3^{bc}	108.7^{Abc}	123.0^{d}	114.4^{Acd}	101.5^{Ab}	100.2^{Ab}				
Water-soluble carbohydrates (g/kg DM)												
30	21.6^{Bb}	25.1^{Bc}	24.4^{Bc}	24.1^{Bc}	38.8^{Bd}	42.1^{Be}	18.8^{Ba}	20.0^{Bab}	1.502	<0.001	<0.001	<0.001
60	13.7^{Ae}	11.5^{Ad}	10.8^{Ad}	9.0^{Ac}	23.8^{Ag}	21.7^{Af}	4.0^{Ab}	6.3^{Aa}				
Neutral detergent fiber (g/kg DM)												
30	633.5^{Bd}	604.0^{cd}	606.0^{cd}	548.5^{b}	582.2^{bc}	504.9^{a}	613.6^{cd}	581.8^{bc}	6.131	<0.001	0.001	0.262
60	570.5^{Aab}	572.2^{ab}	586.1^{b}	544.3^{ab}	521.9^{a}	514.4^{a}	560.7^{ab}	557.5^{ab}				
Acid detergent fiber (g/kg DM)												
30	393.6^{Bb}	363.3^{Bb}	388.7^{Bb}	327.4^{a}	362.5^{Bab}	330.0^{a}	383.6^{Bb}	352.2^{ab}	4.798	0.004	<0.001	0.058
60	342.6^{Aab}	359.1^{b}	343.1^{Aab}	321.6^{ab}	300.6^{Aa}	315.1^{ab}	313.4^{Aab}	350.5^{b}				

FW, fresh weight; DM, dry matter; CK, distilled water; LP, *L. plantarum*; LB, *L. buchneri*; LP + LB, *L. plantarum* and *L. buchneri*; PA, propionic acid; LP + PA, *L. plantarum* and propionic acid; CAP, calcium propionate; LP + CAP, *L. plantarum* and calcium propionate. D = ensilage days effect; T = treatment effect; D × T = the interaction between ensiling days and treatments. Means of treatment within a row followed by different lowercase superscripts differ from each other ($p < 0.05$). Means of ensiling time within a column followed by different uppercase superscripts differ from each other ($p < 0.05$).

TABLE 2 Fermentation characteristics of hybrid *Pennisetum* silage after 30 and 60 days of ensiling.

Ensiling days	Treatments								SEM	*P*		
	CK	LP	LB	LP+LB	PA	LP+PA	CAP	LP+CAP		D	T	D×T
pH												
30	4.14^{Ad}	4.10^{Ad}	4.36^{Be}	3.88^{c}	3.60^{Bb}	3.46^{a}	4.62^{Af}	4.68^{Ag}	0.068	0.045	0.465	0.046
60	4.30^{Bd}	4.19^{Bc}	4.18^{Ac}	3.87^{b}	3.49^{Aa}	3.49^{a}	4.89^{Be}	4.90^{Be}				
Lactic acid (g/kg DM)												
30	8.02^{Ab}	10.31^{Ad}	8.86^{Ac}	13.37^{e}	14.03^{Af}	10.13^{Ad}	8.03^{Ab}	6.85^{Aa}	0.438	<0.001	<0.001	<0.001
60	8.64^{Ba}	11.74^{Bc}	16.58^{Be}	13.07^{d}	17.18^{Bf}	12.95^{Bd}	10.75^{Bb}	8.92^{Bb}				
Acetic acid (g/kg DM)												
30	0.39^{Bb}	0.52^{Bc}	0.69^{Be}	0.62^{Bd}	0.02^{a}	0.02^{Aa}	0.42^{Bb}	0.39^{Ab}	0.030	0.091	0.271	0.002
60	0.17^{Ac}	0.26^{Ad}	0.33^{Af}	0.34^{Af}	0.01^{a}	0.14^{Bb}	0.29^{Ae}	0.51^{Bg}				
Propionic acid (g/kg DM)												
30	2.07^{Ac}	1.67^{a}	1.82^{Ab}	2.32^{Ad}	2.33^{Bd}	2.09^{Ac}	1.82^{Ab}	2.57^{Ae}	0.075	<0.001	<0.001	<0.001
60	2.17^{Bc}	1.61^{a}	2.11^{Bc}	2.48^{Be}	1.77^{Ab}	2.25^{Bd}	3.10^{Bf}	3.64^{Bg}				
Butyric acid (g/kg DM)												
30	0.38^{Ad}	0.19^{c}	0.39^{Bd}	0.44^{Be}	0.04^{a}	0.03^{b}	0.12^{Ab}	0.37^{Bd}	0.022	<0.001	<0.001	<0.001
60	0.50^{Be}	0.16^{b}	0.21^{Ac}	0.22^{Ac}	0.03^{a}	0.01^{a}	0.30^{Bd}	0.29^{Ad}				
Ammonia nitrogen/total nitrogen												
30	40.8^{c}	31.3^{Ab}	30.0^{Ab}	27.3^{Ab}	3.4^{Aa}	3.4^{Aa}	39.8^{Ac}	38.2^{Ac}	2.811	<0.001	<0.001	<0.001
60	43.9^{c}	38.5^{Bb}	39.5^{Bb}	36.7^{Bb}	7.1^{Ba}	6.2^{Ba}	64.1^{Bd}	70.2^{Be}				

CK, distilled water; LP, *L. plantarum*; LB, *L. buchneri*; LP + LB, *L. plantarum* and *L. buchneri*; PA, propionic acid; LP + PA, *L. plantarum* and propionic acid; CAP, calcium propionate; LP + CAP, *L. plantarum* and calcium propionate. D = ensilage days effect; T = treatment effect; D × T = the interaction between ensilage days and treatment. Means of additive treatment within a row followed by different lowercase superscripts differ from each other ($p < 0.05$). Means of ensiling time within a column followed by different uppercase superscripts differ from each other ($p < 0.05$).

the concentration of lactic acid at 30 days was greater ($p < 0.05$) than at 60 days of ensiling in all treatments, except for the PA treatment. Concomitantly, there was a decrease in acetic acid content, which implied that homolatic fermentation dominated. Li et al. (2018) concluded that the addition of *L. plantarum* enhanced the quality of alfalfa silage. At 30 days of ensiling, the LP + LB, PA, and LP + CAP treatments had greater ($p < 0.05$), but the LP, LB and CAP treatments ($p < 0.05$) had lesser concentrations of propionic acid than CK. The increase in propionic acid content was likely a result of lactic acid being consumed by *L. buchneri* (Li et al., 2018). With the prolongation of the ensiling period, the content of propionic acid in the CAP treatment increased ($p < 0.05$), which could be attributed to the dissociation of corresponding organic acid salts (Dai et al., 2022), as was reported by Wen et al. (2017). Kung et al. (2018) reported that butyric acid below 5 g/kg DM was optimal for high quality fermentation, and the butyric acid content of all treatments in the present study met this criterium. After an increase in the ensiling period, the content of butyric acid was lesser ($p < 0.05$) in the LP, PA, LP + PA and CAP treatments than in CK. The production of silage undergoes dynamic enzymatic and microbial processes of which the degradation of proteins is one of the most crucial stages (Wang S. et al., 2022). In the present study, the NH_3-N:TN ratios in the PA and LP + PA treatments were lesser ($p < 0.05$) than in the other treatments, probably because the lower pH inhibited the activity of the protease (Tian H. et al., 2022). In addition, the NH_3-N:TN ratios in the LP, LB and LP + LB treatments were lesser ($p < 0.05$) than in CK. *L. plantarum* inhibits protein degradation through its effect on enzymes and microorganisms (Xian et al., 2022), whereas, *L. buchneri*, through its bacteriostatic effect, reduces the degradation of protein by undesirable microorganisms. The combination of *L. buchneri* and *L. plantarum* had a synergistic effect in reducing the degradation of protein.

3.3. Correlations and principal component analysis of silage indices of hybrid *Pennisetum*

After 30 days of ensiling, CP was correlated positively ($p < 0.05$) with DM content, but negatively ($p < 0.05$) with NDF and ADF contents (Figure 1A). After 60 days of ensiling, CP content was correlated positively ($p < 0.05$) with lactic acid content and negatively ($p < 0.001$) with butyric acid content (Figure 1B). The WSC content was correlated negatively with acetic acid content ($p < 0.05$), pH ($p < 0.01$) and the NH_3-N:TN ratio ($p < 0.001$). Moreover, the WSC content was correlated negatively with pH as a result of the lactic acid produced by LAB, with WSC as a substrate (Filya, 2003). Lactic acid plays the major role in reducing the pH of silage (Fu et al., 2022). After 30 days of ensiling, there was a positive correlation ($p < 0.05$) between pH and the NH_3-N:TN ratio, and this correlation was stronger after 60 days of ensiling. Generally, NH_3-N accumulates continuously during fermentation (Dong et al., 2022a). When LAB dominated in the late stages of ensiling, lactic acid was produced by the fermentation of plant biomass and the pH was reduced to a level that inhibited the activity of ammonia nitrogen producing bacteria (Fan et al., 2022). This could explain the correlation between pH and the NH_3-N:TN ratio.

Principal component analysis (PCA) not only reduces the loss of original information, but also simplifies multiple related indicators into independent components, and, subsequently, assesses the indicators based on the difference in principal component scores (Gallo et al., 2013). In the present study, the PCA of 11 indicators of hybrid *Pennisetum* silage ensiled for different time lengths was carried out. The results of the PCA after 30 days of ensiling are presented in Tables 3, 4. The cumulative contribution of three extracted principal components, based on the characteristic value >1, reached 86.7%, that is, 86.7% of the original index was retained. The positive load value of the NH_3-N:TN ratio and the negative load value of WSC were the greatest in the first principal component in their corresponding eigenvector, indicating that the WSC content could limit the silage quality of hybrid *Pennisetum*. In the second principal component, the positive load value of DM content and the negative load value of lactic acid were the greatest, indicating that the content of lactic acid was the chief factor limiting the quality of hybrid *Pennisetum* silage. The butyric acid content positive load value and the NDF negative load value were the greatest in the third principal component, which meant that the NDF content held a dominant position in limiting the quality of hybrid *Pennisetum* silage. The first principal component was correlated positively with NH_3-N:TN, NDF, ADF and pH, and negatively with CP and WSC. The second principal component was correlated positively with DM, pH and propionic acid, and negatively with lactic acid and WSC, and the third principal component was correlated positively with organic acids such as butyric acid. After 60 days of ensiling, the cumulative contribution rate of the three extracted principal components, based on the characteristic value >1, reached 89.7% (Tables 3, 4). Similar to ensiling for 30 days, WSC, ADF and butyric acid were the main limiting factors of the principal components after ensiling for 60 days.

We concluded that the lower composite scores indicated better silage quality according to the composite of each original index and the proportion of principal components. Therefore, the top three treatments were LP + PA, PA, and LP + LB.

3.4. pH and microbial abundances of hybrid *Pennisetum* after aerobic exposure

The changes in pH and relative abundances of microorganisms during aerobic exposure after 60 days of ensiling are presented in Table 5. The resistance against spoilage varies greatly among silages, and different additives are used to prevent aerobic spoilage (Puntillo et al., 2020; Ferrero et al., 2021). With an increase in aerobic exposure, the pH increased ($p < 0.05$) in all treatments, except for the PA and LP + PA treatments. When the pH of the silage increases by 0.5 after aerobic exposure, it could be regarded as aerobic deterioration (Mu et al., 2021). In the current study, the pH of only the PA and LP + PA treatments did not increase by 0.5 after 6 days of aerobic exposure. The pH of the CAP and LP + CAP treatments were always greater ($p < 0.05$) than that of CK during aerobic exposure. During aerobic exposure, the abundance of LAB in the CK, LP, LB and LP + LB treatments displayed an increasing trend between days 0 and 6 and then a decreasing trend between days 6 and 9 ($p < 0.05$), while the abundance of LAB in the PA, LP + PA, CAP and LP + CAP treatments displayed an increasing trend ($p < 0.05$). When the number of yeasts exceeded 5 $\log_{10}$ cfu/g FW, the silage was prone to aerobic spoilage (Chen et al., 2016). In this study, the number

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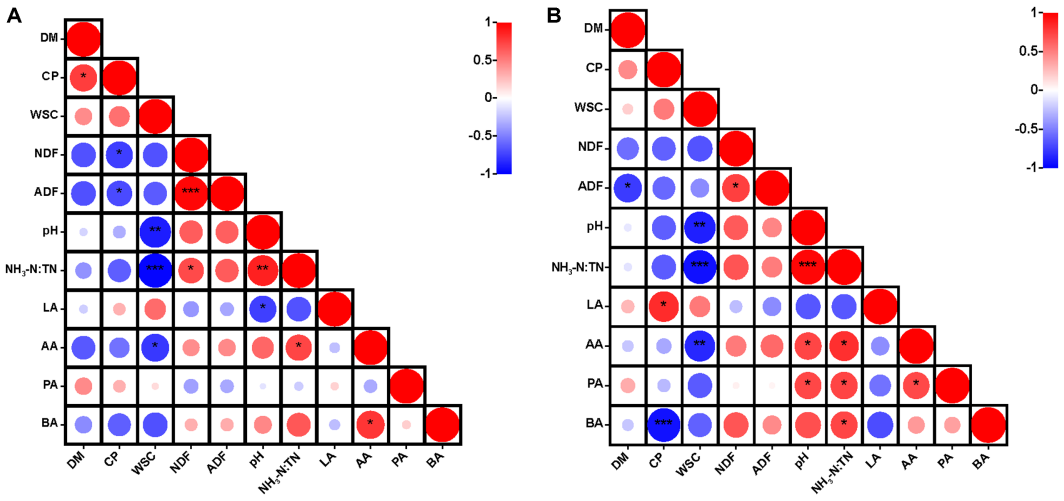

FIGURE 1
Heatmap of Pearson correlations between nutritional quality and fermentation parameters after 30days **(A)** and 60days **(B)** of ensiling hybrid *Pennisetum*. *p<0.05, **p<0.01, ***p<0.001. DM, dry matter; CP, crude protein; WSC, water-soluble carbohydrates; NDF, neutral detergent fiber; ADF, acid detergent fiber; NH_3-N:TN, ammonia nitrogen:total nitrogen ratio; LA, lactic acid; AA, acetic acid; PA, propionic acid; BA, butyric acid.

TABLE 3 Index coefficient and contribution rate of hybrid *Pennisetum* silage.

Item	Principal component (30d)			Principal component (60d)		
	PC1	PC2	PC3	PC1	PC2	PC3
Dry matter	−0.264	0.526	−0.164	−0.143	0.577	−0.136
Crude protein	−0.313	0.267	−0.092	−0.314	0.197	0.476
Water-soluble carbohydrates	−0.365	−0.209	−0.078	−0.345	−0.150	−0.243
Neutral detergent fiber	0.338	−0.175	−0.279	0.294	−0.302	0.235
Acid detergent fiber	0.328	−0.192	−0.245	0.268	−0.399	0.223
pH	0.319	0.370	−0.154	0.357	0.181	0.043
NH_3-N:TN	0.369	0.213	0.003	0.370	0.178	0.085
Lactic acid	−0.218	−0.464	0.357	−0.294	−0.035	0.468
Acetic acid	0.318	−0.039	0.323	0.308	0.155	0.441
Propionic acid	−0.132	0.359	0.467	0.236	0.511	0.013
Butyric acid	0.271	0.112	0.589	0.319	−0.064	−0.410
Eigenvalue	6.375	1.823	1.339	6.618	2.105	1.145
Variance contribution rate (%)	57.95	16.57	12.17	60.16	19.14	10.41
Cumulative contribution rate (%)	57.95	74.53	86.70	60.16	79.30	89.71

NH_3-N:TN, Ammonia nitrogen/total nitrogen.

TABLE 4 Principal component score, comprehensive score and ranking of hybrid *Pennisetum* silage.

Days	Treatment	F1	F2	F3	F	Ranking
30	LP + PA	−4.70	0.69	−0.50	−3.08	1
	PA	−2.85	−1.40	−0.27	−2.21	2
	LP + LB	−0.25	−0.33	2.44	0.10	3
	LP	1.03	−1.25	−0.56	0.37	4
	CAP	1.44	0.68	−1.61	0.87	5
	LP + CAP	0.75	2.75	0.44	1.08	6
	LB	2.06	−0.57	0.10	1.28	7
	CK	2.53	−0.57	−0.05	1.58	8
60	PA	−4.50	0.85	−0.26	−2.57	1
	LP + PA	−3.01	−0.07	−0.36	−1.86	2
	LP + LB	−0.23	0.61	0.33	0.01	3
	LP	0.63	−2.22	0.68	0.03	4
	LB	0.31	−1.07	1.58	0.15	5
	CK	1.85	−1.42	−2.12	0.62	6
	CAP	1.83	1.74	−0.27	1.41	7
	LP + CAP	3.11	1.58	0.42	2.22	8

F1, score of the first principal component; F2, score of the second principal component; F3, score of the third principal component; F, comprehensive score of principal components; CK, distilled water; LP, *L. plantarum*; LB, *L. buchneri*; LP + LB, *L. plantarum* and *L. buchneri*; PA, propionic acid; LP + PA, *L. plantarum* and propionic acid; CAP, calcium propionate; LP + CAP, *L. plantarum* and calcium propionate.

of yeasts in each treatment, except for the organic acid treatment, exceeded this number at 6 days of aerobic exposure, indicating a trend of aerobic spoilage (Wang et al., 2014). The activity of aerobic microorganisms increased after the silage was exposed to air, and they used lactic acid, sugars and amino acids to produce heat continuously, resulting in aerobic putrefaction (Hu et al., 2009; Zhou et al., 2019). In this study, PA and LP + PA inhibited the proliferation of aerobic microorganisms, while the abundance of aerobic bacteria in the other treatments exhibited an increasing

TABLE 5 Changes of pH and microbial quantity of hybrid *Pennisetum* silage during aerobic exposure.

Item and ensiling days	Treatments								SEM	*P*		
	CK	LP	LB	LP+LB	PA	LP+PA	CAP	LP+CAP		D	T	D×T
pH												
0	4.30^{Af}	4.18^{Ad}	4.16^{Ac}	4.28^{Ae}	3.48^{Ab}	3.44^{Aa}	4.86^{Ag}	4.89^{Ah}	0.176	<0.001	<0.001	<0.001
3	5.12^{Bd}	5.59^{Be}	4.86^{Bc}	4.66^{Bb}	3.91^{Ca}	3.89^{Ca}	6.19^{Bg}	5.87^{Bf}				
6	7.14^{Cd}	7.71^{Cf}	7.26^{Ce}	7.04^{Cc}	3.86^{Bb}	3.81^{Ba}	8.16^{Ch}	7.93^{Cg}				
9	7.75^{Dc}	7.88^{Dd}	7.87^{Dd}	8.09^{De}	4.67^{Da}	6.21^{Db}	8.67^{Dg}	8.44^{Df}				
Lactic acid bacteria (log10 cfu/g FW)												
0	2.12^{Aa}	3.63^{Ac}	3.18^{Ab}	3.74^{Ac}	2.19^{Aa}	2.20^{Aa}	4.64^{Ad}	4.59^{Ad}	0.192	<0.001	<0.001	<0.001
3	2.36^{Ba}	5.67^{Bf}	3.79^{Bc}	3.98^{Ad}	2.88^{Bb}	2.40^{Aa}	5.06^{Be}	5.21^{Be}				
6	7.00^{Cc}	7.06^{Ccd}	7.53^{Dg}	7.38^{Cf}	3.21^{Ca}	7.08^{Bd}	6.70^{Cb}	7.18^{Ce}				
9	6.22^{Dc}	5.90^{Bb}	6.68^{Cd}	6.77^{Bd}	3.78^{Da}	7.08^{Be}	6.71^{Cd}	7.46^{Df}				
Yeast (log10 cfu/g FW)												
0	3.42^{Ad}	4.16^{Ae}	3.28^{Ad}	3.41^{Bd}	0.11^{Aa}	0.52^{Ab}	2.49^{Ac}	4.48^{Af}	0.223	<0.001	<0.001	<0.001
3	3.86^{Bc}	5.77^{Be}	3.62^{Ac}	2.63^{Ab}	2.36^{Ba}	2.68^{Bb}	5.09^{Bd}	5.03^{Bd}				
6	7.16^{Cc}	7.25^{Ccd}	7.45_{Be}	7.31^{Dd}	3.04^{Ca}	2.93^{Ba}	6.37^{Cb}	7.28^{Ccd}				
9	8.10^{Df}	8.31^{Df}	7.18^{Be}	6.23^{Cc}	4.52^{Da}	4.98^{Cb}	6.77^{Dd}	7.16^{Ce}				
Aerobic bacteria (log_{10} cfu/g FW)												
0	4.31^{Ac}	4.24^{Ac}	3.77^{Ab}	4.18^{Bc}	2.26^{Aa}	2.09^{Aa}	4.46^{Acd}	4.60_{Ad}	0.204	<0.001	<0.001	<0.001
3	4.97^{Be}	5.76^{Bf}	3.81^{Ab}	3.77^{Ab}	4.79^{Bd}	3.19^{Ba}	4.35^{Ac}	4.74^{Bd}				
6	7.33^{Ccd}	7.66^{Ce}	7.37^{Bd}	7.24^{Cc}	5.79^{Cb}	3.52^{Ca}	7.62^{Be}	7.89^{Cf}				
9	8.86^{Dd}	8.85^{Dd}	8.31^{Cc}	8.33^{Dc}	5.84^{Cb}	5.70^{Da}	8.26^{Cc}	8.35^{Dc}				

CK, distilled water; LP, *L. plantarum*; LB, *L. buchneri*; LP + LB, *L. plantarum* and *L. buchneri*; PA, propionic acid; LP + PA, *L. plantarum* and propionic acid; CAP, calcium propionate; LP + CAP, *L. plantarum* and calcium propionate. D = ensilage days effect; T = treatment effect; D × T = the interaction between ensilage days and treatment. Means of additive treatment within a row followed by different lowercase superscripts differ from each other ($p < 0.05$). Means of ensiling time within a column followed by different uppercase superscripts differ from each other ($p < 0.05$).

trend. The counts of yeast and aerobic bacteria in the PA treatment during aerobic exposure were lower ($p < 0.05$) than in other treatments, while the pH remained stable, and was lower ($p < 0.05$) than in other treatments on day 9, which improved the aerobic stability of the silage. The LB treatment failed to inhibit the proliferation of yeast, resulting in an increase ($p < 0.05$) in pH during aerobic exposure. It is likely that the undesirable microorganisms used the high content of residual lactic acid as a substrate after aerobic exposure (Rabelo et al., 2019).

3.5. Microbial diversity

After sequencing and quality control, a total of 1,044,312 optimized sequences were obtained. According to the 3% difference, a total of 1985 OTUs were obtained by OTU clustering. In total, 88 OTUs were shared by 8 processing units, accounting for 4.43% of all OTUs (Figure 2). CK, LP, LB, LP + LB, PA, LP + PA, CAP and LP + CAP had 11, 1, 2, 3, 63, 102, 5 and 16 OTUs, respectively. Alpha-diversity reflects the microbial abundance and species diversity of a single sample (Xian et al., 2022). Chao1 and ACE diversities are commonly used to measure species richness, while Shannon and Simpson indices are used to measure species diversity (Dong et al., 2020). Compared to CK, except for the LB treatment, the ACE index of all treatments increased, with the LP + CAP treatment being higher than the other treatments (Figure 3). The microbial community within the crop formed in the field, and when the stable environment was disrupted, the microorganisms that were not adapted to the fermentation system were eliminated, and the more adaptable microorganisms dominated in the new environment (Dong et al., 2022b). In this study, the Simpson index of LP was greater ($p < 0.05$) than the other treatments, while the Chao 1 index was greater ($p < 0.05$) and the ACE were lesser ($p < 0.05$) in the LP treatment than CK. Compared to CK, Shannon indices of LP and LB and the Simpson index of LP + CAP were lesser ($p < 0.05$), while the Shannon index of LP + CAP and the Simpson indices of LP, LB and LP + LB were greater ($p < 0.05$) than CK. The Shannon indices of LP + LB, LP + PA and LP + CAP were greater but the indices were lesser ($p < 0.05$) than the LP treatment ($p < 0.05$). The change in alpha diversity among silages was caused by the dynamic response of microorganisms (Feng et al., 2022). The composition and function of bacteria could differ during the period of ensiling (Sepehri and Sarrafzadeh, 2019).

The PCoA based on the Bray–Curtis dissimilarity displayed distinct clusters among the eight silages (Figure 4). Further analysis through ANOSIM revealed that the results were reliable ($R = 0.73$, $p = 0.001$). According to PCoA, CK and LP + LB were clustered in the

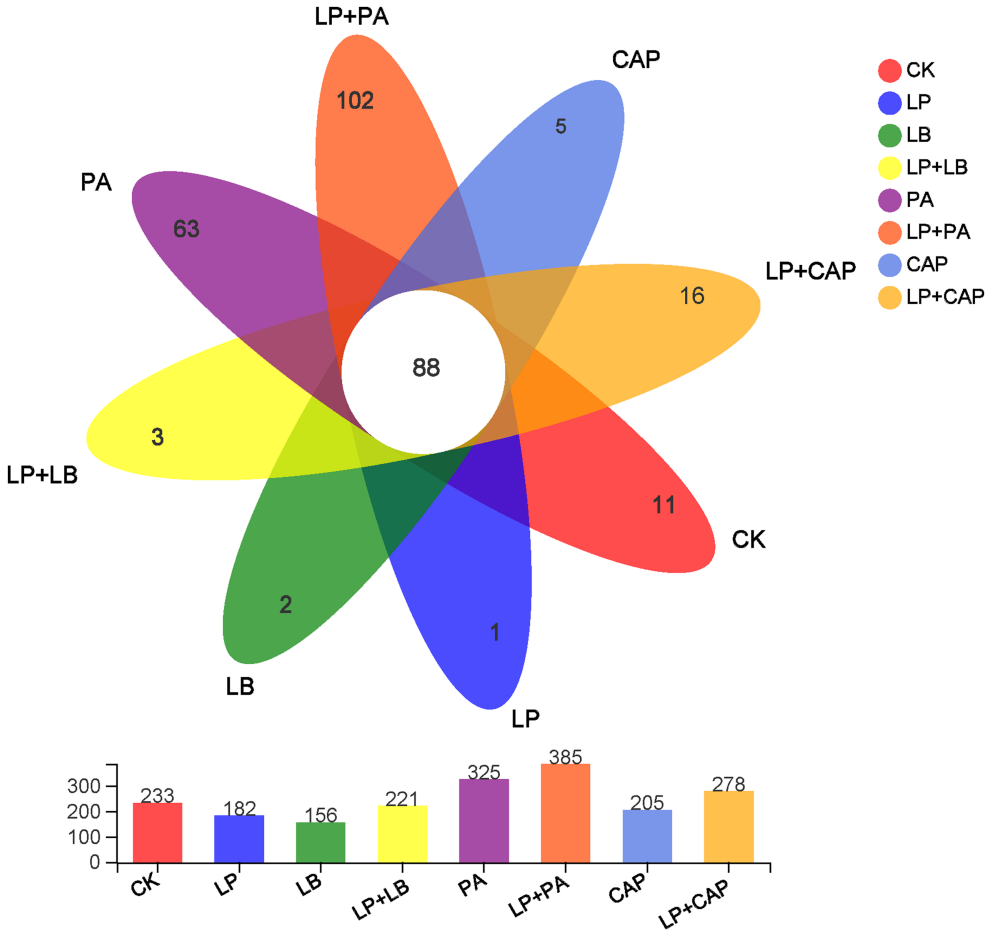

FIGURE 2
Petal diagram illustrating the degree of overlap of bacterial operational taxonomic units (OTUs) in the 8 silages. CK, distilled water; LP, *L. plantarum*; LB, *L. buchneri*; LP+LB, *L. plantarum* and *L. buchneri*; PA, propionic acid; LP+PA, *L. plantarum* and propionic acid; CAP, calcium propionate; LP+CAP, *L. plantarum* and calcium propionate.

second and third quadrants, LP and LB in the third quadrant, PA and LP + PA mainly in the fourth quadrant, and CAP and LP + CAP mainly in the second quadrant. In addition, PA and LP + PA were separated from the other treatments, indicating that there were differences in the composition of species; whereas, CK was relatively close to LP, LB, and LP + LB, indicating that the composition of species was similar among these treatments. These results demonstrated that different additive treatments had significant effects on the bacterial community of hybrid *Pennisetum* silage (Tian J. et al., 2022).

After 60 days of ensiling hybrid *Pennisetum*, the main bacterial phylum was Firmicutes, followed by Proteobacteria and Cyanobacteria (Figure 5A). Liu et al. (2019) reported that Firmicutes and Proteobacteria were the most abundant phyla of barley silage at any point during the ensiling process with or without LAB inoculants, and continued to rise to 99% of the total bacteria at 60 days of ensiling. Most bacteria involved in lactic acid fermentation belong to Firmicutes and Proteobacteria and they play important roles in an anaerobic environment (Yuan et al., 2020). The relative abundance of Firmicutes was lesser ($p < 0.05$) and of Proteobacteria was greater ($p < 0.05$) in PA and LP + PA than in CK, while there was no difference ($p > 0.05$) in the other treatments. The relative abundance of Cyanobacteria in the LP and CAP treatments was lesser ($p < 0.05$), and in the LP + PA treatment was greater ($p < 0.05$) than in CK. Cyanobacteria is often found in tropical herbage and could be replaced by *Lactobacillus* and *Enterobacteria* after fermentation, but further studies are needed to determine their roles in silage production (Li et al., 2019). The relative abundance of Actinobacteriota in LB was lesser ($p < 0.05$), while of Actinobacteriota in CAP and LP + CAP was greater ($p < 0.05$) than in CK. When mixed with *L. plantarum*, the relative abundances of Firmicutes and Cyanobacteria in LP + PA were greater ($p < 0.05$), while the relative abundance of Proteobacteria was lesser ($p < 0.05$) than in LP. The relative abundance of Actinobacteriota in the LP + PA and LP + CAP treatments was greater ($p < 0.05$) than in the LP treatment. It was reported that Actinobacteriota had the potential of bioremediation to degrade pesticides and heavy metals (Alvarez et al., 2017).

The bacterial genera after 60 days of ensiling hybrid *Pennisetum* is presented in Figure 5B. *Lactobacillus* was the dominant genus in each treatment. Xu et al. (2020) reported that *Lactobacillus* was often the most important microbe in the late stages of ensiling, which, together with *Weissella* and *Pediococcus*, were the main producers of lactic acid. The relative abundance of *Lactobacillus* in

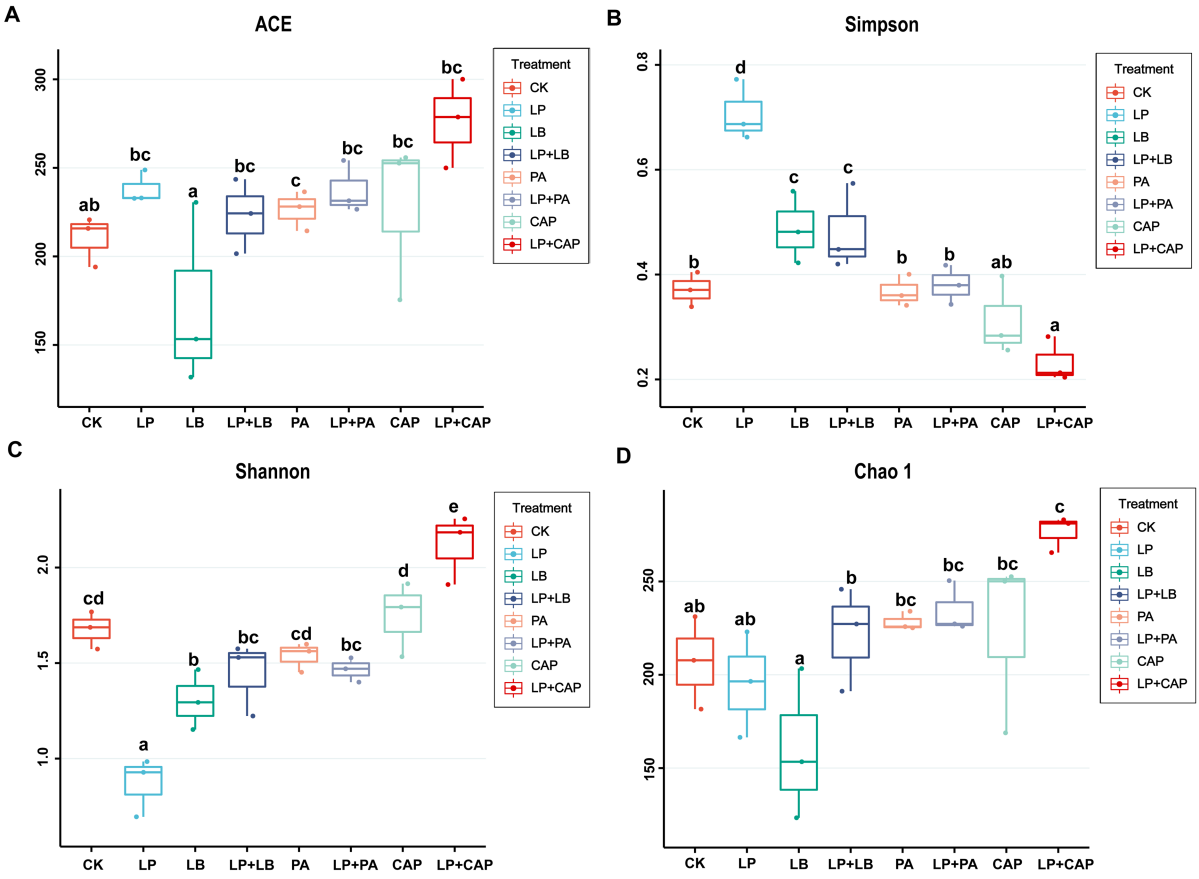

FIGURE 3
Effects of different additives on alpha diversity of hybrid *Pennisetum* silage. ACE **(A)**, Simpson **(B)**, Shannon **(C)** and Chao1 **(D)** indexes are used to reflected alpha diversity. CK, distilled water; LP, *L. plantarum*; LB, *L. buchneri*; LP+LB, *L. plantarum* and *L. buchneri*; PA, propionic acid; LP+PA, *L. plantarum* and propionic acid; CAP, calcium propionate; LP+CAP, *L. plantarum* and calcium propionate. Means with different lowercase letters differ from each other (p<0.05).

LP (85.4%) was greater ($p < 0.05$), but in PA (46.8%) was lesser ($p < 0.05$) than in CK. The relative abundance of *Weissella* in the LP, LB, PA and LP + PA treatments was lesser ($p < 0.05$) than in CK (12.7%). *Weissella* belongs to the heterofermentative bacteria and consumes WSC to produce a mixture of lactic acid and acetic acid (Huang et al., 2021; Sun et al., 2021). The relative abundance of *Delftia* in the PA and LP + PA treatments was greater ($p < 0.05$) than in CK. *Delftia* is often present in soil and plants and promotes the growth and bioremediation of plants (Liu et al., 2018; Bhat et al., 2022). Dong et al. (2022b) concluded that the low pH of the silage might be due to an accumulation of nitrite in the silage. This occurred because *Delftia*, as a reductant of nitrate, could not reduce nitrite further. In the acidic environment of silage, nitrite could be converted into nitrogen oxides to reduce the pH of silage. The addition of organic acids reduced the relative abundances of *Klebsiella*, *Paenibacillus*, and *Enterobacter* in corn silage (Jiang et al., 2020). In the present study, the relative abundances of *Enterobacter* in the PA and LP + PA treatments and of *Enterobacterin* in the LP + LB treatment were greater ($p < 0.05$) than in CK. Guo et al. (2020) reported that *Enterobacter* was one of the dominant bacteria during ensiling, especially in silage treated with LAB. However, *Enterobacter* was unwanted due to nutrient loss caused by acetic acid fermentation (Ni et al., 2017b), although most *Enterobacter* bacteria in silage were considered non-pathogenic (Santos et al., 2016). The relative abundances of *Weissella* and *Pediococcus* in LP + CAP were greater ($p < 0.05$) than in LP, while the relative abundances of *Delftia* and *norank_o__Chloroplast* in LP + PA were greater ($p < 0.05$), but the relative abundance of *Enterobacter* in LP + PA and LP + CAP was lesser ($p < 0.05$) than in CK. The relative abundance of *Methylobactere-methylorubrum* in LP + CAP (1.42%) was greater ($p < 0.05$) than in the other treatments. *Methylobactery-methylorubrum* is a gram negative, rod-shaped, strictly aerobic bacteria that can utilize methanol and other reduced one-carbon compounds via the serine pathway (Dong et al., 2022b). The high pH of the LP + CAP treatment after 60 days of ensiling was consistent with the neutrophilic property of *Methylobacter-methylorubrum* (Knief et al., 2012). *Methylobacterium* was the dominant genus in alfalfa silage (Ni et al., 2017a; Ogunade et al., 2018).

LEfSe was used to analyze the different bacteria species in each treatment. A total of 25 species with relative abundance differences was identified in the 8 treatments (LDA score > 4) (Figure 5C). *Proteobacteria* and *Delftia* were biomarkers of the PA treatment at the phylum and genus levels, respectively, while *Cyanobacteria* and *Enterobacter* were biomarkers of the LP + PA treatment. Firmicutes was the most abundant phylum in the CAP treatment. In addition,

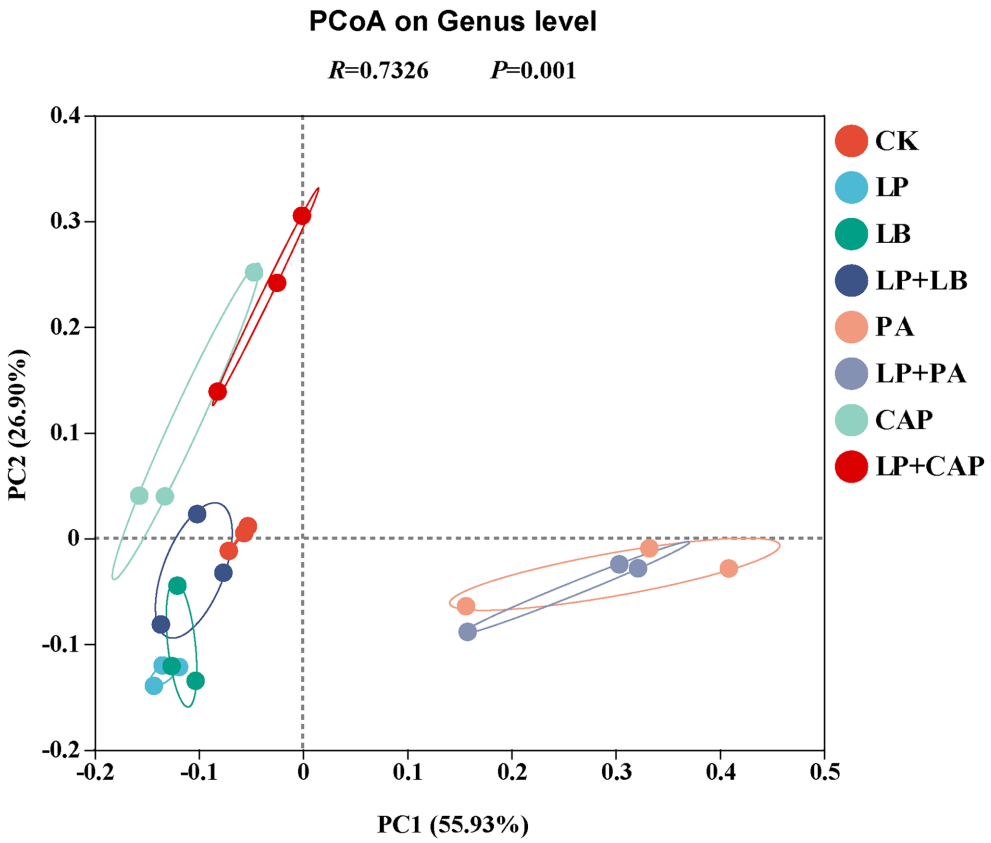

FIGURE 4
Principal coordinate analysis (PCoA) of microbial diversity of hybrid *Pennisetum* silage treated with different additives. CK, distilled water; LP, *L. plantarum*; LB, *L. buchneri*; LP+LB, *L. plantarum* and *L. buchneri*; PA, propionic acid; LP+PA, *L. plantarum* and propionic acid; CAP, calcium propionate; LP+CAP, *L. plantarum* and calcium propionate.

the highest abundances of each treatment at the genus level were as follows: *Lactococcus* in CK, *Lactobacillus* in LP, and *Weissella* and *Pediococcus* in LP + CAP. Previous studies reported that *Pediococcus* was generally highly abundant in silage with high pH (Zhao et al., 2022; Zong et al., 2022), and the treatment with LP + CAP in this study had the highest pH at 60 days of ensiling (Table 2).

Figures 6A,B present the second-level classification of microbial community metabolic functions. The metabolic pathway with the greatest abundance in all treatments was carbohydrate metabolism. This metabolism was greater in the PA and LP + PA treatments than in the other treatments, perhaps due to the their high WSC content (Table 1). In addition, the lactic acid content in the PA and LP + PA treatments was greater ($p < 0.05$) than in CK, which most likely was due to carbohydrate metabolism. The metabolism abundances of the CAP, LP + CAP, PA and LP + PA treatments were greater than CK ($p < 0.05$), which might be due to the response of microorganisms to long-term acid stress in silage (Bai et al., 2022). The metabolic abundances of terpenoids and polyketides were predicted to be relatively high in the PA and LP + PA treatments. Terpenoids are natural compounds, mainly in Chinese herbal medicine, and are reputed to possess antibacterial and antioxidant properties (Elshamy et al., 2016; Tian et al., 2017). Further predictions of the third-level metabolic pathways of carbohydrate metabolism are presented in Figure 6C. The PA and LP + PA treatments had similar metabolic functions in all carbohydrates, and both had greater metabolism of pyruvate, propanoate, butanoate, ascorbate and aldarate than CK. It was reported that pyruvate metabolism was related to the formation of organic acids such as lactic acid, α-acetolactic acid, acetic acid, and formic acid (Dong et al., 2022a). Pyruvate, an intermediate in the glycolytic pathway, is crucial in lactic acid generation by LAB utilizing WSC, and can interconvert sugars, fats, and amino acids through the acetyl CoA and tricarboxylic acid cycles. As mentioned above, the lactic acid contents of the PA and LP + PA treatments were greater than in CK, and the pH was the lowest among all treatments, which might be related to the up-regulation of this pathway. In addition, the increase in the metabolism of ascorbate and aldarate, C5-branched dibasic acid and inositol phosphate, and glyoxylate and dicarboxylate suggests the consumption of sugar (Yin et al., 2022).

Spearman's correlation tested the relationships between microbial communities at the genus level and nutrients and fermentation characteristics of hybrid *Pennisetum* silage. *Lactococcus* correlated positively with ADF ($r = 0.44$, $p < 0.05$), and negatively with CP ($r = -0.53$, $p < 0.01$) (Figure 7). Moreover, WSC correlated positively with *Pseudomonas* ($r = 0.68$, $p < 0.001$) and *Delftia* ($r = 0.66$, $p < 0.001$), and negatively with *Pediococcus*

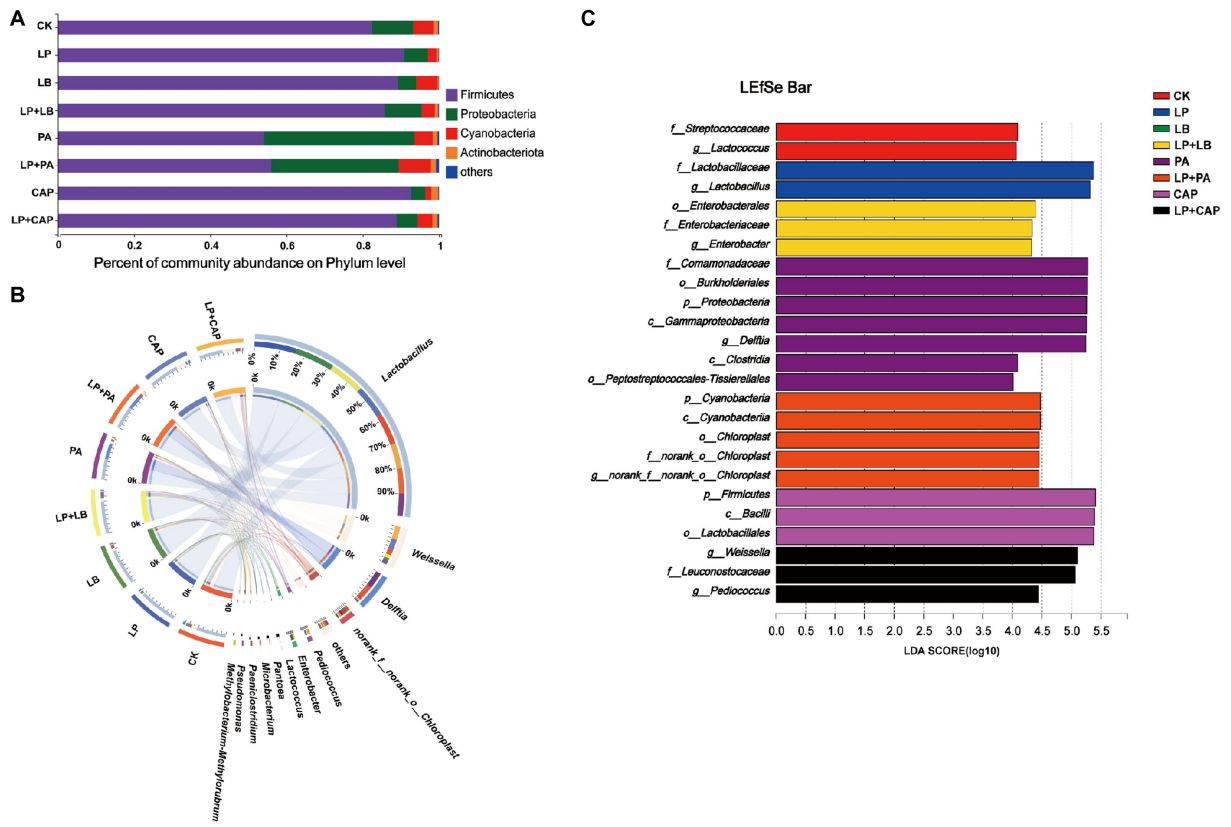

FIGURE 5
Bacterial abundance at phylum **(A)** and genus **(B)** levels of hybrid *Pennisetum* silage. Linear discriminant analysis effect size (LEfSe) of hybrid *Pennisetum* silage treated with different additives **(C)**. CK, distilled water; LP, *L. plantarum*; LB, *L. buchneri*; LP+LB, *L. plantarum* and *L. buchneri*; PA, propionic acid; LP+PA, *L. plantarum* and propionic acid; CAP, calcium propionate; LP+CAP, *L. plantarum* and calcium propionate.

($r = -0.85$, $p < 0.001$) and *Weissella* ($r = -0.76$, $p < 0.001$). WSC could be used by microorganisms as a substrate, so it was not surprising that WSC correlated negatively with microorganisms (Zheng et al., 2022). In addition, *Enterobacter* ($r = -0.52$, $p < 0.01$), *Pantoea* ($r = -0.67$, $p < 0.001$), and *Microbacterium* ($r = -0.65$, $p < 0.001$) were correlated negatively with CP. Previous studies had shown that certain *Enterobacter* were proteolytic, which could cause the loss of protein from silage (Yang et al., 2020). In this study, *Weissella* was correlated negatively with lactic acid content ($r = -0.65$, $p < 0.001$) and positively with acetic acid ($r = 0.62$, $p < 0.01$), propionic acid ($r = 0.68$, $p < 0.001$), butyric acid ($r = 0.87$, $p < 0.001$), pH ($r = 0.87$, $p < 0.001$) and NH_3-N:TN ($r = 0.76$, $p < 0.001$). Results were consistent with those of Zheng et al. (2022) who reported that pH and the concentration of acetic acid were correlated positively with the abundance of *Weissella*. Furthermore, *Pediococcus* was correlated positively with NH_3-N:TN ($r = 0.83$, $p < 0.001$) and acetic acid ($r = 0.86$, $p < 0.001$). Several studies indicated that *Pediococcus* possessed probiotics properties (Fugaban et al., 2021; Jiang et al., 2021). According to Yang et al. (2019), *Pediococcus* plays a major role in the initial stage of ensiling by helping to create an anaerobic environment that is suitable for LAB growth. *Microbacterium* might reduce silage quality as this bacterium correlated negatively with lactic acid and CP and positively with pH, butyric acid and NH_3-N:TN. *Microbacterium* is a gram-positive bacterium belonging to Actinobacteria, and is generally isolated from terrestrial and aquatic ecosystems (Marchant et al., 2006). It was reported *Microbacterium* had the ability to degrade hydrocarbons and complex polysaccharides (Cordovez et al., 2018). However, its specific role in silage production warrants further research.

4. Conclusion

Additives affected the quality of hybrid *Pennisetum* silage by increasing crude protein and lactic acid contents and inhibiting the growth of undesirable bacteria. Principal component analysis revealed that the silage quality of the PA, LP + PA and LB + LP treatments ranked as the top three of the seven treatments. The synergistic effect of *L. plantarum* combined with *L. buchneri* improved the quality of silage more so than any one of them alone. The addition of propionic acid was very beneficial, as it increased the relative abundance of *Delftia*, inhibited the activity of *Enterobacter*, maintained pH, butyric acid and the NH_3-N:TN ratio at low levels and reduced the contents of NDF and ADF. In summary, *L. plantarum*, *L. buchneri*, propionic acid, calcium propionate and their combinations could improve the silage of hybrid *Pennisetum*, which would mitigate the shortage of feed for livestock.

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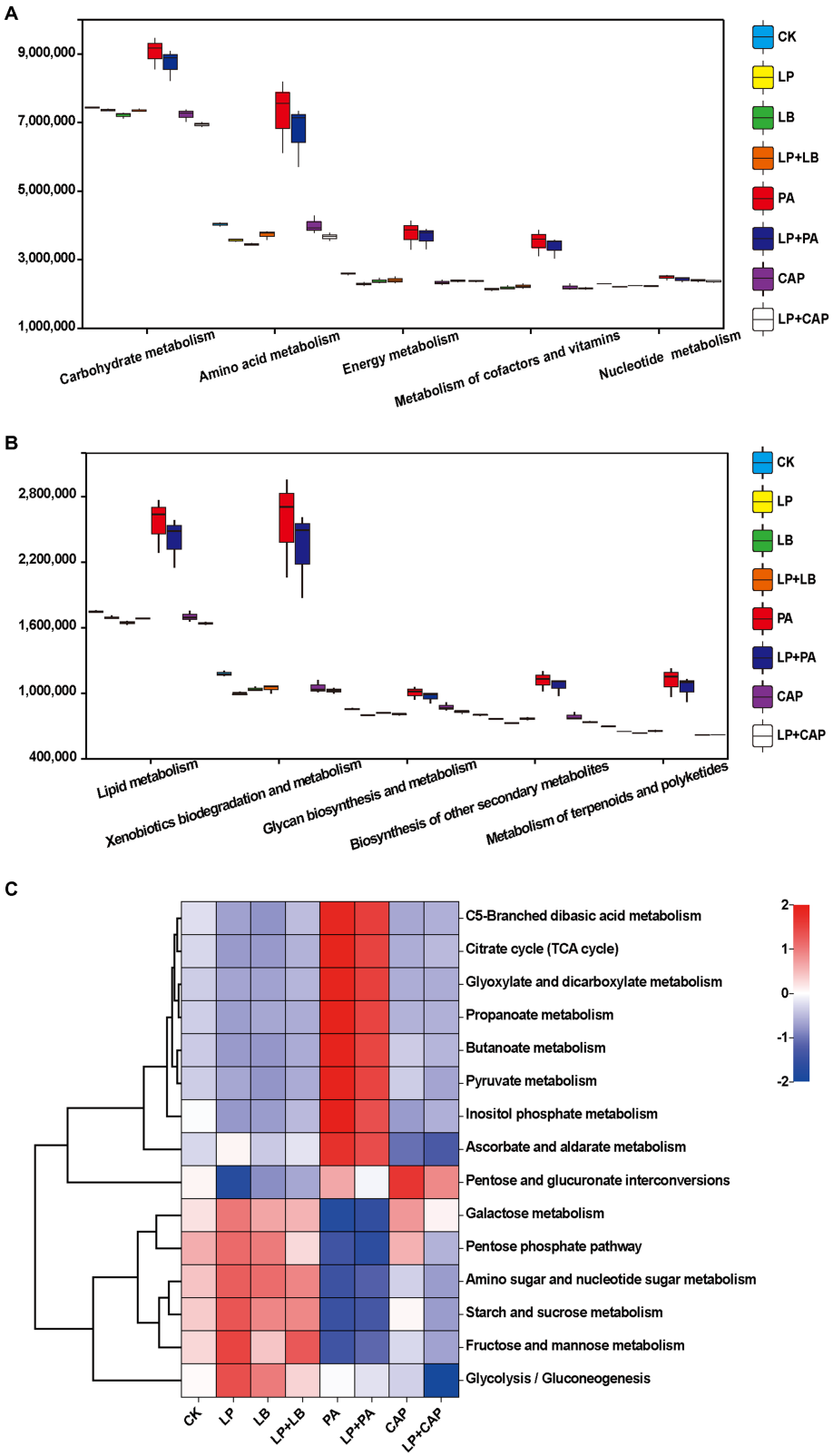

FIGURE 6
Prediction of microbial metabolic functions of hybrid *Pennisetum* silage. The second-level **(A,B)** and third-level **(C)** classification of microbial community metabolic functions. CK, distilled water; LP, *L. plantarum*; LB, *L. buchneri*; LP+LB, *L. plantarum* and *L. buchneri*; PA, propionic acid; LP+PA, *L. plantarum* and propionic acid; CAP, calcium propionate; LP+CAP, *L. plantarum* and calcium propionate.

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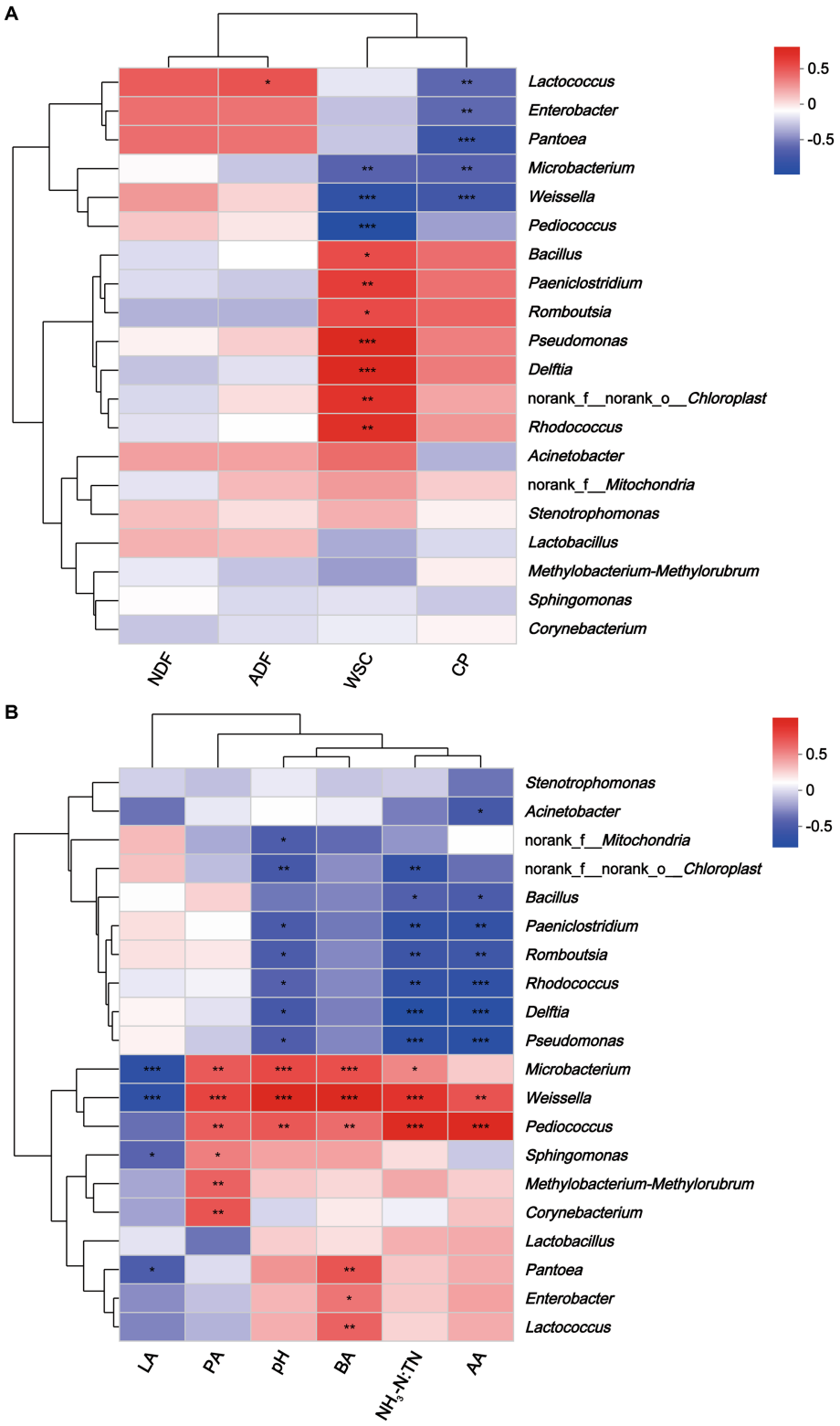

FIGURE 7
Heatmap of Spearman correlations between nutritional composition **(A)**, fermentation parameters **(B)** and bacterial abundance of hybrid *Pennisetum* silage. *p<0.05; **p<0.01, ***p<0.001. NDF, neutral detergent fiber; ADF, acid detergent fiber; WSC, water-soluble carbohydrate; CP, crude protein; LA, lactic acid; AA, acidic acid; PA, propionic acid; BA, butyric acid; NH_3-N:TN, ammonia nitrogen:total nitrogen ratio.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/, PRJNA946341.

Author contributions

QF, JuZ, and WL: writing—original draft, formal analysis, and data curation. AD and CG: writing—review and editing. YZ: formal analysis and data curation. FY and JiZ: funding acquisition, supervision, and writing—review and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

Ali, M. F., and Tahir, M. (2021). An overview on the factors affecting water-soluble carbohydrates concentration during ensiling of silage. *J. Plant Environ.* 3, 63–80. doi: 10.33687/jpe.003.01.3702

Alvarez, A., Saez, J. M., Davila Costa, J. S., Colin, V. L., Fuentes, M. S., Cuozzo, S. A., et al. (2017). Actinobacteria: current research and perspectives for bioremediation of pesticides and heavy metals. *Chemosphere* 166, 41–62. doi: 10.1016/j.chemosphere.2016.09.070

Arthur Thomas, T. (1977). An automated procedure for the determination of soluble carbohydrates in herbage. *J. Sci. Food Agr.* 28, 639–642. doi: 10.1002/jsfa.2740280711

Bai, J., Ding, Z., Su, R., Wang, M., Cheng, M., Xie, D., et al. (2022). Storage temperature is more effective than lactic acid bacteria inoculations in manipulating fermentation and bacterial community diversity, co-occurrence and functionality of the whole-plant corn silage. *Microbiol. Spectr.* 10:e0010122. doi: 10.1128/spectrum.00101-22

Bao, X., Guo, G., Huo, W., Li, Q., Xu, Q., and Chen, L. (2023). Ensiling pretreatment fortified with laccase and microbial inoculants enhances biomass preservation and bioethanol production of alfalfa stems. *Sci. Total Environ.* 857:159442. doi: 10.1016/j.scitotenv.2022.159442

Bhat, S. V., Maughan, H., Cameron, A. D. S., and Yost, C. K. (2022). Phylogenomic analysis of the genus Delftia reveals distinct major lineages with ecological specializations. *Microb. Genom.* 8:mgen000864. doi: 10.1099/mgen.0.000864

Cai, C., Wang, L., Wang, G., Hao, J., Bai, X., Wang, Z., et al. (2020). Effects of dry explosion pretreatment on physicochemical and fuel properties of hybrid Pennisetum (*Pennisetum americanum* × *P. purpureum*). *Bioresour. Technol.* 297:122508. doi: 10.1016/j.biortech.2019.122508

Carvalho, B. F., Ávila, C. L. S., Pinto, J. C., Pereira, M. N., and Schwan, R. F. (2012). Effects of propionic acid and *Lactobacillus buchneri* (UFLA SIL 72) addition on fermentative and microbiological characteristics of sugar cane silage treated with and without calcium oxide. *Grass Forage Sci.* 67, 462–471. doi: 10.1111/j.1365-2494.2012.00863.x

Chen, L., Guo, G., Yuan, X., Zhang, J., Li, J., and Shao, T. (2016). Effects of applying molasses, lactic acid bacteria and propionic acid on fermentation quality, aerobic stability and in vitro gas production of total mixed ration silage prepared with oat–common vetch intercrop on the Tibetan Plateau. *J. Sci. Food Agric.* 96, 1678–1685. doi: 10.1002/jsfa.7271

Cordovez, V., Schop, S., Hordijk, K., Dupré de Boulois, H., Coppens, F., Hanssen, I., et al. (2018). Priming of plant growth promotion by volatiles of root-associated *Microbacterium* spp. *Appl. Environ. Microbiol.* 84, e01865–e01818. doi: 10.1128/AEM.01865-18

Dai, T., Dong, D., Wang, S., Zong, C., Yin, X., Xu, G., et al. (2022). Assessment of organic acid salts on fermentation quality, aerobic stability, and in vitro rumen digestibility of total mixed ration silage. *Trop. Anim. Health Prod.* 54:261. doi: 10.1007/s11250-022-03249-w

Dong, Z., Li, J., Wang, S., Dong, D., and Shao, T. (2022a). Diurnal variation of epiphytic microbiota: an unignorable factor affecting the anaerobic fermentation characteristics of sorghum-sudangrass hybrid silage. *Microbiol. Spectr.* 11:e0340422. doi: 10.1128/spectrum.03404-22

Dong, Z., Li, J., Wang, S., Dong, D., and Shao, T. (2022b). Time of day for harvest affects the fermentation parameters, bacterial community, and metabolic characteristics of sorghum-sudangrass hybrid silage. *mSphere* 7:e0016822. doi: 10.1128/msphere.00168-22

Dong, Z., Shao, T., Li, J., Yang, L., and Yuan, X. (2020). Effect of alfalfa microbiota on fermentation quality and bacterial community succession in fresh or sterile Napier grass silages. *J. Dairy Sci.* 103, 4288–4301. doi: 10.3168/jds.2019-16961

Dong, Z., Yuan, X., Wen, A., Desta, S. T., and Shao, T. (2017). Effects of calcium propionate on the fermentation quality and aerobic stability of alfalfa silage. *Asian Australas J. Anim. Sci.* 30, 1278–1284. doi: 10.5713/ajas.16.0956

Drouin, P., Tremblay, J., Renaud, J., and Apper, E. (2021). Microbiota succession during aerobic stability of maize silage inoculated with *Lentilactobacillus buchneri* NCIMB 40788 and *Lentilactobacillus hilgardii* CNCM-I-4785. *Microbiol. Open* 10:e1153. doi: 10.1002/mbo3.1153

Du, E., Zhao, N., Guo, W., Fan, Q., Wei, J., and Xu, Z. (2022). Effects of konjac flour and *Lactiplantibacillus plantarum* on fermentation quality, aerobic stability, and microbial community of high-moisture forage rape silages. *Fermentation* 8:348. doi: 10.3390/fermentation8080348

Elshamy, A. I., Nassar, M. I., Mohamed, T. A., and Hegazy, M. E. (2016). Chemical and biological profile of Cespitularia species: a mini review. *J. Adv. Res.* 7, 209–224. doi: 10.1016/j.jare.2015.07.003

Fan, X., Xie, Z., Cheng, Q., Li, M., Long, J., Lei, Y., et al. (2022). Fermentation quality, bacterial community, and predicted functional profiles in silage prepared with alfalfa, perennial ryegrass and their mixture in the karst region. *Front. Microbiol.* 13:1062515. doi: 10.3389/fmicb.2022.1062515

Feng, Q., Shi, W., Chen, S., Degen, A. A., Qi, Y., Yang, F., et al. (2022). Addition of organic acids and *Lactobacillus acidophilus* to the leguminous forage *Chamaecrista rotundifolia* improved the quality and decreased harmful bacteria of the silage. *Animals* 12:2260. doi: 10.3390/ani12172260

Ferrero, F., Tabacco, E., and Borreani, G. (2021). *Lentilactobacillus hilgardii* inoculum, dry matter contents at harvest and length of conservation affect fermentation characteristics and aerobic stability of corn silage. *Front. Microbiol.* 12:675563. doi: 10.3389/fmicb.2021.675563

Filya, I. (2003). Nutritive value of whole crop wheat silage harvested at three stages of maturity. *Anim. Feed Sci. Tech.* 103, 85–95. doi: 10.1016/S0377-8401(02)00284-5

Fu, T., and Diao, Q. (2007). The effect of propionic acid on the fermentation and aerobic stability of maize silage. *J. Anim. Feed Sci.* 16, 48–53. doi: 10.22358/jafs/74417/2007

Fu, Z., Sun, L., Hou, M., Hao, J., Lu, Q., Liu, T., et al. (2022). Effects of different harvest frequencies on microbial community and metabolomic properties of annual ryegrass silage. *Front. Microbiol.* 13:971449. doi: 10.3389/fmicb.2022.971449

Fugaban, J. I. I., Vazquez Bucheli, J. E., Kim, B., Holzapfel, W. H., and Todorov, S. D. (2021). Safety and beneficial properties of bacteriocinogenic *Pediococcus acidilactici* and *Pediococcus pentosaceus* isolated from silage. *Lett. Appl. Microbiol.* 73, 725–734. doi: 10.1111/lam.13562

Gallo, A., Moschini, M., Cerioli, C., and Masoero, F. (2013). Use of principal component analysis to classify forages and predict their calculated energy content. *Animal* 7, 930–939. doi: 10.1017/S1751731112002467

Gheller, L. S., Ghizzi, L. G., Takiya, C. S., Grigoletto, N. T., Silva, T. B., Marques, J. A., et al. (2021). Different organic acid preparations on fermentation and microbiological profile, chemical composition, and aerobic stability of whole-plant corn silage. *Anim. Feed Sci. Tech.* 281:115083. doi: 10.1016/j.anifeedsci.2021.115083

Guo, L., Yao, D., Li, D., Lin, Y., Bureenok, S., Ni, K., et al. (2020). Effects of lactic acid bacteria isolated from rumen fluid and feces of dairy cows on fermentation quality, microbial community, and in vitro digestibility of alfalfa silage. *Front. Microbiol.* 10:2998. doi: 10.3389/fmicb.2019.02998

Haas, B. J., Gevers, D., Earl, A. M., Feldgarden, M., Ward, D. V., Giannoukos, G., et al. (2011). Chimeric 16S rRNA sequence formation and detection in sanger and 454-pyrosequenced PCR amplicons. *Genome Res.* 21, 494–504. doi: 10.1101/gr.112730.110

He, L., Li, S., Wang, C., Chen, X., and Zhang, Q. (2021). Effects of vanillic acid on dynamic fermentation parameter, nitrogen distribution, bacterial community, and enzymatic hydrolysis of stylo silage. *Front. Microbiol.* 12:690801. doi: 10.3389/fmicb.2021.690801

He, Q., Zhou, W., Chen, X., and Zhang, Q. (2021). Chemical and bacterial composition of *Broussonetia papyrifera* leaves ensiled at two ensiling densities with or without *Lactobacillus plantarum*. *J. Clean. Prod.* 329:129792. doi: 10.1016/j.jclepro.2021.129792

He, L., Zhou, W., Xing, Y., Pian, R., Chen, X., and Zhang, Q. (2020). Improving the quality of rice straw silage with *Moringa oleifera* leaves and propionic acid: fermentation, nutrition, aerobic stability and microbial communities. *Bioresour. Technol.* 299:122579. doi: 10.1016/j.biortech.2019.122579

Hu, W., Schmidt, R. J., McDonell, E. E., Klingerman, C. M., and Kung, L. (2009). The effect of Lactobacillus buchneri 40788 or *Lactobacillus plantarum* MTD-1 on the fermentation and aerobic stability of corn silages ensiled at two dry matter contents. *J. Dairy Sci.* 92, 3907–3914. doi: 10.3168/jds.2008-1788

Huang, Y., Liang, L., Dai, S., Wu, C., Chen, C., and Hao, J. (2021). Effect of different regions and ensiling periods on fermentation quality and the bacterial community of whole-plant maize silage. *Front. Microbiol.* 12:743695. doi: 10.3389/fmicb.2021.743695

Jaipolsaen, N., Sangsritavong, S., Uengwetwanit, T., Angthong, P., Plengvidhya, V., Rungrassamee, W., et al. (2021). Comparison of the effects of microbial inoculants on fermentation quality and microbiota in Napier grass (*Pennisetum purpureum*) and corn (*Zea mays* L.) silage. *Front. Microbiol.* 12:784535. doi: 10.3389/fmicb.2021.784535

Jiang, S., Cai, L., Lv, L., and Li, L. (2021). *Pediococcus pentosaceus*, a future additive or probiotic candidate. *Microb. Cell Factories* 20:45. doi: 10.1186/s12934-021-01537-y

Jiang, F., Cheng, H., Liu, D., Wei, C., An, W., Wang, Y., et al. (2020). Treatment of whole-plant corn silage with lactic acid bacteria and organic acid enhances quality by elevating acid content, reducing pH, and inhibiting undesirable microorganisms. *Front. Microbiol.* 11:593088. doi: 10.3389/fmicb.2020.593088

Knief, C., Delmotte, N., Chaffron, S., Stark, M., Innerebner, G., Von Wassmann, R., et al. (2012). Metaproteogenomic analysis of microbial communities in the phyllosphere and rhizosphere of rice. *ISME J.* 6, 1378–1390. doi: 10.1038/ismej.2011.192

Kung, L. Jr., Shaver, R., Grant, R., and Schmidt, R. (2018). Silage review: interpretation of chemical, microbial, and organoleptic components of silages. *J. Dairy Sci.* 101, 4020–4033. doi: 10.3168/jds.2017-13909

Langille, M. G., Zaneveld, J., Caporaso, J. G., McDonald, D., Knights, D., Reyes, J. A., et al. (2013). Predictive functional profiling of microbial communities using 16SrRNA marker gene sequences. *Nat. Biotechnol.* 31, 814–821. doi: 10.1038/nbt.2676

Li, D., Ni, K., Zhang, Y., Lin, Y., and Yang, F. (2018). Influence of lactic acid bacteria, cellulase, cellulase-producing Bacillus pumilus and their combinations on alfalfa silage quality. *J. Integr. Agr.* 17, 2768–2782. doi: 10.1016/S2095-3119(18)62060-X

Li, D., Ni, K., Zhang, Y., Lin, Y., and Yang, F. (2019). Fermentation on characteristics, chemical composition and microbial community of tropical forage silage under different temperatures. *Asian Australas J. Anim. Sci.* 32, 665–674. doi: 10.5713/ajas.18.0085

Li, M., Yu, Q., Xu, J., Sun, H., Cheng, Q., Xie, Y., et al. (2022). Effect of different organic acid additives on the fermentation quality and bacterial community of paper mulberry (*Broussonetia papyrifera*) silage. *Front. Microbiol.* 13:1038549. doi: 10.3389/fmicb.2022.1038549

Li, M., Zi, X., Tang, J., Zhou, H., and Cai, Y. (2019). Silage fermentation, chemical composition and ruminal degradation of king grass, cassava foliage and their mixture. *Grassl. Sci.* 65, 210–215. doi: 10.1111/grs.12235

Ling, W., Zhang, L., Feng, Q., Degen, A. A., Li, J., Qi, Y., et al. (2022). Effects of different additives on fermentation quality, microbial communities, and rumen degradation of alfalfa silage. *Fermentation* 8:660. doi: 10.3390/fermentation8110660

Liu, B., Huan, H., Gu, H., Xu, N., Shen, Q., and Ding, C. (2019). Dynamics of a microbial community during ensiling and upon aerobic exposure in lactic acid bacteria inoculation-treated and untreated barley silages. *Bioresour. Technol.* 273, 212–219. doi: 10.1016/j.biortech.2018.10.041

Liu, Y., Tie, B., Li, Y., Lei, M., Wei, X., Liu, X., et al. (2018). Inoculation of soil with cadmium-resistant bacterium *Delftia* sp. B9 reduces cadmium accumulation in rice (*Oryza sativa* L.) grains. *Ecotoxicol. Environ. Saf.* 163, 223–229. doi: 10.1016/j.ecoenv.2018.07.081

Lv, J., Fang, X., Feng, G., Zhang, G., Zhao, C., Zhang, Y., et al. (2020). Effects of sodium formate and calcium propionate additives on the fermentation quality and microbial community of wet brewers grains after short-term storage. *Animals* 10:1608. doi: 10.3390/ani10091608

Magnusson, J., and Schnürer, J. (2001). *Lactobacillus coryniformis* subsp. coryniformis strain Si3 produces a broad-spectrum proteinaceous antifungal compound. *Appl. Environ. Microbiol.* 67, 1–5. doi: 10.1128/AEM.67.1.1-5.2001

Marchant, R., Sharkey, F. H., Banat, I. M., Rahman, T. J., and Perfumo, A. (2006). The degradation of n-hexadecane in soil by thermophilic geobacilli. *FEMS Microbiol. Ecol.* 56, 44–54. doi: 10.1111/j.1574-6941.2006.00061.x

Mu, L., Xie, Z., Hu, L., Chen, G., and Zhang, Z. (2021). *Lactobacillus plantarum* and molasses alter dynamic chemical composition, microbial community, and aerobic stability of mixed (amaranth and rice straw) silage. *J. Sci. Food Agric.* 101, 5225–5235. doi: 10.1002/jsfa.11171

Muck, R. E., Nadeau, E. M. G., McAllister, T. A., Contreras-Govea, F. E., Santos, M. C., and Kung, L. Jr. (2018). Silage review: recent advances and future uses of silage additives. *J. Dairy Sci.* 101, 3980–4000. doi: 10.3168/jds.2017-13839

Ni, K., Minh, T. T., Tu, T. T., Tsuruta, T., Pang, H., and Nishino, N. (2017a). Comparative microbiota assessment of wilted Italian ryegrass, whole crop corn, and wilted alfalfa silage using denaturing gradient gel electrophoresis and next-generation sequencing. *Appl. Microbiol. Biotechnol.* 101, 1385–1394. doi: 10.1007/s00253-016-7900-2

Ni, K., Wang, F., Zhu, B., Yang, J., Zhou, G., Pan, Y., et al. (2017b). Effects of lactic acid bacteria and molasses additives on the microbial community and fermentation quality of soybean silage. *Bioresour. Technol.* 238, 706–715. doi: 10.1016/j.biortech.2017.04.055

Ogunade, I. M., Jiang, Y., Kim, D. H., Cervantes, A. A. P., Arriola, K. G., Vyas, D., et al. (2017). Fate of *Escherichia coli* O157:H7 and bacterial diversity in corn silage contaminated with the pathogen and treated with chemical or microbial additives. *J. Dairy Sci.* 100, 1780–1794. doi: 10.3168/jds.2016-11745

Ogunade, I. M., Jiang, Y., Pech Cervantes, A. A., Kim, D. H., Oliveira, A. S., Vyas, D., et al. (2018). Bacterial diversity and composition of alfalfa silage as analyzed by Illumina MiSeq sequencing: effects of *Escherichia coli* O157:H7 and silage additives. *J. Dairy Sci.* 101, 2048–2059. doi: 10.3168/jds.2017-12876

Puntillo, M., Gaggiotti, M., Oteiza, J. M., Binetti, A., Massera, A., and Vinderola, G. (2020). Potential of lactic acid bacteria isolated from different forages as silage inoculants for improving fermentation quality and aerobic stability. *Front. Microbiol.* 11:586716. doi: 10.3389/fmicb.2020.586716

Queiroz, O. C. M., Ogunade, I. M., Weinberg, Z., and Adesogan, A. T. (2018). Silage review: foodborne pathogens in silage and their mitigation by silage additives. *J. Dairy Sci.* 101, 4132–4142. doi: 10.3168/jds.2017-13901

Rabelo, C. H. S., Härter, C. J., Ávila, C. L. D. S., and Reis, R. A. (2019). Meta-analysis of the effects of *Lactobacillus plantarum* and *Lactobacillus buchneri* on fermentation, chemical composition and aerobic stability of sugarcane silage. *Grassl. Sci.* 65, 3–12. doi: 10.1111/grs.12215

Ren, H., Feng, Y., Pei, J., Li, J., Wang, Z., Fu, S., et al. (2020). Effects of *Lactobacillus plantarum* additive and temperature on the ensiling quality and microbial community dynamics of cauliflower leaf silages. *Bioresour. Technol.* 307:123238. doi: 10.1016/j.biortech.2020.123238

Ren, H., Sun, W., Yan, Z., Zhang, Y., Wang, Z., Song, B., et al. (2021). Bioaugmentation of sweet sorghum ensiling with rumen fluid: fermentation characteristics, chemical composition, microbial community, and enzymatic digestibility of silages. *J. Clean. Prod.* 294:126308. doi: 10.1016/j.jclepro.2021.126308

Ren, H., Wang, C., Fan, W., Zhang, B., Li, Z., and Li, D. (2018). Effects of formic or acetic acid on the storage quality of mixed air-dried corn stover and cabbage waste, and microbial community analysis. *Food Technol. Biotechnol.* 56, 71–82. doi: 10.17113/ftb.56.01.18.5455

Romero, J. J., Zhao, Y., Balseca-Paredes, M. A., Tiezzi, F., Gutierrez-Rodriguez, E., and Castillo, M. S. (2017). Laboratory silo type and inoculation effects on nutritional composition, fermentation, and bacterial and fungal communities of oat silage. *J. Dairy Sci.* 100, 1812–1828. doi: 10.3168/jds.2016-11642

Sandström, V., Chrysafi, A., Lamminen, M., Troell, M., Jalava, M., Piipponen, J., et al. (2022). Food system by-products upcycled in livestock and aquaculture feeds can increase global food supply. *Nat. Food* 3, 729–740. doi: 10.1038/s43016-022-00589-6

Santos, A. O., Ávila, C. L., Pinto, J. C., Carvalho, B. F., Dias, D. R., and Schwan, R. F. (2016). Fermentative profile and bacterial diversity of corn silages inoculated with new tropical lactic acid bacteria. *J. Appl. Microbiol.* 120, 266–279. doi: 10.1111/jam.12980

Sepehri, A., and Sarrafzadeh, M.-H. (2019). Activity enhancement of ammonia-oxidizing bacteria and nitrite-oxidizing bacteria in activated sludge process: metabolite reduction and CO_2 mitigation intensification process. *Appl. Water Sci.* 9:131. doi: 10.1007/s13201-019-1017-6

Shah, A. A., Wu, J., Qian, C., Liu, Z., Mobashar, M., Tao, Z., et al. (2020). Ensiling of whole-plant hybrid pennisetum with natamycin and *Lactobacillus plantarum* impacts on fermentation characteristics and meta-genomic microbial community at low temperature. *J. Sci. Food Agric.* 100, 3378–3385. doi: 10.1002/jsfa.10371

Song, X., Yue, X., Chen, W., Jiang, H., Han, Y., and Li, X. (2019). Detection of cadmium risk to the photosynthetic performance of hybrid Pennisetum. *Front. Plant Sci.* 10:798. doi: 10.3389/fpls.2019.00798

Sun, L., Bai, C., Xu, H., Na, N., Jiang, Y., Yin, G., et al. (2021). Succession of bacterial community during the initial aerobic, intense fermentation, and stable phases of whole-plant corn silages treated with lactic acid bacteria suspensions prepared from other silages. *Front. Microbiol.* 12:655095. doi: 10.3389/fmicb.2021.655095

Tharangani, R. M. H., Yakun, C., Zhao, L. S., Ma, L., Liu, H. L., Su, S. L., et al. (2021). Corn silage quality index: an index combining milk yield, silage nutritional and fermentation parameters. *Anim. Feed Sci. Tech.* 273:114817. doi: 10.1016/j.anifeedsci.2021.114817

Tian, J., Lai, D., and Zhou, L. (2017). Secondary metabolites from acremonium fungi: diverse structures and bioactivities. *Mini Rev. Med. Chem.* 17, 603–632. doi: 10.2174/1389557516666160914194134

Tian, J., Yin, X., and Zhang, J. (2022). Effects of wilting during a cloudy day and storage temperature on the fermentation quality and microbial community of Napier grass silage. *J. Sci. Food Agric.* 102, 4384–4391. doi: 10.1002/jsfa.11792

Tian, H., Zhu, Y., Dai, M., Li, T., Guo, Y., Deng, M., et al. (2022). Additives altered bacterial communities and metabolic profiles in silage hybrid Pennisetum. *Front. Microbiol.* 12:770728. doi: 10.3389/fmicb.2021.770728

Van Soest, P. J., Robertson, J. B., and Lewis, B. A. (1991). Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *J. Dairy Sci.* 74, 3583–3597. doi: 10.3168/jds.S0022-0302(91)78551-2

Wang, S., Li, J., Zhao, J., Dong, Z., Dong, D., and Shao, T. (2022). Dynamics of the bacterial communities and predicted functional profiles in wilted alfalfa silage. *J. Appl. Microbiol.* 132, 2613–2624. doi: 10.1111/jam.15417

Wang, S., Sun, Y., Zhao, J., Dong, Z., Li, J., Nazar, M., et al. (2020). Assessment of inoculating various epiphytic microbiota on fermentative profile and microbial community dynamics in sterile Italian ryegrass. *J. Appl. Microbiol.* 129, 509–520. doi: 10.1111/jam.14636

Wang, N., Xiong, Y., Wang, X., Guo, L., Lin, Y., Ni, K., et al. (2022). Effects of *Lactobacillus plantarum* on fermentation quality and anti-nutritional factors of paper mulberry silage. *Fermentation* 8:144. doi: 10.3390/fermentation8040144

Wang, M., Yang, C., Jia, L., and Yu, K. (2014). Effect of *Lactobacillus buchneri* and *Lactobacillus plantarum* on the fermentation characteristics and aerobic stability of whipgrass silage in laboratory silos. *Grassl. Sci.* 60, 233–239. doi: 10.1111/grs.12065

Wen, A., Yuan, X., Wang, J., Desta, S. T., and Shao, T. (2017). Effects of four short-chain fatty acids or salts on dynamics of fermentation and microbial characteristics of alfalfa silage. *Anim. Feed Sci. Tech.* 223, 141–148. doi: 10.1016/j.anifeedsci.2016.11.017

Wu, P., Li, L., Jiang, J., Sun, Y., Yuan, Z., Feng, X., et al. (2020). Effects of fermentative and non-fermentative additives on silage quality and anaerobic digestion performance of *Pennisetum purpureum*. *Bioresour. Technol.* 297:122425. doi: 10.1016/j.biortech.2019.122425

Xian, Z., Wu, J., Deng, M., Wang, M., Tian, H., Liu, D., et al. (2022). Effects of cellulase and *Lactiplantibacillus plantarum* on the fermentation parameters, nutrients, and bacterial community in *Cassia alata* silage. *Front. Microbiol.* 13:926065. doi: 10.3389/fmicb.2022.926065

Xiong, Y., Meng, Q., Jie, G., Tang, X., and Zhang, H. (2017). Effects of relative humidity on animal health and welfare. *J. Integr. Agric.* 16, 1653–1658. doi: 10.1016/S2095-3119(16)61532-0

Xu, D., Ding, Z., Wang, M., Bai, J., Ke, W., Zhang, Y., et al. (2020). Characterization of the microbial community, metabolome and biotransformation of phenolic compounds of sainfoin (*Onobrychis viciifolia*) silage ensiled with or without inoculation of *Lactobacillus plantarum*. *Bioresour. Technol.* 316:123910. doi: 10.1016/j.biortech.2020.123910

Xu, D., Wang, N., Rinne, M., Ke, W., Weinberg, Z. G., Da, M., et al. (2021). The bacterial community and metabolome dynamics and their interactions modulate fermentation process of whole crop corn silage prepared with or without inoculants. *Microb. Biotechnol.* 14, 561–576. doi: 10.1111/1751-7915.13623

Yang, F., Wang, Y., Zhao, S., and Wang, Y. (2020). *Lactobacillus plantarum* inoculants delay spoilage of high moisture alfalfa silages by regulating bacterial community composition. *Front. Microbiol.* 11:1989. doi: 10.3389/fmicb.2020.01989

Yang, L., Yuan, X., Li, J., Dong, Z., and Shao, T. (2019). Dynamics of microbial community and fermentation quality during ensiling of sterile and nonsterile alfalfa with or without *Lactobacillus plantarum* inoculant. *Bioresour. Technol.* 275, 280–287. doi: 10.1016/j.biortech.2018.12.067

Yin, Q., Ji, J., Zhang, R., Duan, Z., Xie, H., Chen, Z., et al. (2022). Identification and verification of key taste components in wampee using widely targeted metabolomics. *Food Chem. X* 13:100261. doi: 10.1016/j.fochx.2022.100261

Yuan, X., Dong, Z., Li, J., and Shao, T. (2020). Microbial community dynamics and their contributions to organic acid production during the early stage of the ensiling of Napier grass (*Pennisetum purpureum*). *Grass Forage Sci.* 75, 37–44. doi: 10.1111/gfs.12455

Zhang, Y., Li, L., Kang, X., Sun, Y., Yuan, Z., Xing, T., et al. (2019). Improving methane production from Pennisetum hybrid by monitoring plant height and ensiling pretreatment. *Renew. Energ.* 141, 57–63. doi: 10.1016/j.renene.2019.03.084

Zhang, G., Wang, J., and Zeng, Y. (2020). A modified rabbit model of tracheal stenosis and a household endoscope. More simplicity and accessibility. *Acta Cir. Bras.* 35:e351104. doi: 10.1590/ACB351104

Zhao, M., Zhang, H., Pan, G., Yin, H., Sun, J., Yu, Z., et al. (2022). Effect of exogenous microorganisms on the fermentation quality, nitrate degradation and bacterial community of sorghum-sudangrass silage. *Front. Microbiol.* 13:1052837. doi: 10.3389/fmicb.2022.1052837

Zheng, Y., Li, M., Xu, J., Sun, H., Cheng, Q., Xie, Y., et al. (2022). Effects of different cutting methods and additives on the fermentation quality and microbial community of *Saccharum arundinaceum* silage. *Front. Microbiol.* 13:999881. doi: 10.3389/fmicb.2022.999881

Zheng, P., Yang, J., Li, Y., Wu, J., Liang, W., Yin, B., et al. (2020). Gut microbial signatures can discriminate unipolar from bipolar depression. *Adv. Sci.* 7:1902862. doi: 10.1002/advs.201902862

Zhou, Y., Chen, Y., Guo, J., Shen, Y., and Yang, J. (2019). The correlations and spatial characteristics of microbiome and silage quality by reusing of citrus waste in a family-scale bunker silo. *J. Clean. Prod.* 226, 407–418. doi: 10.1016/j.jclepro.2019.04.075

Zhu, Y., Xiong, H., Wen, Z., Tian, H., Chen, Y., Wu, L., et al. (2022). Effects of different concentrations of *Lactobacillus plantarum* and *Bacillus licheniformis* on silage quality, in vitro fermentation and microbial community of hybrid *Pennisetum*. *Animals* 12:1752. doi: 10.3390/ani12141752

Zong, C., Wu, Q., Dong, Z., Wu, A., Wu, J., Shao, T., et al. (2022). Recycling deteriorated silage to remove hazardous mycotoxins and produce a value-added product. *J. Hazard. Mater.* 424:127627. doi: 10.1016/j.jhazmat.2021.127627

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Antimicrobial resistance and virulence profiles of staphylococci isolated from clinical bovine mastitis

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Staphylococci, mainly including *Staphylococcus aureus* and coagulase-negative staphylococci (CNS), are one of the most common pathogens causing bovine mastitis worldwide. In this study, we investigated the antimicrobial resistance and virulence profiles of staphylococci from clinical bovine mastitis in Ningxia Hui Autonomous Region of China. Antimicrobial resistance was determined by disc diffusion combined with *E*-test method. Genes of antimicrobial resistance and virulence factors were determined by PCR. A total of 332 staphylococcal isolates were confirmed from 1,519 mastitic milk samples, including 172 *S. aureus* and 160 CNS isolates. Fifteen CNS species were identified, with *S. chromogenes* being the most frequent found (49.4%), followed by *S. equorum* (13.8%). Noticeably, 2 *S. agnetis* isolates were found among the CNS isolates. To our knowledge, this is the first report documenting the presence of *S. agnetis* from bovine mastitis in China. The *S. aureus* and CNS isolates showed high resistance against penicillin, followed by erythromycin and tetracycline. Multidrug resistance was found in 11.6 and 16.3% of the *S. aureus* and CNS isolates, respectively. Resistance to penicillin was attributed to the presence of *bla*Z, erythromycin resistance to *ermC* (alone or combined with *ermB*) and tetracycline resistance to *tet*K (alone or combined with *tetM*). Notably, one *S. equorum* isolate and one *S. saprophyticus* isolate were both methicillin-resistant and *mecA* positive. Additionally, all *S. aureus* isolates carried the adhesin genes *fnbpA*, *clfA*, *clfB*, and *sdrC*, and most of them contained *cna* and *sdrE*. Conversely, only a few of the CNS isolates carried *clf*A, *cna*, and *fnb*A. Regarding toxin genes, all *S. aureus* isolates harbored *hlb*, and most of them were *hlg* positive. The *lukE-lukD*, *lukM*, *sec*, *sed*, *sei*, *sen*, *seo*, *tst*, *seg*, *seh*, and *sej* were also detected with low frequencies. However, no toxin genes were observed in CNS isolates. This study reveals high species diversity of staphylococci from clinical bovine mastitis in Ningxia Hui Autonomous Region of China. The findings for the genetic determinants of antimicrobial resistance and virulence factor provide valuable information for control and prevention of staphylococcal bovine mastitis.

KEYWORDS

***Staphylococcus aureus*, coagulase-negative staphylococci, antimicrobial resistance, virulence, bovine mastitis**

1. Introduction

Bovine mastitis remain the most frequent and costly disease affecting dairy cattle due to its effects on health, welfare, and productivity. Staphylococci, mainly including *Staphylococcus aureus* and coagulase-negative staphylococci (CNS), are one of the most common etiological agents causing bovine mastitis worldwide. *S. aureus* is generally considered major mastitis pathogen and mainly induce clinical mastitis, while CNS have traditionally considered minor mastitis-causing pathogen and usually cause subclinical mastitis (Naranjo-Lucena and Slowey, 2023). Currently, however, reports of subclinical and clinical mastitis cases caused by different CNS species have surfaced largely and they have emerged as an important pathogen (Li et al., 2015; De Visscher et al., 2017; Mahato et al., 2017; Ferreira et al., 2022). Among the group of CNS commonly isolated from bovine milk samples, *S. chromogenes*, *S. epidermidis*, *S. haemolyticus*, *S. simulans*, and *S. xylosus* have been identified as the CNS species most likely to cause mastitis (Leroy et al., 2015).

Mastitis is the most common reason for antimicrobials use to control or prevent staphylococcal infections in dairy cattle. Unfortunately, the selective pressure from antimicrobial agents significantly contributes to the dissemination of resistant strains, which greatly attenuate the therapeutic effectiveness of antimicrobial therapy (Isaac et al., 2017; Ahmed et al., 2020). Antimicrobial resistance of staphylococci are mainly attributed to various resistant determinants, such as genes *blaZ* and *mecA* for β-lactams resistance, *tet*s for tetracyclines resistance, and *erm*s for macrolides resistance. The reduced susceptibility of staphylococci against these commonly used antimicrobials in veterinary medicine might promote their persistence in the dairy herd (Kot et al., 2012; Piessens et al., 2012). Therefore, surveillance of antimicrobial resistance is important to ensure optimal results of antimicrobial use and minimize the risk for development and spread of antimicrobial resistance (Waller et al., 2011).

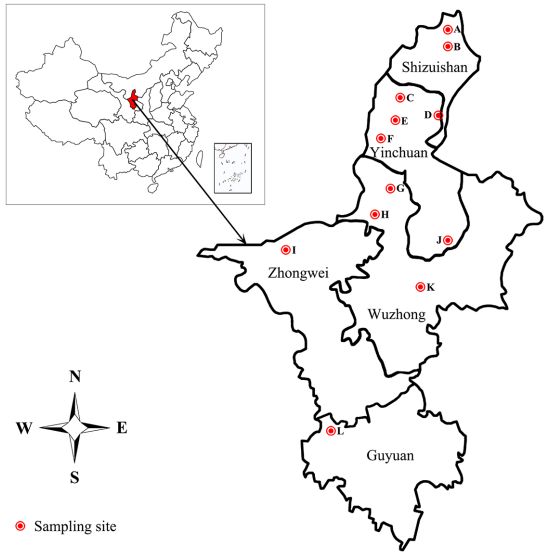

FIGURE 1
Sampling areas of mastitic milk in Ningxia Hui Autonomous Region, China.

Staphylococci possess a wide variety of virulence factors, including different cell wall-associated adhesins and toxins, that facilitate the bacteria to avoid the immune system and contribute to increased severity of infections. Although most of these factors are originally identified in *S. aureus*, they have also been detected in CNS, including the isolates from bovine origin (González-Martín et al., 2020). In the last decades, the virulence factors in *S. aureus* isolates from bovine mastitis had been frequently reported worldwide. However, despite the emergence of CNS as pathogens, the knowledge regarding their virulence as well antimicrobial resistance in CNS is still poorly understood and is not usually identified at species level, especially the isolates from bovine mastitis in China, which makes it difficult to control infection because a great diversity of species have their own characteristics. Thus, this study was designed to investigate the antimicrobial resistance and virulence profiles of staphylococci isolated from clinical bovine mastitis cases in Ningxia Hui Autonomous Region of China.

2. Materials and methods

2.1. Bacterial isolation and identification

The 332 staphylococcal isolates tested in this study were isolated from 1,519 clinical mastitic milk samples from cows from 12 commercial dairy herds located in Ningxia Hui Autonomous Region in China during July 2021 to Aug 2022 (Figure 1; Supplementary Table S1). Bovine udder showing obvious signs, such as oedema, lumps, increase in temperature, hardening or pain, and milk samples showing any visual evidence of abnormality, such as the presence of clots, flakes or blood, were classified as clinical mastitis (Schmidt et al., 2015; Pérez et al., 2020). Before sampling, teats were disinfected using hydrophilic cotton saturated with 70% ethanol. The first milk squirts were discarded, and 5–10 mL of milk was collected in sterile tubes and transported to the laboratory under refrigeration in cool boxes with ice packs. After transportation to the laboratory, 100 μL of milk was inoculated onto blood agar plates supplemented with 5% defibrinated sheep blood and incubated at 37°C for 48 h. Colonies were initially identified as staphylococcal isolates by appearance (shape, color, and size), Gram staining, catalase and coagulase testing. The suspected isolates were further confirmed by PCR and sequencing as described in our previous study (Yang et al., 2019). Briefly, the genomic DNA was extracted through the Bacterial DNA Kit (Omega Bio-Tek, Norcross, GA) according to the manufacturer's instructions.[1] The 16S rRNA gene was amplified by the 16S rDNA Bacterial Identification PCR Kit (Takara, Shiga, Japan) in accordance with the manufacturer's recommendation.[2] The PCR products were purified and sequenced by Sanger sequencing by Sangon Biotech (Shanghai) Co., Ltd. in China. Nucleotide sequences were analyzed with the program NCBI-BLAST.[3] Sequences with 99 to 100% identity to sequences deposited in public domain databases were considered to be positive identification. Confirmed isolates were kept into tryptic soy broth with 20% glycerol at −70°C for molecular testing.

1 https://www.omegabiotek.com/product/e-z-n-a-bacterial-dna-kit/
2 https://www.takarabiomed.com.cn/DownLoad/RR176.pdf
3 http://www.ncbi.nlm.nih.gov

2.2. Antimicrobial susceptibility testing

Antimicrobial susceptibility was determined by disc diffusion method on Mueller-Hinton agar (Oxoid, United Kingdom) according to the protocol of Clinical and Laboratory Standards Institute (CLSI, 2018). The panel of antimicrobial agents (Oxoid) included penicillin (10 U), cefoxitin (30 μg), gentamicin (10 μg), erythromycin (15 μg), tetracycline (30 μg), ciprofloxacin (5 μg), nitrofurantoin (300 μg), trimethoprim-sulfamethoxazole (1.25/23.75 μg), chloramphenicol (30 μg), quinupristin/dalfopristin (15 μg), and linezolid (30 μg). Susceptibility to cefoxitin was used to detect the methicillin-resistance phenotype. The *E*-test strips (Liofilchem, Roseto, Italy) were used to detect the vancomycin (0.016 to 256 μg/mL) susceptibility of the staphylococcal isolates. *S. aureus* ATCC 25923 was used as quality control strain. Multidrug-resistant (MDR) isolates were defined as an isolate being resistant to at least 3 antimicrobial agents belonging to different antimicrobial categories (Magiorakos et al., 2012).

2.3. Detection of antimicrobial resistance and virulence genes

The resistance genes for penicillin (*blaZ*), methicillin (*mecA* and *mecC*), tetracycline (*tetK* and *tetM*), and erythromycin (*ermA*, *ermB*, and *ermC*) were tested by PCR as described previously using specific primer sets in Supplementary Table S2 (Paterson et al., 2012; Yang et al., 2016). Similarly, the adhesins encoding genes *fnbpA* (fibronectin bind protein), *clfA* and *clfB* (clumping factor), *cna* (collagen binding protein), *sdrC*, *sdrD* and *sdrE* (serine-aspartic acid repeat proteins), *bbp* (bone sialoprotein-binding protein), *ebpS* (elastin-binding protein) and *map*/*eap* (major histocompatibility complex class II analogous protein/extracellular adherence protein), as well as toxins encoding genes *sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, *sej*, *sen*, *seo*, and *sem* (staphylococcal enterotoxins), *tst* (toxic shock syndrome toxin-1), *eta* and *etb* (exfoliative toxins), *lukS*/*lukF-PV*, *lukE-lukD*, and *lukM* (leukocidins), *hla*, *hlb*, *hld*, and *hlg* (hemolysins) and *edin* (epidermal cell differentiation inhibitor) were evaluated through PCR (Supplementary Table S2; Jarraud et al., 2002; Peacock et al., 2002). The PCR products were analyzed using 1.0% agarose gel electrophoresis.

3. Results

3.1. Bacterial identification

Overall, 172 *S. aureus* and 160 CNS isolates were identified from the 332 staphylococcal isolates. Among the CNS isolates, a total of 15 species were identified. The predominant species were *S. chromogenes* (49.4%), followed by *S. equorum* (13.8%), *S. succinus* (9.4%), *S. xylosus* (6.3%), *S. simulans* (5.0%), *S. haemolyticus* (4.4%), *S. hominis* (2.5%), *S. saprophyticus* (1.9%), *S. lugdunensis* (1.9%), *S. gallinarum* (1.9%), *S. agnetis* (1.3%), *S. auricularis* (0.6%), *S. cohnii* (0.6%), *S. epidermidis* (0.6%), and *S. hyicus* (0.6%) (Table 1).

3.2. Antimicrobial susceptibility testing

The antimicrobial susceptibility of the staphylococcal isolates against 12 antimicrobial agents were evaluated. The *S. aureus* isolates showed highest resistance rate to penicillin (101, 58.7%), followed by erythromycin (38, 22.1%), tetracycline (26, 15.1%), gentamicin (18, 10.5%), ciprofloxacin (15, 8.7%), and chloramphenicol (10, 5.8%). In addition, 20 (11.6%) *S. aureus* isolates exhibited MDR. Similar to the antimicrobial resistance profile of *S. aureus*, the CNS isolates displayed high resistance to penicillin (114, 71.3%), followed by erythromycin (46, 28.8%), tetracycline (31, 19.4%), gentamicin (15, 9.4%), chloramphenicol (9, 7.9%), ciprofloxacin (4, 2.5%), and cefoxitin (2, 1.3%). Methicillin-resistant phenotype was detected in 1 *S. equorum* and 1 *S. saprophyticus* isolates based on their susceptibility to cefoxitin. Antimicrobial resistance rates varied by CNS species. Multidrug resistance was found in 26 (16.3%) CNS isolates, including *S. chromogenes* (12, 15.2%), *S. equorum* (5, 22.7%), *S. succinus* (1, 6.7%), *S. xylosus* (2, 20.0%), *S. simulans* (1, 12.5%), *S. haemolyticus* (3, 42.9%), *S. saprophyticus* (1, 33.3%), and *S. epidermidis* (1, 100.0%). None of the staphylococcal isolates showed resistance to nitrofurantoin, trimethoprim-sulfamethoxazole, quinupristin/dalfopristin, linezolid or vancomycin in this study (Table 1, Supplementary Table S3).

3.3. Genetic determinants for antimicrobial resistance

The staphylococcal isolates showed higher resistance to penicillin, erythromycin and tetracycline compared to other tested antimicrobial agents in this study. Hence, the resistance encoding genes for these antimicrobial agents as well as methicillin resistant genes *mecA* and *mecC* were tested and shown in Table 2 and Supplementary Table S3. In *S. aureus* isolates, the *blaZ* was detected in 105 (61.0%) isolates. All penicillin-resistant *S. aureus* isolates carried *blaZ*. Besides, 4 penicillin-susceptible isolates also contained this gene. The *tetK* and *tetM* were determined in 21 (12.2%) and 17 (9.9%) isolates, respectively. All *tetK* positive (alone or combined with *tetM*) isolates showed resistance to tetracycline. Five tetracycline-resistant *S. aureus* isolates were negative for *tetK* or *tetM*. Additionally, genes *ermC* and *ermB* were found in 38 (22.1%) and 23 (13.4%) *S. aureus* isolates, respectively. And all erythromycin-resistant isolates harbored *ermC* alone or in combination with *ermB*. None of the isolates were positive for the *mecA*, *mecC* or *ermA*.

Among the 160 CNS isolates evaluated, the *blaZ* was found in 111 (69.4%) isolates and all of them showed resistance to penicillin. Two *S. equorum* and 1 *S. simulans* that were resistant against penicillin were negative for *blaZ*. Importantly, both of the methicillin-resistant isolates, 1 *S. equorum* and 1 *S. saprophyticus*, carried *mecA*. The *tetK* and *tetM* were determined in 28 (17.5%) and 20 (12.5%) CNS isolates, respectively. All *tetK*-carrying (alone or combined with *tetM*) isolates showed resistance to tetracycline. Three tetracycline-resistant isolates, including 1 *S. chromogenes*, 1 *S. haemolyticus* and 1 *S. saprophyticus*, did not harbored *tetK* or *tetM*. Moreover, *ermC* and *ermB* were detected in 45 (28.1%) and 27 (16.9%) CNS isolates, respectively. All *ermC*-carrying (alone or combined with *ermB*) isolates displayed resistance to erythromycin. One erythromycin-resistant *S. equorum*

TABLE 1 Distribution and the antimicrobial resistance of staphylococci isolated from clinical bovine mastitis[a].

Species (No./%)		Antimicrobial resistance (No./%)												MDR (No./%)
		PEN	FOX	VAN	GEN	ERM	TET	CIP	NIT	COT	CHL	QDA	LZD	
S. aureus	172/100.0	101/58.7	0/0.0	0/0.0	18/10.5	38/22.1	26/15.1	15/8.7	0/0.0	0/0.0	10/5.8	0/0.0	0/0.0	20/11.6
S. chromogenes	79/49.4	60/75.9	0/0.0	0/0.0	2/2.5	25/31.6	14/17.7	1/1.3	0/0.0	0/0.0	2/2.5	0/0.0	0/0.0	12/15.2
S. equorum	22/13.8	18/81.8	1/4.5	0/0.0	2/9.1	4/18.2	6/27.3	0/0.0	0/0.0	0/0.0	1/4.5	0/0.0	0/0.0	5/22.7
S. succinus	15/9.4	9/60.0	0/0.0	0/0.0	0/0.0	4/26.7	2/13.3	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	1/6.7
S. xylosus	10/6.3	9/90.0	0/0.0	0/0.0	1/10.0	3/30.0	2/20.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	2/20.0
S. simulans	8/5.0	5/62.5	0/0.0	0/0.0	4/50.0	2/25.0	1/12.5	1/12.5	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	1/12.5
S. haemolyticus	7/4.4	5/71.4	0/0.0	0/0.0	4/57.1	3/42.9	2/28.6	2/28.6	0/0.0	0/0.0	2/28.6	0/0.0	0/0.0	3/42.9
S. hominis	4/2.5	2/50.0	0/0.0	0/0.0	0/0.0	1/25.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0
S. saprophyticus	3/1.9	2/66.7	1/33.3	0/0.0	1/33.3	1/33.3	1/33.3	0/0.0	0/0.0	0/0.0	1/33.3	0/0.0	0/0.0	1/33.3
S. lugdunensis	3/1.9	1/33.3	0/0.0	0/0.0	0/0.0	1/33.3	1/33.3	0/0.0	0/0.0	0/0.0	2/66.7	0/0.0	0/0.0	0/0.0
S. gallinarum	3/1.9	1/33.3	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0
S. agnetis	2/1.3	1/50.0	0/0.0	0/0.0	0/0.0	1/50.0	1/50.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0
S. auricularis	1/0.6	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0
S. cohnii	1/0.6	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0
S. epidermidis	1/0.6	1/100.0	0/0.0	0/0.0	1/100.0	1/100.0	1/100.0	0/0.0	0/0.0	0/0.0	1/100.0	0/0.0	0/0.0	1/100.0
S. hyicus	1/0.6	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0
Total CNS	160/100.0	114/71.3	2/1.3	0/0.0	15/9.4	46/28.8	31/19.4	4/2.5	0/0.0	0/0.0	9/7.9	0/0.0	0/0.0	26/16.3

[a]PEN, penicillin; FOX, cefoxitin; VAN, vancomycin; GEN, gentamicin; ERM, erythromycin; TET, tetracycline; CIP, ciprofloxacin; NIT, nitrofurantoin; COT, trimethoprim-sulfamethoxazole; CHL, chloramphenicol; QDA, quinupristin-dalfopristin; LZD, linezolid.

was negative for *ermC* or *ermB*. None of the CNS isolates were positive for the *mecC* or *ermA* (Table 2, Supplementary Table S3).

3.4. Genetic determinants for virulence factors

The presence and distribution of adhesin and toxin genes in staphylococcal isolates were presented in Table 3 and Supplementary Table S3. All *S. aureus* isolates harbored the adhesin genes *fnbpA*, *clfA*, *clfB*, and *sdrC*. Most of them contained *cna* (137, 79.7%) and *sdrE* (118, 68.6%), while genes *ebpS*, *sdrD* and *map*/*eap* were only found in 34.3% (59), 12.8% (22), and 18.6% (32) of the isolates, respectively. For the toxin genes, *hlb* was present in all *S. aureus* isolates, followed by *hlg* (118, 68.6%), *lukE-lukD* (59, 34.3%), and *lukM* (38, 22.1%). Genes *sec*, *sed*, *seg*, *seh*, *sei*, *sej*, *sen*, *seo* and *tst* were only observed in 15.7% (27), 15.7% (27), 6.4% (11), 6.4% (11), 15.7% (27), 6.4% (11), 15.7% (27), 15.7% (27), and 15.7% (27) of the isolates, respectively. None of the *S. aureus* isolates were positive for *bbp*, *sea*, *seb*, *see*, *sem*, *eta*, *etb*, *lukS/lukF-PV*, *hla*, *hld*, and *edin*. In contrast to *S. aureus*, virulence genes were detected in a small number of the CNS isolates. Only 10.0% (16) of the CNS isolates were positive for *clfA*, 1.9% (3) for *cna* and 0.6% (1) for *fnbA*. The toxin-encoding genes were not observed in any of the CNS isolates.

4. Discussion

A variety of bacteria have been implicated in bovine mastitis, with staphylococci being considered one of the most significant and prevalent causative agents in China and other countries (Gao et al., 2017). Understanding the pathogen profile for mastitis is critical to management (Dyson et al., 2022). In routine mastitis diagnostic laboratories, CNS are usually not identified to the species level but are reported as a single group. Consequently, limited knowledge is available regarding the epidemiology and relative importance of different species in this group (Ruegg et al., 2015; Schmidt et al., 2015; Dyson et al., 2022; Zigo et al., 2022). Although a protective effect against clinical mastitis has been postulated (Addis et al., 2020), ascribing the beneficial effect to the CNS as a group is probably inaccurate and still a topic of debate; such effect will rather be situated at the species or even strain level (Vanderhaeghen et al., 2014). The CNS group isolated from bovine milk samples consists of more than 50 different species and subspecies (Locatelli et al., 2013), and the distribution of CNS species change over time and vary between different regions (Dyson et al., 2022). In our study, 172 *S. aureus* and 160 CNS isolates were identified from the 332 staphylococcal isolates through 16S rRNA gene sequencing. A total of 15 species were confirmed among the CNS isolates. These species were frequently observed in both clinical and subclinical mastitis with slight differences among herds worldwide (Frey et al., 2013; Condas et al., 2017; El-Razik et al., 2017; Lianou et al., 2021), but the proportion of different *Staphylococcus* species varied between studies carried out in different countries (Schmidt et al., 2015; Xu et al., 2015). In accordance with the previous reports (Rall et al., 2014; Dos Santos et al., 2016; Valckenier et al., 2021), the predominant CNS species analyzed in this study was *S. chromogenes*. Normally, *S. equorum* was a less frequently detected species among CNS from dairy cattle (Adkins et al., 2018; Mahmmod et al., 2018; Jenkins et al., 2019; Valckenier et al., 2021). However, the *S. equorum* was the second most prevalent CNS species in our study, similar to the report that high proportion of this species was found in bulk milk (De Visscher et al., 2017), suggesting that this

TABLE 2 Resistance genes of staphylococci isolated from clinical bovine mastitis.

Species (No.)	Resistance genes (No./%)							
	PEN	OXA		TET		ERM		
	blaZ	*mecA*	*mecC*	*tetK*	*tetM*	*ermA*	*ermB*	*ermC*
S. aureus (172)	105/61.0	0/0.0	0/0.0	21/12.2	17/9.9	0/0.0	23/13.4	38/22.1
S. chromogenes (79)	60/75.9	0/0.0	0/0.0	13/16.5	13/16.9	0/0.0	17/21.5	25/31.6
S. equorum (22)	16/72.7	1/4.5	0/0.0	6/27.3	5/22.7	0/0.0	3/13.6	3/13.6
S. succinus (15)	9/66.7	0/0.0	0/0.0	2/13.3	1/6.7	0/0.0	1/6.7	4/26.7
S. xylosus (10)	9/90.0	0/0.0	0/0.0	2/20.0	0/0.0	0/0.0	1/10.0	3/30.0
S. simulans (8)	4/50.0	0/0.0	0/0.0	1/12.5	0/0.0	0/0.0	0/0.0	2/25.0
S. haemolyticus (7)	5/71.4	0/0.0	0/0.0	1/14.3	0/0.0	0/0.0	2/28.6	3/42.9
S. hominis (4)	2/50.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	1/25.0
S. saprophyticus (3)	2/66.7	1/33.3	0/0.0	0/0.0	0/0.0	0/0.0	1/33.3	1/33.3
S. lugdunensis (3)	1/33.3	0/0.0	0/0.0	1/33.3	1/33.3	0/0.0	1/33.3	1/33.3
S. gallinarum (3)	1/33.3	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0
S. agnetis (2)	1/50.0	0/0.0	0/0.0	1/50.0	0/0.0	0/0.0	0/0.0	1/50.0
S. auricularis (1)	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0
S. cohnii (1)	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0
S. epidermidis (1)	1/100.0	0/0.0	0/0.0	1/100.0	0/0.0	0/0.0	1/100.0	1/100.0
S. hyicus (1)	0/0.0	0/0.0	0/0.0	0/0	0/0.0	0/0.0	0/0.0	0/0.0
Total CNS (160)	111/69.4	2/1.3	0/0.0	28/17.5	20/12.5	0/0.0	27/16.9	45/28.1

species might be relevant for udder health in the sampling site. Additionally, although *S. epidermidis* and *S. haemolyticus* commonly presents a high prevalence among CNS from bovine origin (Lee and Lee, 2022), the *S. epidermidis* and *S. haemolyticus* in our study were only observed in few isolates. This may indicate that these two species are not significant causative agent of mastitis in our studied area. Management practices, origin and strategy of samples, housing systems, climate and herd size used in the studies could probably explain some of the differences. Moreover, the distribution of the most common species has been shown to change over time (Koop et al., 2012; Nyman et al., 2018). Notably, although the prevalence was low, *S. agnetis* isolates were identified among the CNS isolates in the current study. This *Staphylococcus* species, an emerging pathogen, was described as a separate species in 2012 and frequently isolated from mastitic milk samples in other countries (Condas et al., 2017; Mahato et al., 2017; Poulsen et al., 2017; Rahmdel et al., 2018; Szafraniec et al., 2020). To our knowledge, this is the first report documenting the occurrence of *S. agnetis* from bovine mastitis in China. Further sampling is required to ascertain the true prevalence and significance of this species in local dairy herds.

Antimicrobial therapy has been used as a successful strategy for controlling staphylococcal mastitis. β-Lactams, tetracyclines and macrolides were commonly used to treat staphylococcal mastitis. But the therapeutic effects are hampered by the increasing number of drug-resistant strains (Kim et al., 2019; Achek et al., 2020). In the present study, the most resistance was observed against penicillin in both *S. aureus* and CNS isolates, followed by erythromycin and tetracycline. Meanwhile, low resistance rates of gentamicin, ciprofloxacin and chloramphenicol were also found in the staphylococcal isolates tested in this study. Resistance to these antimicrobials was also frequently reported by other authors (Cheng et al., 2019; Vasileiou et al., 2019; Francisco et al., 2021; Lianou et al., 2021; Mostafa Abdalhamed et al., 2022). In agreement with other recent studies (Fernandes Dos Santos et al., 2016; Taponen et al., 2016), resistance to the tested antimicrobials was higher in CNS than that in *S. aureus* with the exception of gentamicin and ciprofloxacin in the present study. Nevertheless, our results were similar to previous studies reporting low-level resistance to gentamicin and ciprofloxacin in both *S. aureus* and CNS isolates from bovine mastitis (Frey et al., 2013; Mahato et al., 2017; Martins et al., 2017; Klibi et al., 2018), probably due to the low frequent use of these antimicrobials in dairy farm in comparison with penicillin, erythromycin and tetracycline. Notably, 1 *S. equorum* and 1 *S. saprophyticus* isolates were resistant to methicillin in the current study. The occurrence of methicillin resistance in these 2 CNS species isolated from humans, livestock and farm environment has been previously described (Cicconi-Hogan et al., 2014; Teeraputon et al., 2017; Lu et al., 2020; Bonvegna et al., 2021; Garbacz et al., 2021). However, to the best of the available knowledge, there are no reports of the methicillin resistance in *S. equorum* and *S. saprophyticus* causing bovine mastitis in China. These resistant bacteria have been reported as an emerging problem in veterinary medicine and pose a threat to public health due to their transfer from animals to the humans caring for them (Kim et al., 2019). Moreover, our findings were in accord with previous study found that CNS often exhibit greater tendency to develop multidrug resistance (MDR) than *S. aureus* (Schmidt et al., 2015). The high phenotypic resistance could be explained by the frequent use of these antimicrobials for the treatment of mastitis or other diseases such as lameness, respiratory, or reproductive problems. An augmented exposure to antimicrobials can lead to an increase in resistant strains

TABLE 3 Virulence genes of staphylococci isolated from clinical bovine mastitis.

Virulence genes	Species (No./%)																Total CNS (No./%)
	S. aureus	*S. chromogenes*	*S. equorum*	*S. succinus*	*S. xylosus*	*S. simulans*	*S. haemolyticus*	*S. hominis*	*S. saprophyticus*	*S. lugdunensis*	*S. gallinarum*	*S. agnetis*	*S. auricularis*	*S. cohnii*	*S. epidermidis*	*S. hyicus*	
fnbA	172/100.0	1/1.3	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	1/0.6
clfA	172/100.0	1/1.3	1/4.6	6/40.0	0/0.0	3/37.5	1/14.3	1/25.0	1/33.3	0/0.0	1/33.3	0/0.0	0/0.0	1/100.0	0/0.0	0/0.0	16/10.0
clfB	172/100.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0
cna	137/79.7	0/0.0	3/13.6	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	3/1.9
sdrC	172/100.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0
sdrD	22/12.8	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0
sdrE	118/68.6	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0
bbp	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0
ebpS	59/34.3	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0
map/eap	32/18.6	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0
sea	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0
seb	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0
sec	27/15.7	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0
sed	27/15.7	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0
see	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0
seg	11/6.4	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0
seh	11/6.4	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0
sei	27/15.7	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0
sej	11/6.4	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0
sen	27/15.7	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0
seo	27/15.7	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0
sem	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0
tst	27/15.7	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0
eta	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0
etb	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0
lukS/lukF-PV	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0
lukE-lukD	59/34.3	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0
lukM	38/22.1	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0
hla	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0
hlb	172/100.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0
hld	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0
hlg	118/68.6	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0
edin	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0	0/0.0

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and consequently to the diversity we observed in the resistance profile of the isolates (Fernandes Dos Santos et al., 2016; Osman et al., 2016). Furthermore, we found very large differences in antimicrobial resistance between different CNS species, possibly due to the limited number of isolates at the species level.

In this study, the most commonly antimicrobial resistance determined was against penicillin, erythromycin and tetracycline. Thus, the genes conferring resistance to these antimicrobials as well as methicillin were detected. Corresponding to the phenotypic resistance, *blaZ* showed high prevalence and was found in all penicillin-resistant *S. aureus* isolates in this study. However, 4 *blaZ*-containing isolates were susceptible to penicillin. Previous studies also found the phenomenon that some *blaZ*-positive *S. aureus* isolates were susceptible to penicillin (Ruegg et al., 2015; Andrade et al., 2021). The discrepancy may be attributable to the lack of *blaZ* expression (Hammad et al., 2014). In CNS, all *blaZ*-positive isolates were resistant to penicillin. But 3 penicillin-resistant CNS isolates were negative for this gene. This may be attributed to the fact that mechanisms such as efflux pump or biofilm other than expression of the *blaZ* gene can cause penicillin resistance because multiple mechanisms of resistance often exist in these isolates (Osman et al., 2015; Addetia et al., 2019; Francisco et al., 2021). Moreover, the *mecA* was observed in both of the methicillin-resistant isolates (one isolate each of *S. equorum* and *S. saprophyticus*), which confirmed the phenotypic resistance to methicillin. To date, at least 38 tetracycline resistance genes have been found, and the genes *tetK* and *tetM* has been commonly found in species of staphylococci (Ruegg et al., 2015). In this study, despite the low occurrence of *tetM*, the *tetK* was determined in most of the tetracycline-resistant staphylococcal isolates. This may not be surprising because *tetK* is very frequent in staphylococci species from cows with clinical mastitis (Klibi et al., 2018). Furthermore, all *tetK*-carrying (alone or combined with *tetM*) isolates showed resistance to tetracycline. A few staphylococcal isolates showed phenotypic resistance to tetracycline but were negative for *tetM* or *tetK*. Additionally, 10 genes have been identified encoding resistance to erythromycin until now, being *ermA*, *ermB*, and *ermC* the major mechanism in staphylococci for erythromycin resistance (Sun et al., 2018). But in our study, aside from 1 erythromycin-resistant *S. equorum* that was negative for *ermC* or *ermB*, the *ermC* alone or in combination with *ermB* were detected in all erythromycin-resistant staphylococcal isolates, which is supported by previous research indicating that *ermC* is the most prevalent *erm* gene recovered from cases of staphylococcal bovine mastitis and most of the isolates exhibited phenotypic resistance to erythromycin (Ruegg et al., 2015). The coexistence of these tetracyclines and macrolides resistance genes has been frequently reported in *S. aureus* or CNS isolates from bovine mastitis in China and other countries (Klibi et al., 2018; Naranjo-Lucena and Slowey, 2023). The discrepancies observed between the phenotypic susceptibility and resistance genes could be due to the presence of other resistance-encoding genes, such as *tetL* or *tetO* for tetracyclin and *ermE*, *ermT*, *mefA*, or *mefE* for erythromycin, or due to a mutation in the primer-annealing site (DiPersio et al., 2008; Schmidt et al., 2015).

The pathogenicity of staphylococci is mainly related to its capacity to encode and produce a multitude of virulence factors, facilitating their adhesion and invasion of the host cells and establishment of infection (Klibi et al., 2018). The initial attachment of staphylococci to epithelial cells of the teat canal depends on the interaction of bacterial adhesins with host surface proteins, peptides and molecules located in the basement membrane (Stutz et al., 2011). In the current study, the *S. aureus* isolates exhibited high prevalence of *fnbpA*, *clfA*, *clfB*, *sdrC*, *cna*, and *sdrE*. Similar observations have also been reported in our previous study and by other reports (Yang et al., 2020; Avila-Novoa et al., 2022; Ibrahim et al., 2022). However, the frequencies of *ebpS*, *sdrD*, and *map*/*eap* found in our study was lower than those reported by other authors (Cheraghi et al., 2017; Kot et al., 2022). Our findings indicated that a diversity of adhesins were involved in the initial attachment of host cells and colonization of the mammary gland by *S. aureus* in Ningxia Hui Autonomous Region. In addition, this group of isolates was also evaluated for toxin genes related to the invasion of host cells and the evasion of immune response. Hemolysins are pore-forming toxins that attack cell membranes and cause platelet damage, lysosome destruction, ischemia, and necrosis (Abril et al., 2020). Most *S. aureus* isolates from bovine and human origins have been reported to primarily possess the *hla*, which causes incomplete or partial hemolysis (Zhang et al., 2018; Khan et al., 2021). However, the *hlb* and *hlg* were the predominant hemolysin genes in this study, and none of the isolates contained *hla*. Leukocidins are also pore-forming two-component toxins that specifically attack immune cells (Abril et al., 2020). Similar to previous studies (Haveri et al., 2007, 2008; Thomas et al., 2021), *lukE-lukD* was the most prevalent leukocidin-encoding gene in our study, followed by *lukM*. Moreover, enterotoxins and toxic shock syndrome toxin-1 are pyrogenic toxins known as staphylococcal superantigens causing staphylococcal food poisoning and are able to interrupt host immune responses (Podkowik et al., 2013; Abril et al., 2020). In the present study, enterotoxin-encoding genes *sec*, *sed*, *seg*, *seh*, *sei*, *sej*, *sen*, and *seo* as well as toxic shock syndrome toxin-1-encoding gene *tst* were detected with low frequencies. These findings were in accordance with those of other reports involved in bovine *S. aureus* (Hummerjohann et al., 2014; Rall et al., 2014; Mello et al., 2016; Vaughn et al., 2020). The variation in the prevalence of the tested virulence factors could be associated with the genetic diversity of strains, the source and sizes of samples or their geographic locations (Avila-Novoa et al., 2022). Given that certain virulence genes are overrepresented in some clonal lineages and that some combinations are correlated with high pathogenic potential (Achek et al., 2020), further investigations need to be performed to explore the diversity of virulence factors combination in *S. aureus* pathogenesis.

Consistent with other studies (Supré et al., 2011; Xu et al., 2015; França et al., 2021), the virulence genes in CNS were significantly less prevalent than that in the *S. aureus* in our study. Previous study indicated that collagen binding protein (*cna*) and fibronectin binding protein (*fnbA*) were often associated with CNS attachment in bovines (Pizauro et al., 2019). However, in the current study, only few of the CNS isolates carried *cna* and *fnbA*. Similar results were obtained from subclinical mastitis milk in China (Xu et al., 2015). Additionally, a few of the CNS isolates were positive for *clfA* in this study, in line with the report by Felipe et al. that 12.2% of the CNS isolates contained this gene (Felipe et al., 2017). The capacity to adhere to bovine mammary epithelial cells strongly differs among the different CNS isolates and potentially reflects intra-species diversity in ecology and epidemiological behavior (Souza et al., 2016). Recent studies have provided strong evidence for the presence of toxin genes and production of the corresponding toxins in CNS in China and other countries, especially the enterotoxins (Rall et al., 2014; Salaberry et al.,

2015; Mahato et al., 2017; Martins et al., 2017; Pizauro et al., 2019). However, the toxin-encoding genes were not observed in any of the CNS isolates in our study. Our results were similar to those presented by other authors that all CNS species were negative for the toxin genes, even though a wide range of genes were tested (Nemati et al., 2008; Klempt et al., 2022). This may be attributed to the fact that the low prevalence of toxin-producing CNS isolates in the studied area, or the presence of other toxin genes which were not tested (Xu et al., 2015). Another possible reason is the requirement of different primer sets to detect the target genes (Vanderhaeghen et al., 2014). Furthermore, previous studies demonstrated that the use of antimicrobial drugs influenced the expression of virulence genes in staphylococci. The connection between genetic elements conferring resistance to antimicrobials and expression of virulence factors is intricately linked to the ability of bacteria to communicate through two-component system and quorum sensing system and has not yet been fully elucidated (Pérez et al., 2020). In further research, large sample size and sufficient numbers of isolates of each species are needed to explore the species-specific association between antimicrobial resistance and virulence factors in staphylococci.

5. Conclusion

This study provides high species diversity of staphylococci from clinical bovine mastitis in Ningxia Hui Autonomous Region in China. Noticeably, to our knowledge, we first describe the occurrence of *S. agnetis* from bovine mastitis in China. The *S. aureus* and CNS isolates displayed high frequencies of phenotypic and genotypic resistance to penicillin, erythromycin and tetracycline, which remind the government to pay continuous attention to the commonly used antimicrobial agents in dairy industry. Moreover, the high occurrence of the adhesin genes *fnbpA*, *clfA*, *clfB*, *sdrC*, *cna*, and *sdrE* tested in this study as well as the toxin genes *hlb* and *hlg* in *S. aureus* indicate their pathogenic potential causing bovine mastitis in the studied area. Further investigation is necessary to explore the diversity of virulence factors combination in *S. aureus* pathogenesis. Furthermore, despite the absence of toxin genes in CNS in this study, a more extensive examination is needed to demonstrate the true toxigenic potential of this organisms group in mastitis.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was reviewed and approved by Animal Welfare and Ethics Committee of Northwest A&F University. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

FY conceptualized the study, designed the methodology, conducted the tests, and wrote the original paper. WS, NM, and YZ helped to conduct the experimental tests. XD and QL provided resources, made the review, as well as were the leadership and responsible for funding acquisition. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1190790/full#supplementary-material

References

Achek, R., El-Adawy, H., Hotzel, H., Tomaso, H., Ehricht, R., Hamdi, T. M., et al. (2020). Short communication: diversity of staphylococci isolated from sheep mastitis in northern Algeria. *J. Dairy Sci.* 103, 890–897. doi: 10.3168/jds.2019-16583

Addetia, A., Greninger, A. L., Adler, A., Yuan, S., Makhsous, N., Qin, X., et al. (2019). A novel, widespread *qac*A allele results in reduced chlorhexidine susceptibility in *Staphylococcus epidermidis*. *Antimicrob. Agents Chemother.* 63, e02607–e02618. doi: 10.1128/AAC.02607-18

Addis, M. F., Maffioli, E. M., Ceciliani, F., Tedeschi, G., Zamarian, V., Tangorra, F., et al. (2020). Influence of subclinical mastitis and intramammary infection by coagulase-negative staphylococci on the cow milk peptidome. *J. Proteome* 226:103885. doi: 10.1016/j.jprot.2020.103885

Adkins, P. R. F., Dufour, S., Spain, J. N., Calcutt, M. J., Reilly, T. J., Stewart, G. C., et al. (2018). Cross-sectional study to identify staphylococcal species isolated from teat and inguinal skin of different-aged dairy heifers. *J. Dairy Sci.* 101, 3213–3225. doi: 10.3168/jds.2017-13974

Ahmed, W., Neubauer, H., Tomaso, H., El Hofy, F. I., Monecke, S., Abdeltawab, A. A., et al. (2020). Characterization of staphylococci and streptococci isolated from milk of bovides with mastitis in Egypt. *Pathogens* 9:381. doi: 10.3390/pathogens9050381

Andrade, N. C., Laranjo, M., Costa, M. M., and Queiroga, M. C. (2021). Virulence factors in *Staphylococcus* associated with small ruminant mastitis: biofilm production and antimicrobial resistance genes. *Antibiotics (Basel)* 10:633. doi: 10.3390/antibiotics10060633

Avila-Novoa, M. G., Solis-Velazquez, O. A., Guerrero-Medina, P. J., González-Gómez, J. P., González-Torres, B., Velázquez-Suárez, N. Y., et al. (2022). Genetic and compositional analysis of biofilm formed by *Staphylococcus aureus* isolated from food contact surfaces. *Front. Microbiol.* 13:1001700. doi: 10.3389/fmicb.2022.1001700

Bonvegna, M., Grego, E., Sona, B., Stella, M. C., Nebbia, P., Mannelli, A., et al. (2021). Occurrence of methicillin-resistant coagulase-negative staphylococci (MRCoNS) and methicillin-resistant *Staphylococcus aureus* (MRSA) from pigs and farm environment in northwestern Italy. *Antibiotics (Basel)* 10:676. doi: 10.3390/antibiotics10060676

Cheng, J., Qu, W., Barkema, H. W., Nobrega, D. B., Gao, J., Liu, G., et al. (2019). Antimicrobial resistance profiles of 5 common bovine mastitis pathogens in large Chinese dairy herds. *J. Dairy Sci.* 102, 2416–2426. doi: 10.3168/jds.2018-15135

Cheraghi, S., Pourgholi, L., Shafaati, M., Fesharaki, S. H., Jalali, A., Nosrati, R., et al. (2017). Analysis of virulence genes and accessory gene regulator (*agr*) types among methicillin-resistant *Staphylococcus aureus* strains in Iran. *J. Glob. Antimicrob. Resist.* 10, 315–320. doi: 10.1016/j.jgar.2017.06.009

Cicconi-Hogan, K. M., Belomestnykh, N., Gamroth, M., Ruegg, P. L., Tikofsky, L., and Schukken, Y. H. (2014). Short communication: prevalence of methicillin resistance in coagulase-negative staphylococci and *Staphylococcus aureus* isolated from bulk milk on organic and conventional dairy farms in the United States. *J. Dairy Sci.* 97, 2959–2964. doi: 10.3168/jds.2013-7523

CLSI (2018). *Performance standards for antimicrobial susceptibility testing. 29* (Wayne, PA: CLSI), M100–MS29.

Condas, L. A. Z., De Buck, J., Nobrega, D. B., Carson, D. A., Roy, J. P., Keefe, G. P., et al. (2017). Distribution of non-aureus staphylococci species in udder quarters with low and high somatic cell count, and clinical mastitis. *J. Dairy Sci.* 100, 5613–5627. doi: 10.3168/jds.2016-12479

De Visscher, A., Piepers, S., Haesebrouck, F., Supré, K., and De Vliegher, S. (2017). Coagulase-negative *Staphylococcus* species in bulk milk: prevalence, distribution, and associated subgroup- and species-specific risk factors. *J. Dairy Sci.* 100, 629–642. doi: 10.3168/jds.2016-11476

DiPersio, L. P., DiPersio, J. R., Frey, K. C., and Beach, J. A. (2008). Prevalence of the *erm*(T) gene in clinical isolates of erythromycin-resistant group D *Streptococcus* and *Enterococcus*. *Antimicrob. Agents Chemother.* 52, 1567–1569. doi: 10.1128/AAC.01325-07

Dos Santos, D. C., Lange, C. C., Avellar-Costa, P., Dos Santos, K. R., Brito, M. A., and Giambiagi-deMarval, M. (2016). *Staphylococcus chromogenes*, a coagulase-negative *Staphylococcus* species that can clot plasma. *J. Clin. Microbiol.* 54, 1372–1375. doi: 10.1128/JCM.03139-15

Dyson, R., Charman, N., Hodge, A., Rowe, S. M., and Taylor, L. F. (2022). A survey of mastitis pathogens including antimicrobial susceptibility in southeastern Australian dairy herds. *J. Dairy Sci.* 105, 1504–1518. doi: 10.3168/jds.2021-20955

El-Razik, K. A. A., Arafa, A. A., Hedia, R. H., and Ibrahim, E. S. (2017). Tetracycline resistance phenotypes and genotypes of coagulase-negative staphylococcal isolates from bubaline mastitis in Egypt. *Vet. World.* 10, 702–710. doi: 10.14202/vetworld.2017.702-710

Felipe, V., Morgante, C. A., Somale, P. S., Varroni, F., Zingaretti, M. L., Bachetti, R. A., et al. (2017). Evaluation of the biofilm forming ability and its associated genes in *Staphylococcus* species isolates from bovine mastitis in Argentinean dairy farms. *Microb. Pathog.* 104, 278–286. doi: 10.1016/j.micpath.2017.01.047

Fernandes Dos Santos, F., Mendonça, L. C., Reis, D. R. L., Guimarães, A. S., Lange, C. C., Ribeiro, J. B., et al. (2016). Presence of *mec*A-positive multidrug-resistant *Staphylococcus epidermidis* in bovine milk samples in Brazil. *J. Dairy Sci.* 99, 1374–1382. doi: 10.3168/jds.2015-9931

Ferreira, E. M., Romero, L. C., Cunha, M. L. R. S. D., Malagó Junior, W., Camargo, C. H., Barioni Júnior, W., et al. (2022). Persistence of *Staphylococcus* spp. in milk from cows undergoing homeopathy to control subclinical mastitis. *BMC Vet. Res.* 18:273. doi: 10.1186/s12917-022-03364-8

França, A., Gaio, V., Lopes, N., and Melo, L. D. R. (2021). Virulence factors in coagulase-negative staphylococci. *Pathogens* 10:170. doi: 10.3390/pathogens10020170

Francisco, M. S., Rossi, C. C., Brito, M. A. V. P., Laport, M. S., Barros, E. M., and Giambiagi-deMarval, M. (2021). Characterization of biofilms and antimicrobial resistance of coagulase-negative *Staphylococcus* species involved with subclinical mastitis. *J. Dairy Res.* 88, 179–184. doi: 10.1017/S0022029921000285

Frey, Y., Rodriguez, J. P., Thomann, A., Schwendener, S., and Perreten, V. (2013). Genetic characterization of antimicrobial resistance in coagulase-negative staphylococci from bovine mastitis milk. *J. Dairy Sci.* 96, 2247–2257. doi: 10.3168/jds.2012-6091

Abril, A. G., Villa, T. G., Barros-Velázquez, J., Cañas, B., Sánchez-Pérez, A., Calo-Mata, P., et al. (2020). *Staphylococcus aureus* exotoxins and their detection in the dairy industry and mastitis. *Toxins (Basel)* 12:537. doi: 10.3390/toxins12090537

Gao, J., Barkema, H. W., Zhang, L., Liu, G., Deng, Z., Cai, L., et al. (2017). Incidence of clinical mastitis and distribution of pathogens on large Chinese dairy farms. *J. Dairy Sci.* 100, 4797–4806. doi: 10.3168/jds.2016-12334

Garbacz, K., Wierzbowska, M., Kwapisz, E., Kosecka-Strojek, M., Bronk, M., Saki, M., et al. (2021). Distribution and antibiotic-resistance of different *Staphylococcus* species identified by matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) isolated from the oral cavity. *J. Oral Microbiol.* 13:1983322. doi: 10.1080/20002297.2021.1983322

González-Martín, M., Corbera, J. A., Suárez-Bonnet, A., and Tejedor-Junco, M. T. (2020). Virulence factors in coagulase-positive staphylococci of veterinary interest other than *Staphylococcus aureus*. *Vet. Q.* 40, 118–131. doi: 10.1080/01652176.2020.1748253

Hammad, A. M., Shimamoto, T., and Shimamoto, T. (2014). Genetic characterization of antibiotic resistance and virulence factors in *Enterococcus* spp. from Japanese retail ready-to-eat raw fish. *Food Microbiol.* 38, 62–66. doi: 10.1016/j.fm.2013.08.010

Haveri, M., Hovinen, M., Roslöf, A., and Pyörälä, S. (2008). Molecular types and genetic profiles of *Staphylococcus aureus* strains isolated from bovine intramammary infections and extramammary sites. *J. Clin. Microbiol.* 46, 3728–3735. doi: 10.1128/JCM.00769-08

Haveri, M., Roslöf, A., Rantala, L., and Pyörälä, S. (2007). Virulence genes of bovine *Staphylococcus aureus* from persistent and nonpersistent intramammary infections with different clinical characteristics. *J. Appl. Microbiol.* 103, 993–1000. doi: 10.1111/j.1365-2672.2007.03356.x

Hummerjohann, J., Naskova, J., Baumgartner, A., and Graber, H. U. (2014). Enterotoxin-producing *Staphylococcus aureus* genotype B as a major contaminant in Swiss raw milk cheese. *J. Dairy Sci.* 97, 1305–1312. doi: 10.3168/jds.2013-7643

Ibrahim, E. S., Arafa, A. A., Dorgam, S. M., Eid, R. H., Atta, N. S., El-Dabae, W. H., et al. (2022). Molecular characterization of genes responsible for biofilm formation in *Staphylococcus aureus* isolated from mastitic cows. *Vet. World* 15, 205–212. doi: 10.14202/vetworld.2022.205-212

Isaac, P., Bohl, L. P., Breser, M. L., Orellano, M. S., Conesa, A., Ferrero, M. A., et al. (2017). Commensal coagulase-negative *Staphylococcus* from the udder of healthy cows inhibits biofilm formation of mastitis-related pathogens. *Vet. Microbiol.* 207, 259–266. doi: 10.1016/j.vetmic.2017.05.025

Jarraud, S., Mougel, C., Thioulouse, J., Lina, G., Meugnier, H., Forey, F., et al. (2002). Relationships between Staphylococcus aureus genetic background, virulence factors, agr groups (alleles), and human disease. *Infect. immun.* 70, 631–641. doi: 10.1128/IAI.70.2.631-641.2002

Jenkins, S. N., Okello, E., Rossitto, P. V., Lehenbauer, T. W., Champagne, J., Penedo, M. C. T., et al. (2019). Molecular epidemiology of coagulase-negative *Staphylococcus* species isolated at different lactation stages from dairy cattle in the United States. *PeerJ.* 7:e6749. doi: 10.7717/peerj.6749

Khan, S., Marasa, B. S., Sung, K., and Nawaz, M. (2021). Genotypic characterization of clinical isolates of *Staphylococcus aureus* from Pakistan. *Pathogens* 10:918. doi: 10.3390/pathogens10080918

Kim, S. J., Moon, D. C., Park, S. C., Kang, H. Y., Na, S. H., and Lim, S. K. (2019). Antimicrobial resistance and genetic characterization of coagulase-negative staphylococci from bovine mastitis milk samples in Korea. *J. Dairy Sci.* 102, 11439–11448. doi: 10.3168/jds.2019-17028

Klempt, M., Franz, C. M. A. P., and Hammer, P. (2022). Characterization of coagulase-negative staphylococci and macrococci isolated from cheese in Germany. *J. Dairy Sci.* 105, 7951–7958. doi: 10.3168/jds.2022-21941

Klibi, A., Maaroufi, A., Torres, C., and Jouini, A. (2018). Detection and characterization of methicillin-resistant and susceptible coagulase-negative staphylococci in milk from cows with clinical mastitis in Tunisia. *Int. J. Antimicrob. Agents* 52, 930–935. doi: 10.1016/j.ijantimicag.2018.07.026

Koop, G., De Vliegher, S., De Visscher, A., Supré, K., Haesebrouck, F., Nielen, M., et al. (2012). Differences between coagulase-negative *Staphylococcus* species in persistence and in effect on somatic cell count and milk yield in dairy goats. *J. Dairy Sci.* 95, 5075–5084. doi: 10.3168/jds.2012-5615

Kot, B., Piechota, M., Jakubczak, A., Gryzińska, M., Witeska, M., Grużewska, A., et al. (2022). The prevalence of virulence determinants in methicillin-resistant *Staphylococcus aureus* isolated from different infections in hospitalized patients in Poland. *Sci. Rep.* 12:5477. doi: 10.1038/s41598-022-09517-x

Kot, B., Piechota, M., Wolska, K. M., Frankowska, A., Zdunek, E., Binek, T., et al. (2012). Phenotypic and genotypic antimicrobial resistance of staphylococci from bovine milk. *Pol. J. Vet. Sci.* 15, 677–683. doi: 10.2478/v10181-012-0105-4

Lee, Y. J., and Lee, Y. J. (2022). Characterization of biofilm producing coagulase-negative staphylococci isolated from bulk tank milk. *Vet. Sci.* 9:430. doi: 10.3390/vetsci9080430

Leroy, F., Van Coillie, E., Braem, G., Piessens, V., Verbist, B., De Vuyst, L., et al. (2015). Short communication: subtyping of *Staphylococcus haemolyticus* isolates from milk and corresponding teat apices to verify the potential teat-skin origin of intramammary infections in dairy cows. *J. Dairy Sci.* 98, 7893–7898. doi: 10.3168/jds.2015-9415

Li, L., Feng, W., Zhang, Z., Xue, H., and Zhao, X. (2015). Macrolide-lincosamide-streptogramin resistance phenotypes and genotypes of coagulase-positive *Staphylococcus aureus* and coagulase-negative staphylococcal isolates from bovine mastitis. *BMC Vet. Res.* 11:168. doi: 10.1186/s12917-015-0492-8

Lianou, D. T., Petinaki, E., Cripps, P. J., Gougoulis, D. A., Michael, C. K., Tsilipounidaki, K., et al. (2021). Antibiotic resistance of staphylococci from bulk-tank

milk of sheep flocks: prevalence, patterns, association with biofilm formation, effects on milk quality, and risk factors. *Biology* 10:1016. doi: 10.3390/biology10101016

Locatelli, C., Piepers, S., De Vliegher, S., Barberio, A., Supré, K., Scaccabarozzi, L., et al. (2013). Effect on quarter milk somatic cell count and antimicrobial susceptibility of *Staphylococcus rostri* causing intramammary infection in dairy water buffaloes. *J. Dairy Sci.* 96, 3799–3805. doi: 10.3168/jds.2012-6275

Lu, Y., Lu, Q., Cheng, Y., Wen, G., Luo, Q., Shao, H., et al. (2020). High concentration of coagulase-negative staphylococci carriage among bioaerosols of henhouses in Central China. *BMC Microbiol.* 20:21. doi: 10.1186/s12866-020-1709-y

Magiorakos, A. P., Srinivasan, A., Carey, R. B., Carmeli, Y., Falagas, M. E., Giske, C. G., et al. (2012). Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin. Microbiol. Infect* 18, 268–281. doi: 10.1111/j.1469-0691.2011.03570.x

Mahato, S., Mistry, H. U., Chakraborty, S., Sharma, P., Saravanan, R., and Bhandari, V. (2017). Identification of variable traits among the methicillin resistant and sensitive coagulase negative staphylococci in milk samples from mastitic cows in India. *Front. Microbiol.* 8:1446. doi: 10.3389/fmicb.2017.01446

Mahmmod, Y. S., Klaas, I. C., Svennesen, L., Pedersen, K., and Ingmer, H. (2018). Communications of *Staphylococcus aureus* and non-aureus *Staphylococcus* species from bovine intramammary infections and teat apex colonization. *J. Dairy Sci.* 101, 7322–7333. doi: 10.3168/jds.2017-14311

Martins, K. B., Faccioli, P. Y., Bonesso, M. F., Fernandes, S., Oliveira, A. A., Dantas, A., et al. (2017). Characteristics of resistance and virulence factors in different species of coagulase-negative staphylococci isolated from milk of healthy sheep and animals with subclinical mastitis. *J. Dairy Sci.* 100, 2184–2195. doi: 10.3168/jds.2016-11583

Mello, P. L., Moraes Riboli, D. F., Pinheiro, L., de Almeida Martins, L., Vasconcelos Paiva Brito, M., and Ribeiro de Souza da Cunha, M. (2016). Detection of enterotoxigenic potential and determination of clonal profile in *Staphylococcus aureus* and coagulase-negative staphylococci isolated from bovine subclinical mastitis in different Brazilian states. *Toxins (Basel)* 8:104. doi: 10.3390/toxins8040104

Mostafa Abdalhamed, A., Zeedan, G. S. G., Ahmed Arafa, A., Shafeek Ibrahim, E., Sedky, D., and Abdel Nabey Hafez, A. (2022). Detection of methicillin-resistant *Staphylococcus aureus* in clinical and subclinical mastitis in ruminants and studying the effect of novel green synthetized nanoparticles as one of the alternative treatments. *Vet. Med. Int.* 2022:6309984. doi: 10.1155/2022/6309984

Naranjo-Lucena, A., and Slowey, R. (2023). Invited review: antimicrobial resistance in bovine mastitis pathogens: a review of genetic determinants and prevalence of resistance in European countries. *J. Dairy Sci.* 106, 1–23. doi: 10.3168/jds.2022-22267

Nemati, M., Hermans, K., Vancraeynest, D., De Vliegher, S., Sampimon, O. C., Baele, M., et al. (2008). Screening of bovine coagulase-negative staphylococci from milk for superantigen-encoding genes. *Vet. Rec.* 163, 740–743. doi: 10.1016/j.zool.2007.04.002

Nyman, A. K., Fasth, C., and Waller, K. P. (2018). Intramammary infections with different non-aureus staphylococci in dairy cows. *J. Dairy Sci.* 101, 1403–1418. doi: 10.3168/jds.2017-13467

Osman, K. M., Abd El-Razik, K. A., Marie, H. S., and Arafa, A. (2015). Relevance of biofilm formation and virulence of different species of coagulase-negative staphylococci to public health. *Eur. J. Clin. Microbiol. Infect. Dis.* 34, 2009–2016. doi: 10.1007/s10096-015-2445-3

Osman, K. M., Abd El-Razik, K. A., Marie, H. S., and Arafa, A. (2016). Coagulase-negative staphylococci collected from bovine milk: species and antimicrobial gene diversity. *J. Food Saf.* 36, 89–99. doi: 10.1111/jfs.12216

Paterson, G. K., Larsen, A. R., Robb, A., Edwards, G. E., Pennycott, T. W., Foster, G., et al. (2012). The newly described *mecA* homologue, $mecA_{LGA251}$, is present in methicillin-resistant *Staphylococcus aureus* isolates from a diverse range of host species. *J. Antimicrob. Chemother.* 67, 2809–2813. doi: 10.1093/jac/dks329

Peacock, S. J., Moore, C. E., Justice, A., Kantzanou, M., Story, L., Mackie, K., et al. (2002). Virulent combinations of adhesin and toxin genes in natural populations of Staphylococcus aureus. *Infect. immun.* 70, 4987–4996. doi: 10.1128/IAI.70.9.4987-4996.2002

Pérez, V. K. C., Costa, G. M. D., Guimarães, A. S., Heinemann, M. B., Lage, A. P., and Dorneles, E. M. S. (2020). Relationship between virulence factors and antimicrobial resistance in *Staphylococcus aureus* from bovine mastitis. *J. Glob. Antimicrob. Resist.* 22, 792–802. doi: 10.1016/j.jgar.2020.06.010

Piessens, V., De Vliegher, S., Verbist, B., Braem, G., Van Nuffel, A., De Vuyst, L., et al. (2012). Characterization of coagulase-negative *Staphylococcus* species from cows' milk and environment based on *bap*, *ica*A, and *mec*A genes and phenotypic susceptibility to antimicrobials and teat dips. *J. Dairy Sci.* 95, 7027–7038. doi: 10.3168/jds.2012-5400

Pizauro, L. J. L., de Almeida, C. C., Soltes, G. A., Slavic, D., de Ávila, F. A., Zafalon, L. F., et al. (2019). Short communication: detection of antibiotic resistance, *mec*A, and virulence genes in coagulase-negative *Staphylococcus* spp. from buffalo milk and the milking environment. *J. Dairy Sci.* 102, 11459–11464. doi: 10.3168/jds.2018-15920

Podkowik, M., Park, J. Y., Seo, K. S., Bystroń, J., and Bania, J. (2013). Enterotoxigenic potential of coagulase-negative staphylococci. *Int. J. Food Microbiol.* 163, 34–40. doi: 10.1016/j.ijfoodmicro.2013.02.005

Poulsen, L. L., Thøfner, I., Bisgaard, M., Olsen, R. H., Christensen, J. P., and Christensen, H. (2017). *Staphylococcus agnetis*, a potential pathogen in broiler breeders. *Vet. Microbiol.* 212, 1–6. doi: 10.1016/j.vetmic.2017.10.018

Rahmdel, S., Hosseinzadeh, S., Shekarforoush, S. S., Torriani, S., Gatto, V., and Pashangeh, S. (2018). Safety hazards in bacteriocinogenic *Staphylococcus* strains isolated from goat and sheep milk. *Microb. Pathog.* 116, 100–108. doi: 10.1016/j.micpath.2018.01.016

Rall, V. L., Miranda, E. S., Castilho, I. G., Camargo, C. H., Langoni, H., Guimarães, F. F., et al. (2014). Diversity of *Staphylococcus* species and prevalence of enterotoxin genes isolated from milk of healthy cows and cows with subclinical mastitis. *J. Dairy Sci.* 97, 829–837. doi: 10.3168/jds.2013-7226

Ruegg, P. L., Oliveira, L., Jin, W., and Okwumabua, O. (2015). Phenotypic antimicrobial susceptibility and occurrence of selected resistance genes in gram-positive mastitis pathogens isolated from Wisconsin dairy cows. *J. Dairy Sci.* 98, 4521–4534. doi: 10.3168/jds.2014-9137

Salaberry, S. R., Saidenberg, A. B., Zuniga, E., Melville, P. A., Santos, F. G., Guimarães, E. C., et al. (2015). Virulence factors genes of *Staphylococcus* spp. isolated from caprine subclinical mastitis. *Microb. Pathog.* 85, 35–39. doi: 10.1016/j.micpath.2015.05.007

Schmidt, T., Kock, M. M., and Ehlers, M. M. (2015). Diversity and antimicrobial susceptibility profiling of staphylococci isolated from bovine mastitis cases and close human contacts. *J. Dairy Sci.* 98, 6256–6269. doi: 10.3168/jds.2015-9715

Souza, F. N., Piepers, S., Della Libera, A. M. M. P., Heinemann, M. B., Cerqueira, M. M. O. P., and De Vliegher, S. (2016). Interaction between bovine-associated coagulase-negative staphylococci species and strains and bovine mammary epithelial cells reflects differences in ecology and epidemiological behavior. *J. Dairy Sci.* 99, 2867–2874. doi: 10.3168/jds.2015-10230

Stutz, K., Stephan, R., and Tasara, T. (2011). SpA, ClfA, and FnbA genetic variations lead to Staphaurex test-negative phenotypes in bovine mastitis *Staphylococcus aureus* isolates. *J. Clin. Microbiol.* 49, 638–646. doi: 10.1128/JCM.01148-10

Sun, X., Lin, Z. W., Hu, X. X., Yao, W. M., Bai, B., Wang, H. Y., et al. (2018). Biofilm formation in erythromycin-resistant *Staphylococcus aureus* and the relationship with antimicrobial susceptibility and molecular characteristics. *Microb. Pathog.* 124, 47–53. doi: 10.1016/j.micpath.2018.08.021

Supré, K., Haesebrouck, F., Zadoks, R. N., Vaneechoutte, M., Piepers, S., and De Vliegher, S. (2011). Some coagulase-negative *Staphylococcus* species affect udder health more than others. *J. Dairy Sci.* 94, 2329–2340. doi: 10.3168/jds.2010-3741

Szafraniec, G. M., Szeleszczuk, P., and Dolka, B. (2020). A review of current knowledge on *Staphylococcus agnetis* in poultry. *Animals (Basel)* 10:1421. doi: 10.3390/ani10081421

Taponen, S., Nykäsenoja, S., Pohjanvirta, T., Pitkälä, A., and Pyörälä, S. (2016). Species distribution and *in vitro* antimicrobial susceptibility of coagulase-negative staphylococci isolated from bovine mastitic milk. *Acta Vet. Scand.* 58:12. doi: 10.1186/s13028-016-0193-8

Teeraputon, S., Santanirand, P., Wongchai, T., Songjang, W., Lapsomthob, N., Jaikrasun, D., et al. (2017). Prevalence of methicillin resistance and macrolide-lincosamide-streptogramin B resistance in *Staphylococcus haemolyticus* among clinical strains at a tertiary-care hospital in Thailand. *New Microbes. New Infect.* 19, 28–33. doi: 10.1016/j.nmni.2017.05.007

Thomas, A., Chothe, S., Byukusenge, M., Mathews, T., Pierre, T., Kariyawasam, S., et al. (2021). Prevalence and distribution of multilocus sequence types of *Staphylococcus aureus* isolated from bulk tank milk and cows with mastitis in Pennsylvania. *PLoS One* 16:e0248528. doi: 10.1371/journal.pone.0248528

Valckenier, D., Piepers, S., Schukken, Y. H., De Visscher, A., Boyen, F., Haesebrouck, F., et al. (2021). Longitudinal study on the effects of intramammary infection with non-aureus staphylococci on udder health and milk production in dairy heifers. *J. Dairy Sci.* 104, 899–914. doi: 10.3168/jds.2020-18685

Vanderhaeghen, W., Piepers, S., Leroy, F., Van Coillie, E., Haesebrouck, F., and De Vliegher, S. (2014). Invited review: effect, persistence, and virulence of coagulase-negative *Staphylococcus* species associated with ruminant udder health. *J. Dairy Sci.* 97, 5275–5293. doi: 10.3168/jds.2013-7775

Vasileiou, N. G. C., Sarrou, S., Papagiannitsis, C., Chatzopoulos, D. C., Malli, E., Mavrogianni, V. S., et al. (2019). Antimicrobial agent susceptibility and typing of staphylococcal isolates from subclinical mastitis in ewes. *Microb. Drug Resist.* 25, 1099–1110. doi: 10.1089/mdr.2019.0009

Vaughn, J. M., Abdi, R. D., Gillespie, B. E., and Kerro Dego, O. (2020). Genetic diversity and virulence characteristics of *Staphylococcus aureus* isolates from cases of bovine mastitis. *Microb. Pathog.* 144:104171. doi: 10.1016/j.micpath.2020.104171

Waller, K. P., Aspán, A., Nyman, A., Persson, Y., and Andersson, U. G. (2011). CNS species and antimicrobial resistance in clinical and subclinical bovine mastitis. *Vet. Microbiol.* 152, 112–116. doi: 10.1016/j.vetmic.2011.04.006

Xu, J., Tan, X., Zhang, X., Xia, X., and Sun, H. (2015). The diversities of staphylococcal species, virulence and antibiotic resistance genes in the subclinical mastitis milk from a single Chinese cow herd. *Microb. Pathog.* 88, 29–38. doi: 10.1016/j.micpath.2015.08.004

Yang, F., Wang, Q., Wang, X., Wang, L., Li, X., Luo, J., et al. (2016). Genetic characterization of antimicrobial resistance in *Staphylococcus aureus* isolated from

bovine mastitis cases in Northwest China. *J. Integr. Agric.* 15, 2842–2847. doi: 10.1016/S2095-3119(16)61368-0

Yang, F., Zhang, S., Shang, X., Li, H., Zhang, H., Cui, D., et al. (2020). Short communication: detection and molecular characterization of methicillin-resistant *Staphylococcus aureus* isolated from subclinical bovine mastitis cases in China. *J. Dairy Sci.* 103, 840–845. doi: 10.3168/jds.2019-16317

Yang, F., Zhang, S., Shang, X., Wang, X., Yan, Z., Li, H., et al. (2019). Short communication: antimicrobial resistance and virulence genes of *Enterococcus faecalis* isolated from subclinical bovine mastitis cases in China. *J. Dairy Sci.* 102, 140–144. doi: 10.3168/jds.2018-14576

Zhang, L., Gao, J., Barkema, H. W., Ali, T., Liu, G., Deng, Y., et al. (2018). Virulence gene profiles: alpha-hemolysin and clonal diversity in *Staphylococcus aureus* isolates from bovine clinical mastitis in China. *BMC Vet. Res.* 14:63. doi: 10.1186/s12917-018-1374-7

Zigo, F., Farkašová, Z., Výrostková, J., Regecová, I., Ondrašovičová, S., Vargová, M., et al. (2022). Dairy cows' udder pathogens and occurrence of virulence factors in staphylococci. *Animals (Basel)* 12:470. doi: 10.3390/ani12040470

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The abundance and diversity of antibiotic resistance genes in layer chicken ceca is associated with farm enviroment

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Industrialized layer chicken feedlots harbor complex environmental microbial communities that affect the enrichment and exchange of gut bacteria and antibiotic resistance genes (ARGs). However, the contribution of different environmental sources to the gut ARGs of layer chickens is not clear. Here, layer chicken gut and environmental samples (air, water, feed, cage, feather, maternal hen feces, uropygial glands) were collected during the early 3month period before the laying of eggs, and the source and characteristics of the gut microorganisms and ARGs were analyzed by performing 16S rRNA and metagenomic sequencing. The results showed that the abundances of Bacteroidetes and Actinobacteria in cecum of layer chickens gradually increased, while that of Proteobacteria decreased with age, and the number and relative abundance of ARGs decreased significantly with age. On day 5, 57% of the layer chicken cecal ARGs were from feather samples, and 30% were from cage samples. Subsequently, the contribution of cage ARGs became progressively more prominent over time. At days 30 and 57, the contribution of cage ARGs to the chick cecal ARGs reached 63.3 and 69.5%, respectively. The bacterial community composition (especially the abundances of *Klebsiella pneumoniae* and *Escherichia coli*) was the major factor impacting the ARG profile. *K. pneumoniae* and *E. coli* were mainly transmitted from feathers to the layer chicken cecum, and the contribution rates were 32 and 3.4%, respectively. In addition, we observed the transmission of ARG-carrying bacteria (*Bacteroides fragilis*) from the cage to the gut, with a contribution rate of 11.5%. It is noteworthy that *B. fragilis* is an opportunistic pathogen that may cause diarrhea in laying hens. These results can provide reference data for the healthy breeding of layer chickens and the prevention and control of ARG pollution.

KEYWORDS

industrialized feedlot, lohmann layer chicken, cecal microbiome, antibiotic resistance genes, source tracker

1. Introduction

Antibiotic resistance genes (ARGs) are emerging environmental contaminants that have raised serious public health concerns. Approximately 700,000 people die every year because of

resistant infections, and by 2050, that number will increase to 10 million deaths a year (Chin et al., 2018). It has been reported that approximately 73% of antibiotics use occurs in the farming industry (Van Boeckel et al., 2019). Antibiotics are administered to animals, usually through feed or drinking water for the prevention and treatment of diseases (Hu and Cheng, 2016). Therefore, the gut microbes of food animals likely constitute a large reservoir for ARGs, significantly increasing the risk of ARGs transmission through the food chain (Wang et al., 2019). However little attention has been paid to the gut microbial composition and associated ARGs in laying chicken, in contrast to the extensive studies of pig and broiler microbiomes and ARGs (He et al., 2014; Zhao et al., 2018). Consequently, a comprehensive study on the structure and function of the bacterial community composition and its associated ARGs in laying chickens is urgently needed.

The ARGs in the gut microbiota are mainly affected by bacterial community composition (Li et al., 2021). As the primary host of ARGs, bacterial communities account for 4.8–32.2% of the variation in the ARG profile (Pu et al., 2020; Yang et al., 2021). The establishment of the laying chicken gut microbiota is a complex and dynamic process (Diaz Carrasco et al., 2019). Age is an important factor affecting the composition of the gut microbiota. Early life is a critical window period for the colonization of gut microbiota in animals (Liu et al., 2019). The gut microbiota that is established during this early stage has an important influence on the growth of the animals and the development of the immune system (Tamburini et al., 2016). Therefore, it is helpful to further understand the association between changes in the gut microbiota and ARGs in laying chicken by studying the changes in gut microbiota over time in early life.

Despite evidence suggesting that newborn laying chicks acquire their initial microbial community from their maternal hen and immediate environment, the impact and relative contribution of different microbial sources in shaping the gut microbiota in layer chickens remains poorly understood (Lee et al., 2019; Maki et al., 2020). Research has shown that *Halomonas* and *Ochrobactrum* are dominant genera in embryos, and there was a moderate correlation (0.4) between the maternal hen and the embryo (Ding et al., 2017). The results suggest that the maternal hen fecal microbiota on eggshells may contribute to the establishment of gut microbiota in chicks. In addition, the rearing environment also exerts a sustained influence on the development of infant gut microbiota. The microbiota of indoor hens consists of a higher number of bacterial species than the microbiota of outdoor hens (Seidlerova et al., 2020). However, no longitudinal studies have been conducted to analyze the relative contributions of these bacterial sources to the colonization of newborn laying chicks.

Thus, a large-scale study was conducted to investigate the spatial and temporal development of cecal bacteria and ARGs in healthy newborn laying chicks, and to explore the relationship between bacterial transmission and the changes in ARGs within the gut microbiota. In addition, relative contributions of different microbial sources from the maternal hen fecal and the rearing environment (air, water, chicken cages, chicken feed, uropygial glands, and feathers) were also assessed.

2. Materials and methods

2.1. Sample collection

The layer chicken management was performed according to our previous study, we chose to sample more frequently during the early stages of rapid development and changes in the gut microbiota (0–7 days), and then transition to less frequent sampling during the later stages of community stabilization (Xiao et al., 2021). Random samples (5 g) of feed (day 0, 12, 43) were taken from the feed trough using sterile spoons and placed into sterile sampling bags. Six layer chickens were randomly obtained from the flock in the middle of house at days 0, 1, 3, 5, 7, 12, 18, 24, 30, 36, 40, 43, 50, and 57 after hatching, with a total of 84 layer chickens used for this study (Supplementary Figure S1). Using sterile scissors, 8–10 feathers were cut from the neck, back, abdomen, wings, and tail of the chickens, and mixed together in a sterile bag. And than the layer chicken selected for each time point were killed by cervical dislocation. After the abdomen was opened; the caecum was removed from each chicken, and immediately placed in liquid nitrogen and collected, transferred to the laboratory and stored at −80°C until DNA extraction. At the same time, maternal fecal samples were collected rectally from each laying hen using a sterile cotton swab (Hua Chen Yang, Shen Zhen, China) premoistened with sterile phosphate-buffered saline (PBS), and then the swab head was placed in a 5 mL sterile screw top collection tube (Corning, NY, United States). The uropygial gland was cut off with sterilized scissors and placed in a 5 mL sterile screw-top collection tube. Three water samples (approximately 3 L each) were collected from the water trough and placed into sterile containers at each sampling timepoint. Indoor air samples were collected using liquid-based air samplers, which were placed ~50 cm above the floor. Three replicate air samples were collected at 8:00, 12:00 and 16:00 by drawing air for 1 h at a rate of 13 L per min through impingers filled with sterile molecular-grade water. For each replicate, the air sample was collected three times simultaneously at five points indoors, and the resulting samples (30 mL each) were pooled (150 mL total volume). Cage surface samples from each coop were collected from six sites. During this procedure, a swab premoistened with sterile PBS was rubbed back and forth several times at each sampling site, and then the swab head was placed in a 5-mL sterile screw-top collection tube. Samples were immediately placed in liquid nitrogen after collection, transferred to the laboratory and stored at-80°C until DNA extraction.

2.2. Sample pretreatment and DNA extraction

For swab-collected samples (cage surface samples), the six-swab head was vortexed at maximum speed in a bead tube with sterile PBS. After discarding the swabs, the tube was centrifuged and the pellets were suspended in PowerBead solution and C1 buffer from the DNA isolation kit. Zirconium glass beads (400 mg; diameter, 0.1 mm) (BioSpec, Bartlesville, OK, United States) were added, and the mixture was shaken vigorously using an automatic rapid sample grinding instrument (JXFSTPRP-48, Shanghai Jingxin). The mixture was then incubated at 95°C for 5 min to maximize bacterial DNA extraction. All subsequent steps followed the manufacturer's protocol for the PowerFecal DNA Kit (Qiagen, Germany). For DNA extraction with a PowerWater DNA Isolation Kit (Qiagen, Germany), the water samples and impinger liquid from the air samples were vacuum filtered onto sterile 0.22 μM polycarbonate filters (Sigma, St. Louis, MO, United States), transferred to 0.7 mm garnet bead tubes containing 1 mL of PW1 solution, and vortexed at maximum speed for 10 min. The remaining steps were performed according to the manufacturer's

protocol. To obtain microbial samples from the feather surface, the feather sample was divided into three parts and placed into a centrifuge tube containing 30 mL of PBS (0.1 mol/L, pH 7.0). After vortex oscillation at 180 r/min at 4°C for 3 h and ultrasonication at 4°C for 30 min, the feather sample cleaning solution was vacuum filtered onto sterile 0.22-μM polycarbonate filters (Sigma, St. Louis, MO, United States) and transferred to 0.7-mm garnet bead tubes containing 1 mL of PW1 solution. The mixture was shaken vigorously using a FastPrep-24 instrument, and the remaining steps were performed according to the manufacturer's protocol. Uropygial glands (0.02 mg) were hydrolyzed with proteinase K before being processed with a DNA Mini Kit (Qiagen, Germany). Microbial DNA was extracted from the chicken feed using a DNeasy PowerFood Microbial Kit (Qiagen, Germany). DNA quality control was performed on the samples using several steps. Firstly, DNA degradation and potential contamination were monitored on 1% agarose gels. Secondly, DNA purity was assessed by determining the ratios of OD260/OD280 and OD260/OD230 using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington). Finally, DNA concentration was measured using the Qubit® dsDNA Assay Kit and Qubit 2.0 fluorometer (Invitrogen, Thermo Fisher Scientific, MA). DNA samples with OD values ranging from 1.8 to 2.0 and DNA contents above 1 μg were deemed suitable for library construction.

2.3. 16S rRNA sequencing

To analyze the microbiota community composition, specific V4 regions of the 16S rRNA gene were amplified with barcoded primers (F:5′-GTGCCAGCMGCCGCGGTAA-3′; R:5′-GGACTACHVGG GTWTCTAAT-3′) and PremixTaq (TaKaRa) was used for PCR amplification. PCR conditions were as follows: (1) 94°C for 3 min; (2) 30 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 30 s; and (3) a final extension at 72°C for 5 min. The amplicons were purified using a QIAquick PCR Purification Kit (Qiagen, Germany), and 250-bp read sequencing was performed on the Illumina HiSeq platform. The raw reads from 16S rRNA gene sequencing were demultiplexed and quality-filtered using Quantitative Insights into Microbial Ecology (QIIME2–2020.6) (Bokulich et al., 2013). Clean data were clustered using the Divisive Amplicon Denoising Algorithm 2 (DADA2) method with amplicon sequence variant (ASV) levels. The feature sequences were taxonomically assigned at the kingdom, phylum, class, order, family and genus levels. The α-diversity analysis, including the Chao1 and Shannon indexes, was conducted using QIIME2. For β-diversity analysis, the principal coordinates analysis (PCoA) was performed using QIIME2 to investigate the dissimilarities in bacterial community structure among samples. The 16S rRNA gene sequences in this study were deposited into the National Center for Biotechnology Information (NCBI) database (PRJNA855329).

2.4. Metagenomic sequencing and ARG analysis

The DNA extracted from the layer chicken cecal, feather, uropygial gland and cage samples on days 5, 30, and 57 for 16S rRNA gene sequencing was simultaneously used for metagenomic sequencing analysis. Sequencing libraries were generated with the NEBNext® Ultra™ DNA Library Prep Kit (E7645S, NEB, United States) according to the manufacturer's recommendations. Then, the metagenomic libraries were sequenced on the Illumina HiSeq platform (Novogene, Beijing, China) using the 150-bp paired-end module. The raw reads were filtered and trimmed to obtain high-quality reads (clean reads), and quality control was performed using the following criteria: (1) reads with >10% unidentified nucleotides (*N*), (2) reads with ≥50% bases with mass fraction ≤20, and (3) reads aligned with barcodes were removed. The metagenomic sequences in this study were deposited into the NCBI database (PRJNA855329). We identified the taxonomic profiles (including kingdom, phylum, class, order, family, genus, and species information) of the metagenomic samples using kraken2. ARG-OAP v2.0 was applied to determine the ARG profiles (Yang et al., 2016). Briefly, potential ARG reads and 16S rRNA genes were extracted, and ARG-like reads were identified and annotated using the Perl package Ublastx_stageone X by combining the Comprehensive Antibiotic Resistance Database (CARD) and Antibiotic Resistance Gene Database (ARDB) (Yin et al., 2018). The normalized abundances of ARGs were expressed as "copies of ARGs per bacterial cell" (Jia et al., 2020). Species attribution analysis of resistance genes and resistance mechanism analysis were also conducted (McArthur et al., 2013; Jia et al., 2017).

2.5. SourceTracker

We used SourceTracker to evaluate the possible sources and proportions of microbial communities in the layer chicken cecum in the early stage. ASVs present in less than 1% of samples were first filtered, and the resultant ASV table was imputed with default parameters (Knights et al., 2011), with the layer chicken cecum at different days as the "sink" and the samples from different sources (feathers, uropygial gland, air, water, chicken feed and cage) identified as the "source." The SourceTracker algorithm was then used to estimate the probability that the species in the intestinal samples came from the source environment (probability>80%).

To identify transfer events involving ARGs, SourceTracker was run with the default settings using the feather and cage ARGs as the source.

2.6. Statistical analysis

Data preparation was performed in Microsoft Excel 2019 (Microsoft, United States). SPSS 20.0 (IBM Corp, USA) was used to assess statistical significance, and the results were visualized with GraphPad Prism 8.0 software. The threshold for significance was set at $p < 0.05$. PCoA was performed using the coverage correlation matrix of the ARG subtypes. Venn diagrams were drawn with the Venn Diagram package. Network plots of the ARGs and bacterial communities (species level) were generated with Cytoscape 3.9.1 software.

3. Results

3.1. ARG distribution characteristics and differences among layer chicken cecal, feather, and cage samples

A total of 620 ARG subtypes were detected in the layer chicken cecal, feather and cage samples, even though no antibiotics were used

on the farm. These ARGs were associated with 19 antibiotic types (350 β-lactam, 74 multidrug, 40 macrolide-lincosamide-streptogramin (MLS), 39 tetracycline, and 36 aminoglycoside resistance genes, among others) (Figure 1). The number of ARG subtypes in each group varied, ranging from 205 to 416. The 10 most abundant ARG subtypes in the different groups are summarized in Supplementary Table S1. The feathers on day 5 contained the most ARG types (416), and the total abundance was also the highest. The layer chicken cecum contained the fewest types (205), and there was a significant decrease in the total abundance of ARGs in the chick cecum over time (Figure 2A). These changes may be related to changes in gut microbes. Notably, no resistance genes were detected in the uropygial gland microbiome samples.

We then assessed the global similarity of ARG composition in each group based on principal coordinates analysis (PCoA). The results showed that the layer chicken cecum on day 5 differed from that on day 30 and day 57 in terms of ARGs, and the cage ARGs from different days clustered together, indicating that the ARGs composition in different groups changed over time. In addition, the results showed that the layer chicken cecum ARGs on day 5 were overall more similar to the feather ARGs than to the cage ARGs (Figure 2B), implying that the feather ARGs might have been transmitted to the layer chicken cecum on day 5.

A total of 123 ARGs belonging to 17 types were shared by the layer chicken cecal, feather and cage samples on different days (Figure 2C, summarized in Supplementary Table S2). In terms of total coverage, these 123 shared ARGs contributed to 97.01 ± 0.79%, 95.13 ± 0.08%, and 91.15 ± 0.10% of the total ARGs detected in the chick cecal and feather samples on day 5; 84.80 ± 3.4%, 63.17 ± 0.29%, and 93.30 ± 0.83% on day 30; and 82.2 ± 4.3%, 67.89 ± 0.36%, and 94.34 ± 0.17% on day 57 (Supplementary Figure S2). These shared ARGs included 34 multidrug resistance genes (*emrA*, *emrB*, *mdtA*, *TolC*, etc.) and 13 tetracycline resistance genes (*tetA*, *tetW*, *totO*, *tet44*, etc.). There were more unique ARGs in the cage and feather samples than in the layer chicken cecal samples.

3.2. Microbial community composition in different microbial sources

Supplementary Figure S3 shows the alpha diversity of different groups. Both the richness index and Shannon index revealed an increase in microbial diversity in the layer chicken cecum over time, and there was a significant difference in alpha diversity among the microbial sample types from different sources (uropygial gland>cage>feather; $p < 0.05$).

The results showed that Proteobacteria (48.31 ± 9.18%) and Firmicutes (47.32 ± 7.62%) were the major phyla in the layer chicken cecum on day 5 (Figure 3); at the genus level, they were dominated by *Escherichia* (36.83 ± 8.53%) and *Flavonifractor* (15.44 ± 4.61%) (Supplementary Figure S4). Similarly, increased relative abundances of Bacteroidetes and Actinobacteria and a

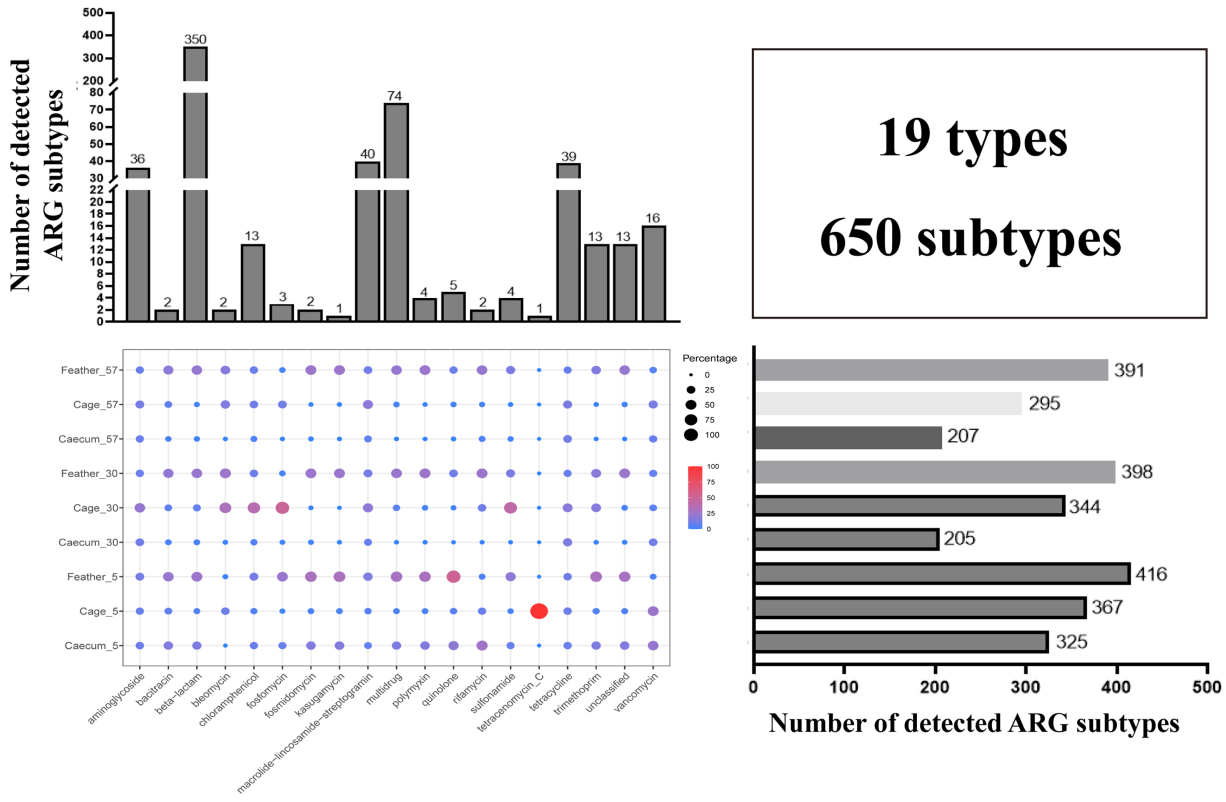

FIGURE 1
Statistics of ARG diversity between groups. The total number of detected ARG subtypes in different ARG types in all samples is shown on top, the total number of detected ARG subtypes in different groups is shown on the right, and the bubble chart shows the relative abundance of each ARG type in different groups.

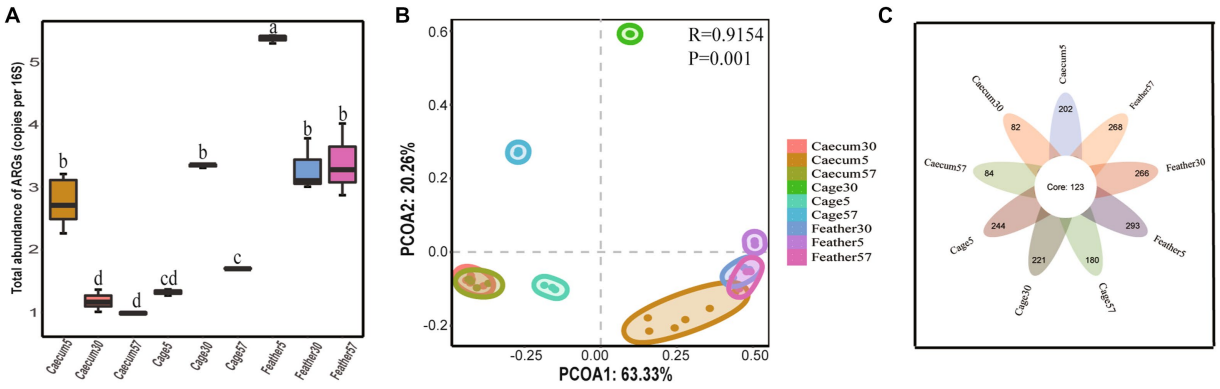

FIGURE 2
ARG distribution characteristics. **(A)** The total abundance of ARGs in different groups. In the box chart, "□" represent the means, boxes represent the upper and lower quartiles, lines represent the medians, and whiskers represent ranges. **(B)** PCoA plots showing the ARG composition differences among groups. **(C)** Venn diagram showing the number of shared and unique ARGs among groups.

decreased relative abundance of Proteobacteria were observed in the layer chicken cecum ($p < 0.05$) on day 30 and day 57. *Bacteroides* and *Alistipes* were the major genera in the layer chicken cecum on days 30 and 57. Furthermore, among the different microbial sources, Proteobacteria, Firmicutes and Actinobacteria were the major phyla in the uropygial gland samples, Firmicutes were dominated by *Escherichia* in the feather samples, and Firmicutes were dominated by *Lactobacillus* in the cage samples.

Further analysis showed shared and unique bacteria among the layer chicken cecal, feather, uropygial gland and cage samples (Supplementary Figure S5). The results showed that between days 5 and 57, the number of microbial species increased from 2,998 to 4,708, indicating that the gut microbes early in life are derived from a dramatic and complex transition from a near-sterile state to extremely dense colonization. More bacterial species (2885) in the layer chicken cecum were shared with the uropygial gland than with the other groups on day 5. At days 30 and 57, the layer chicken cecum shared a high number (2,869 and 3,379 respectively) of microbial species with the cage samples, and some opportunistic pathogens, such as *Escherichia* and *Enterococcus* species, were also shared among the cage, feather, uropygial gland and layer chicken cecal samples.

3.3. Layer chicken microbial sources and estimated proportions of the early gut microbial communities

To investigate the development and potential sources of gut microbiota in layer chickens, we collected cecal contents and corresponding environmental samples, including feather, uropygial gland, water, air, feed, and cage microbiota samples at post-hatching days 0, 1, 3, 5, 7, 12, 18, 24, 30, 36, 40, 43, 50, and 57. The PCoA ordination based on Bray–Curtis dissimilarity showed that the early layer chicken cecal samples (at days 0 and 7) clustered with the layer chicken uropygial gland and feather samples, but they gradually diverged with age, eventually showing some similarities with the cage sample (Figure 4). These results suggest that the uropygial gland, feather, and cage microbiomes may play a role in the early colonization of the cecal microbiome in layer chickens, but further investigation is needed to determine the extent and mechanisms of transmission.

SourceTracker, a Bayesian probability tool, was used to further investigate how different microbial sources contributed to the cecal community assembly of hatchlings. The results revealed that the feather and uropygial gland microbiota contributed the most to the cecal (day 0) microorganisms compared with other sources, with contributions of 47.0 and 38.6%, respectively, but their contributions gradually declined with age. Interestingly, the relative contribution of the cage microbiota to the chick cecal microbiome was more than 12% at all stages, indicating that the cage may be the primary environmental source of bacterial communities in layer chickens, especially seven days after hatching (Figure 5). To our surprise, there was little evidence that water and air microbes colonized hatchlings that had a microbiota from largely unknown sources.

Next, we sought to identify specific genera transmitted from different microbial sources to the layer chicken cecum. These transmission events included diverse taxa from Firmicutes, Proteobacteria and Bacteroidetes, some of which (*Escherichia*, *Enterococcus*, *Ruminococcaceae*, and *Helicobacter*) include opportunistic pathogenic strains responsible for zoonotic infections (Supplementary Figure S6). Among them, *Escherichia* was the main bacterial taxon transmitted from feathers to the layer chicken cecum at day 0, with a contribution rate of 40.8%, and *Ruminococcaceae* was mainly transmitted to the cecum through the cage, with a contribution rate of 3.0%. The transmission of these opportunistic pathogenic strains indicates that the surroundings during feeding may pose underappreciated occupational hazards in industrialized farming. To confirm that the species and their resistance genes were environmentally acquired, we performed metagenomic analysis of the layer chicken cecal, feather, uropygial gland and cage microbiome samples at days 5, 30, and 57.

3.4. Transmission of microbes and ARGs from cages and feathers to the layer chicken cecum

The results of the SourceTracker algorithm analysis showed that, on day 5, 30% of the layer chicken cecal ARGs were from the cage,

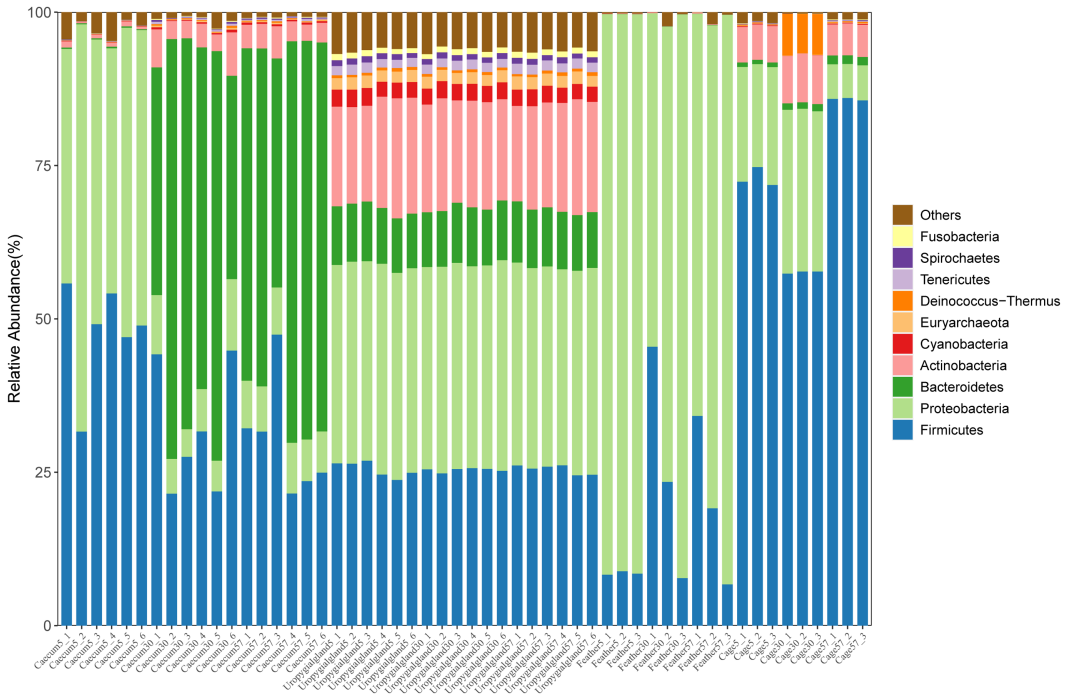

FIGURE 3
Relative abundances of the top 10 phyla in the layer chicken cecal, feather, uropygial gland and cage samples.

57% were from feathers and 13% were from unknown sources. The contribution of cage ARGs became progressively more prominent with age, and cage ARGs contributed 63.3 and 69.5% of the layer chicken cecal ARGs between days 30 and 57 (Figure 6A). In addition, we identified 290 ARGs involved in transfer from cage and feather samples to the layer chicken cecum, including 152 β-lactam resistance genes, 45 multidrug resistance genes, 21 tetracycline resistance genes and 17 MLS resistance genes. Among them, 240 ARGs contributed more than 0.1% (summarized in Supplementary Table S3).

Members of the bacterial community are the main hosts of ARGs. To identify the ARG hosts more accurately, metagenomics was used to analyze the species attributions of ARGs. A total of 4,364 species were the main 240 ARG hosts. However, not all ARG-carrying bacteria can be transmitted from cages and feathers to the chick cecum. The SourceTracker algorithm was again used to assess bacterial transmission from the cage and feather to the layer chicken cecal samples (Figure 6B). The results showed that on day 5, 54.4% of the layer chicken cecal microbes originated from cages, 41% from feathers, 0.32% from the uropygial gland and 4.2% from unknown sources. The contribution of cage microbes gradually increased to 77.3% on day 30, and that of the uropygial gland microbes to cecal microbes increased to 11.8%, while the contribution of feathers to the cecum decreased sharply and was negligible. At day 57, the contribution of the cage microbes to cecal microbes decreased slightly to 63%, and the contribution of the uropygial gland was 7.1%. A ternary plot was used to more intuitively reflect the contribution of various bacterial sources to each cecal microbiome of layer chickens on different days. As shown in the plot, at day 5, almost all the layer chicken samples showed a uniform distribution between feather and cage samples, and the layer chicken cecal samples gradually became increasingly closely related to the cage samples over time (Supplementary Figure S7), which was similar to the results of the ARG source analysis.

Among them, we identified 85 species that contributed more than 0.1% (Figure 6C). The cage contributed the greatest number of bacterial species (57) on day 5, and the highest contributions were *Bacteroides fragilis* (11.5%), *Bacteroides dorei* (6.5%) and *Odoribacter splanchnicus* (4.8%). The feathers contributed 15 kinds of bacteria, and the highest contributions were *Escherichia coli* (32.0%) and *Klebsiella pneumoniae* (3.4%). The uropygial gland contributed only one bacterium, *Clostridium butyricum*, and the contribution was low (0.12%). On day 30, the cage contributed the highest number of bacterial species (56), including *B. fragilis* (38.0%), *Flavonifractor plautii* (3.2%) and *Clostridiales bacterium CCNA10* (3.0%), and the uropygial gland contributed 8 bacterial species, including *B. fragilis* (3.1%) and *Bacteroides ovatus* (0.6%). On day 57, the number of bacterial species contributed by the cage increased to 59, including *B. fragilis* (11.5%), *B. dorei* (6.5%), *O. splanchnicus* (4.8%) and *Faecalibacterium prausnitzii* (3.1%). The number of bacterial species contributed by the uropygial gland decreased to 4, namely, *B. fragilis* (1.1%), *Alistipes finegoldii* (0.6%), *O. splanchnicus* (0.5%) and *B. dorei* (0.4%).

Next, we analyzed the correlations between 85 bacterial species and 240 ARGs, and the results indicated that 63 bacterial species were speculated to be possible hosts of 60 ARGs (Figure 7A). For instance, *K. pneumoniae* was a potential host for 32 ARG subtypes, 23 of which were multidrug resistance genes (*acrA*, *emrK*, *mdfA*, *mdtB*, *mexA*, *mtrE*, etc.), 3 were tetracycline resistance genes (*tetQ*, *tetS* and *tetX*), 3 were MLS resistance genes (*lmrB*, *mscrC* and *vgaD*), 2 were vancomycin resistance genes (*vanA* and *vanC*) and 1 was a quinolone resistance gene (*norB*). *E. coli* was a potential host for 24 ARG subtypes, 16 of which were multidrug resistance genes

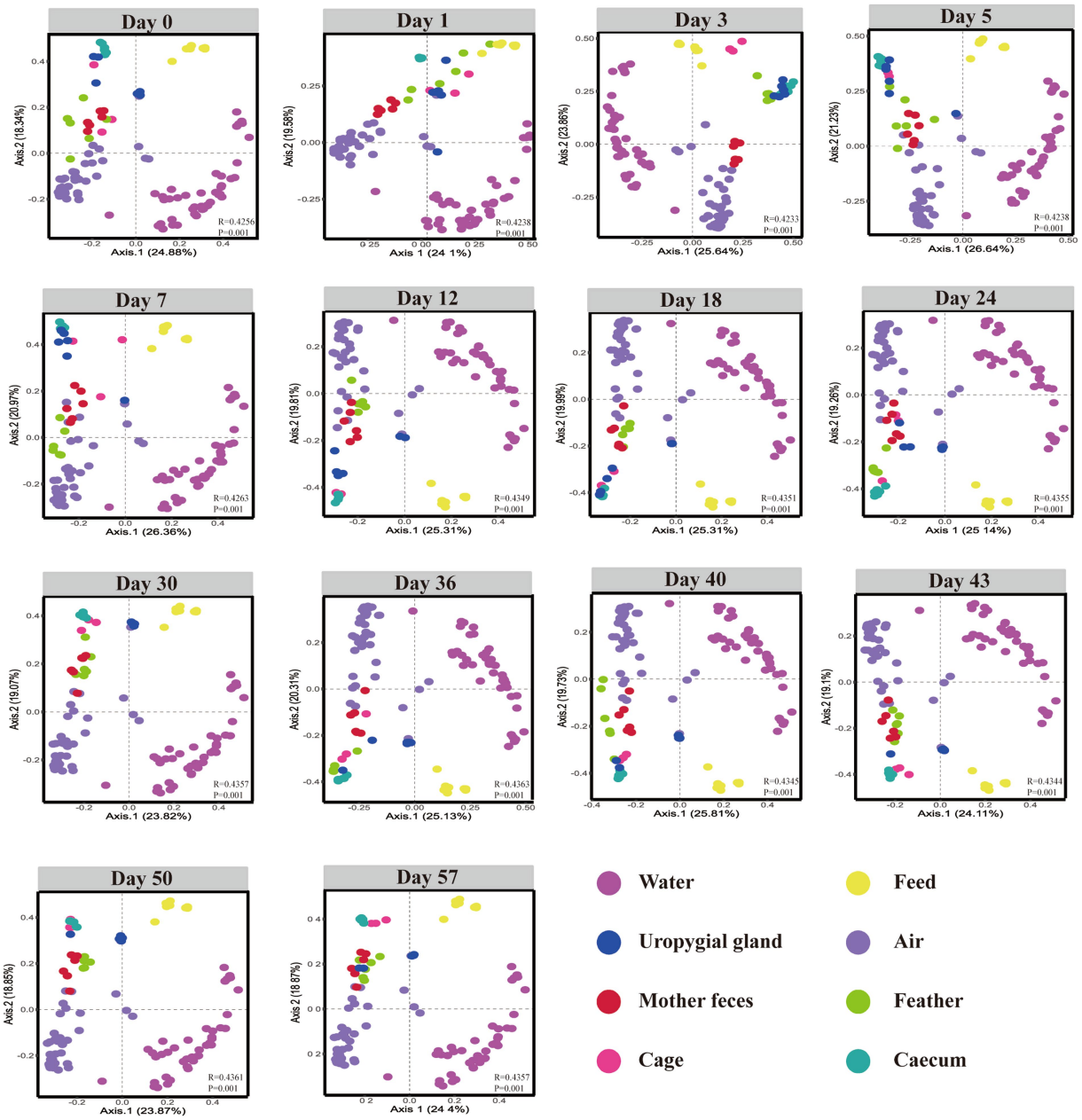

FIGURE 4
β-Diversity of the cecal, feed, maternal fecal, uropygial gland, feather, water, air and cage samples. Bray–Curtis dissimilarity was calculated using the abundance of ASVs.

(*acrA*, *emrK*, *mdtA*, etc.), 3 were MLS resistance genes (*msrC*, *vatD*, *vatE* and *vgaD*), 2 were tetracycline resistance genes (*tetQ* and *tetX*), 1 was a β-lactam resistance gene (*ampC*) and 1 was a vancomycin resistance gene (*vatC*). *B. fragilis* was a potential host for 13 ARG subtypes, such as *acrA* and *emrB*. In addition, *Intestinimonas butyriciproducens* and *Clostridioides difficile* were the predominant hosts and harbored most of the diverse ARG subtypes for multidrug resistance. Figures 7B-D shows the 11 bacteria with the highest number of resistance gene types. The results show that the relative abundances of *K. pneumoniae* and *E. coli* gradually decreased, but those of *L. salivarius L. bacterium KGMB03038* gradually increased. These results suggest an extensive exchange of antibiotic-resistant bacteria between layer chickens and their surrounding environments.

4. Discussion

By the metagenomic shotgun sequencing method, we identified 650 ARGs across layer chicken cecal, feather and cage samples on days 5, 30, and 57, and more diverse ARGs were observed in the cage and feather samples than in the layer chicken cecal sample. A total of 123 ARGs were shared among all samples, and these were mainly multidrug resistance genes and tetracycline resistance genes. The

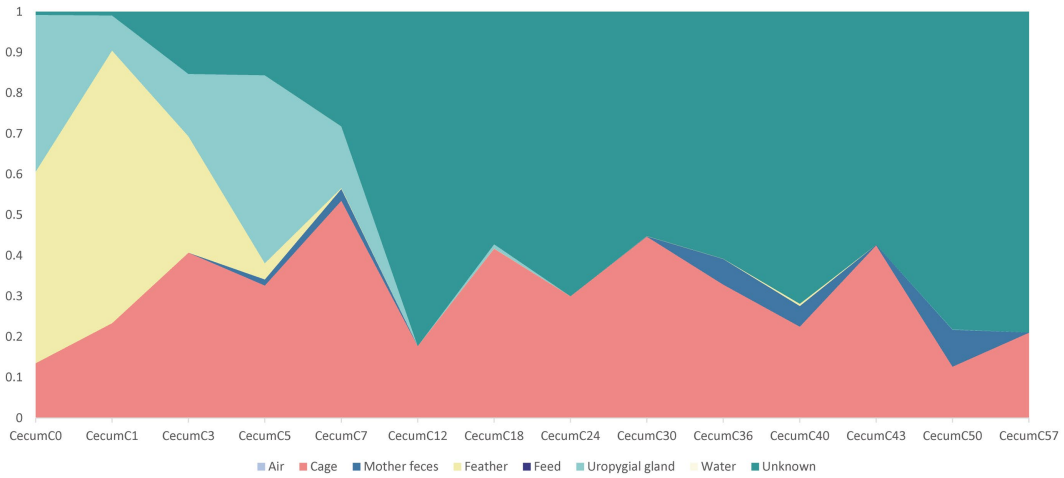

FIGURE 5
Dynamic contributions of different microbial sources to the layer chickens cecal microbiota during the first 57 days. The proportion of the microbiota from cecal samples of layer chicken was estimated as originating from different sources (colored regions) using bacterial source tracking.

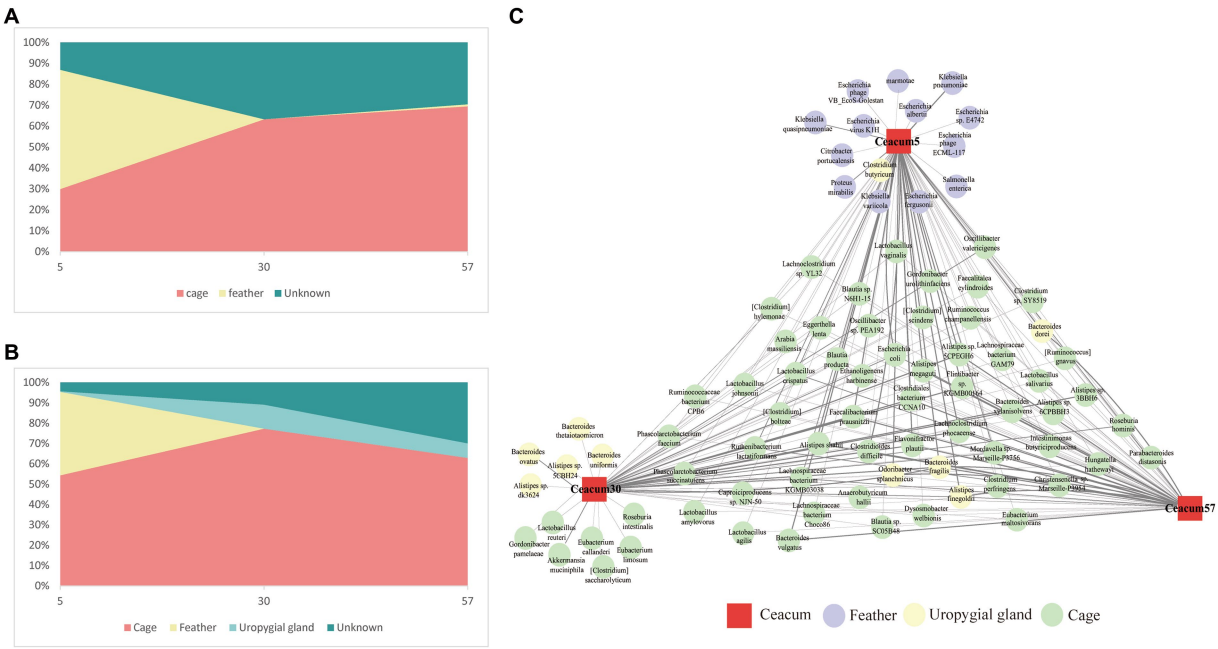

FIGURE 6
Transmission of microbes and ARGs from feathers and cages to the layer chicken cecum. **(A)** Predicted proportions of ARGs in the layer chicken gut microbiota at different days after hatching (5, 30, and 57) that originated from their feathers and cages. **(B)** Proportion of the microbiota from the cecal samples of layer chickens at different days estimated as having originated from the uropygial gland, feather and cage. **(C)** Microbial species transmission network from the feather and cage to the layer chicken cecum. The red square depicts the intestine (different days are displayed in the center of each node), circles indicate transmitted species, connecting arrows represent the transmission events, and the edge thickness is equivalent to the magnitude of the contribution.

contribution rates of 123 shared ARGs to the gut ARGs of layer chickens were all more than 82%, suggesting that the environment is an important contributor to the gut ARGs in layer chickens (Ding et al., 2022).

The number and total abundance of gut ARGs in layer chicken decreased significantly over time, consistent with previous studies showing that the fecal ARG abundance in the brooding period was higher than that in the growing period (Zhu et al., 2021). The microbial community was the key factor that directly affected the ARGs (Wang et al., 2020). We identified ARG hosts using metagenomic assembly-based host-tracking analysis. The results showed that *K. pneumoniae*, *E. coli*, *I. butyriciproducens* and *C. difficile* were the main host bacteria of ARGs. The relative abundance of these ARG-carrying bacteria gradually decreased in the layer chickens, which may explain the decrease in total ARG abundance. It has been suggested that *K. pneumoniae* has a flexible ability to accumulate and

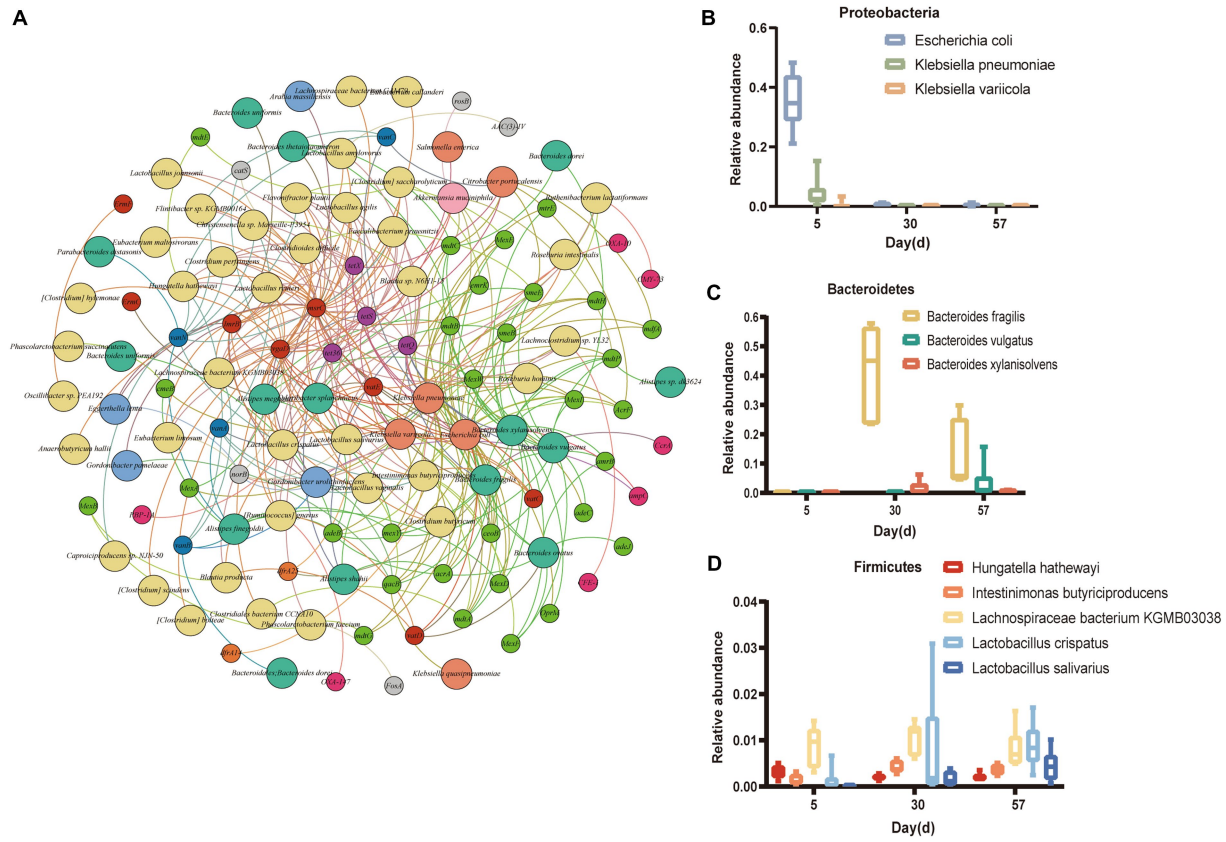

FIGURE 7
Microbial (species-level) attributions of ARGs. **(A)** The connecting line between two points indicates an affiliation. For example, emrk is linked to *Escherichia coli*, indicating that *Escherichia coli* contained *emrk*. The large nodes represent bacterial phyla, and the small nodes represent ARG types. The nodes are colored in accordance with the ARG types or phyla. **(B-D)** Relative abundances of the top 11 ARG-carrying bacteria with the largest number of ARG type variations over time [Proteobacteria **(B)**, Bacteroidetes **(C)** and Firmicutes **(D)**].

switch resistance and is the host of many ARGs; together with other highly important multidrug-resistant pathogens, it has been classified as an ESKAPE organism (Navon-Venezia et al., 2017). In our study, we found that *K. pneumoniae* was the host of 32 ARGs. In addition, *E. coli* is also a common host of ARGs; antibiotic-resistant *E. coli* strains carrying different ARGs have been widely studied as possible environmental pollutants in recent years, and their dissemination poses potential risks to human health (Power et al., 2016; Chika et al., 2019; Zhang et al., 2020). We found that *E. coli* harbored 24 ARGs, including *acrA*, *tetX*, and *lmrB*. Zhou et al. (2020) found that the poultry microbial community shifts after antibiotic administration were mainly induced by increased abundances of the families *Escherichia/Shigella* and *Klebsiella*. However, no antibiotics were used during the whole experiment, and the relative abundance of *Escherichia* peaked at day 5, which was gradually replaced by the probiotic *Bacteroides*. This suggests that banning the use of antibiotics plays a positive role in maintaining gut microbial homeostasis and reducing ARG levels in laying hens. It is worth noting that this study found that probiotics (e.g., *B. fragilis*) are also potential hosts. It has been shown that polysaccharide A (PSA) of *B. fragilis* is the archetypical example of a commensal molecule that can modulate the host immune system in health and disease (Erturk-Hasdemir and Kasper, 2018). In this experiment, we found that *B. fragilis* is the host for *tetQ* and *tetS*. This suggests that probiotics may contain ARGs. Feeding with probiotics carrying a high abundance of ARGs may further increase the risk of ARG transmission in the environment.

Furthermore, through SourceTracker analysis of the sources of related ARGs and microbes, it was found that the environment contributed more to the shaping of the gut microbiota, and the contribution of adult hen to the layer chickens only 2.5% at day7. However the cecal microbiota of chicks remaining in contact with an adult hen developed quickly and within a week reached a composition similar to that observed in adult hen (Kubasova et al., 2019). This was mainly because in commercial laying hen production, the microbes attached to the surface of the eggshell are killed because of the treatment of eggs (disinfection and fumigation) before hatching (Videnska et al., 2014). Direct contact between chicks and parents after hatching does not occur as in wild birds, so the external environment plays a vital role in shaping the gut microbial community in layer chickens (Maki et al., 2020). In this study, beta and SourceTracker analysis revealed that layer chicken gut microbes were more similar to feather and uropygial gland microbes after hatching, which has not been reported previously. We speculate that this may be related to the commercial production mode, in which layer chickens that hatch at the same time are usually fed together and are afraid of the cold, easily frightened and have a clustering habit (Appleby et al., 2004). In addition, compared with free layer chickens, caged layer chickens feed daily due to their appetite, so the nutritional needs of layer chickens

can be satisfied sooner, while the feeding density is large and the activity space is restricted, thus causing layer chicken psychological tension and anxiety, which can easily induce feather pecking and anal pecking (Tahamtani et al., 2022). Therefore, feather and uropygial gland microbes, including *E. coli*, *K. pneumoniae* and *C. butyricum*, are more likely to be transmitted to the gut for colonization. Among them, *K. pneumoniae* and *E. coli* are the main hosts of ARGs. It is worth noting that the SourceTracker results of the ARGs were similar to those of microorganisms. Early ARGs came mainly from feathers. These results suggest that the early gut ARGs in layer chickens may originate *via* the transmission of microbes from feathers.

However, cage microbes gradually became the major source of gut microbes in layer chickens. Although the cage was cleaned and disinfected before raising chickens, the frequent activities of layer chickens in the cage created a unique microbial environment. Studies have shown that feather pecking is negatively correlated with ground pecking and that a decrease in feather pecking is associated with an increase in ground pecking (Aneja et al., 2008). Frequent ground pecking in poultry leads to horizontal transmission of microbes, and cage microbes may originate from the feces of layer chickens, dust in the air, water and feed residues. However, the contribution of air and water bacteria to the colonization of the layer chicken cecum was very low in our study. The reason for this may be that the aerobic bacteria in the air did not adapt well to the anaerobic environment of the layer chicken gut. Volf et al. (2021) found that the feed microbiota and water microbiota were not the major sources of gut anaerobes for chickens in commercial production. Litter microbes have a great influence on poultry gut microbes in commercial production (Wang et al., 2016). Cressman et al. (2010) showed the cycling of certain bacteria between the litter and gut of poultry. The same results were found in commercially produced lambs and piglets (Bi et al., 2019; Chen et al., 2020). The results showed that the early gut was more easily colonized by cage microbes when the chicks were not in contact with their parents. The frequent exchange of ARG-carrying bacteria between the gut and cage of layer chickens may lead to the accumulation of ARGs in the gut and the environment, posing a threat to animal and environmental safety.

Although, in this study, we carried out comprehensive sampling of commercial layer chickens, the samples were used for SourceTracker analysis of gut microbes and ARGs in layer chickens, and the study still had several limitations. For example, since the experimental site was a brood farm, samples could not be collected during the laying period for a more comprehensive and systematic assessment of microbial and ARG transmission.

Despite these shortcomings, we can still provide some suggested management strategies for layer chickens. In daily management, (1) attention should be given to reducing the stimulation of layer chickens and reducing rearing density to reduce the feather pecking and anal pecking behavior of layer chickens. (2) The cages should be kept clean and hygienic, and any remaining feces and food residues in the cages should be promptly removed. This will not only reduce the transmission of ARGs and ARG host bacteria from the environment to the layer chicken gut but also help improve animal welfare.

5. Conclusion

There was a high relative abundance of ARGs in the layer chicken cecal, feather and cage samples, including ARGs that confer resistance to multiple drugs, such as tetracycline, aminoglycosides, and β-lactams. The layer chicken cecal ARGs originated mainly from cage. The variations in ARG profiles in layer chicken cecal samples and identified the bacterial species that primarily influence these changes, including *K. pneumoniae*, *E. coli*, *B. fragilis*, and *B. dorei* Our findings indicate that these bacteria are mainly transmitted from the cage to the layer chicken cecum. The cycling of ARG-carrying bacteria between these two environments may result in the accumulation of ARGs in the gut and cage environment, ultimately posing a potential risk to animal health. It is noteworthy that *B. fragilis* is an opportunistic pathogen that may cause diarrhea in laying hens, suggesting that the transmission of opportunistic pathogens may pose a dual risk. However, further experiments are needed to verify this risk. These results can provide reference data for the healthy breeding of layer chickens and the prevention and control of ARG pollution.

Data availability statement

The data presented in the study are deposited in the National Center for Biotechnology Information (NCBI) repository, accession number PRJNA855329 can be found in the article/Supplementary material.

Ethics statement

The present study followed the institutional guidelines for the care and use of animals, and all experimental procedures involving animals were approved by the Animal Experimental Committee of South China Agricultural University (Ethics Approval Code: SYXK 2014–0136).

Author contributions

SX: conceptualization, data curation, formal analysis, methodology, writing—original draft, and writing—review and editing. JM: data curation, conceptualization, methodology, and supervision. YC: conceptualization, investigation, methodology, formal analysis, and data curation. KF and LM: methodology, data curation. XL and YW: conceptualization and supervision. Yan Wang: conceptualization, investigation, writing—review and editing, resources, supervision, and funding. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1177404/full#supplementary-material

References

Aneja, V. P., Blunden, J., Roelle, P. A., Schlesinger, W. H., Knighton, R., Niyogi, D., et al. (2008). Workshop on agricultural air quality: state of the science. *Atmos. Environ.* 42, 3195–3208. doi: 10.1016/j.atmosenv.2007.07.043

Appleby, M. C., Mench, J. A., and Hughes, B. O. (2004). Poultry behaviour and welfare. Cambridge: CABI Publishing.

Bi, Y., Cox, M. S., Zhang, F., Suen, G., Zhang, N., Tu, Y., et al. (2019). Feeding modes shape the acquisition and structure of the initial gut microbiota in newborn lambs. *Environ. Microbiol.* 21, 2333–2346. doi: 10.1111/1462-2920.14614

Bokulich, N., Subramanian, S., Faith, J., Gevers, D., Gordon, J., Knight, R., et al. (2013). Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. *Nat. Methods* 10, 57–59. doi: 10.1038/nmeth.2276

Chen, C., Chen, C., Chen, Y., Fang, A., Shaw, G., Hung, C., et al. (2020). Maternal gut microbes shape the early-life assembly of gut microbiota in passerine chicks via nests. *Microbiome* 8, 1–13. doi: 10.1186/s40168-020-00896-9

Chika, F., and Odum, O. N. (2019). Freshwater environments as reservoirs of antibiotic resistant bacteria and their role in the dissemination of antibiotic resistance genes. *Environ. Pollut.* 254:113067. doi: 10.1016/j.envpol.2019.113067

Chin, C. Y., Tipton, K. A., Farokhyfar, M., Burd, E. M., Weiss, D. S., and Rather, P. N. (2018). A high-frequency phenotypic switch links bacterial virulence and environmental survival in *Acinetobacter baumannii*. *Nat. Microbiol.* 3, 563–569. doi: 10.1038/s41564-018-0151-5

Cressman, M. D., Yu, Z., Nelson, M. C., Moeller, S. J., Lilburn, M. S., and Zerby, H. N. (2010). Interrelations between the microbiotas in the litter and in the intestines of commercial broiler chickens. *Appl. Environ. Microbiol.* 76, 6572–6582. doi: 10.1128/AEM.00180-10

Diaz Carrasco, J. M., Casanova, N. A., and Fernández Miyakawa, M. E. (2019). Microbiota, gut health and chicken productivity: what is the connection? *Microorganisms.* 7:374. doi: 10.3390/microorganisms7100374

Ding, J., Dai, R., Yang, L., He, C., Xu, K., Liu, S., et al. (2017). Inheritance and establishment of gut microbiota in chickens. *Front. Microbiol.* 8:1967. doi: 10.3389/fmicb.2017.01967

Ding, D., Zhu, J., Gao, Y., Yang, F., Ma, Y., Cheng, X., et al. (2022). Effect of cattle farm exposure on oropharyngeal and gut microbial communities and antibiotic resistance genes in workers. *Sci. Total Environ.* 806:150685. doi: 10.1016/j.scitotenv.2021.150685

Erturk-Hasdemir, D., and Kasper, D. L. (2018). Finding a needle in a haystack: *Bacteroides fragilis* polysaccharide a as the archetypical symbiosis factor. *Ann. N. Y. Acad. Sci.* 1417, 116–129. doi: 10.1111/nyas.13660

He, L.-Y., Liu, Y.-S., Su, H.-C., Zhao, J.-L., Liu, S.-S., Chen, J., et al. (2014). Dissemination of antibiotic resistance genes in representative broiler feedlots environments: identification of indicator ARGs and correlations with environmental variables. *Environ. Sci. Technol.* 48, 13120–13129. doi: 10.1021/es5041267

Hu, Y., and Cheng, H. (2016). Health risk from veterinary antimicrobial use in China's food animal production and its reduction. *Environ. Pollut.* 219, 993–997. doi: 10.1016/j.envpol.2016.04.099

Jia, S., Bian, K., Shi, P., Ye, L., and Liu, C. H. (2020). Metagenomic profiling of antibiotic resistance genes and their associations with bacterial community during multiple disinfection regimes in a full-scale drinking water treatment plant. *Water Res.* 176:115721. doi: 10.1016/j.watres.2020.115721

Jia, B., Raphenya, A., Alcock, B., Waglechner, N., Guo, P., Tsang, K., et al. (2017). CARD 2017: expansion and model-centric curation of the comprehensive antibiotic resistance database. *Nucleic Acids Res.* 45, D566–D573. doi: 10.1093/nar/gkw1004

Knights, D., Kuczynski, J., Charlson, E., Zaneveld, J., Mozer, M., Collman, R., et al. (2011). Bayesian community-wide culture-independent microbial source tracking. *Nat. Methods* 8, 761–763. doi: 10.1038/nmeth.1650

Kubasova, T., Kollarcikova, M., Crhanova, M., Karasova, D., Cejkova, D., Sebkova, A., et al. (2019). Contact with adult hen affects development of caecal microbiota in newly hatched chicks. *PLoS One* 14:e0212446. doi: 10.1371/journal.pone.0212446

Lee, S., La, T.-M., Lee, H.-J., Choi, I.-S., Song, C.-S., Park, S.-Y., et al. (2019). Characterization of microbial communities in the chicken oviduct and the origin of chicken embryo gut microbiota. *Sci. Rep.* 9, 1–11. doi: 10.1038/s41598-019-43280-w

Li, X., Stokholm, J., Brejnrod, A., Vestergaard, G. A., Russel, J., Trivedi, U., et al. (2021). The infant gut resistome associates with *E. coli*, environmental exposures, gut microbiome maturity, and asthma-associated bacterial composition. *Cell Host Microbe* 29:e974, 975–987. doi: 10.1016/j.chom.2021.03.017

Liu, H., Zeng, X., Zhang, G., Hou, C., Li, N., Yu, H., et al. (2019). Maternal milk and fecal microbes guide the spatiotemporal development of mucosa-associated microbiota and barrier function in the porcine neonatal gut. *BMC Biol.* 17, 1–15. doi: 10.1186/s12915-019-0729-2

Maki, J. J., Bobeck, E. A., Sylte, M. J., and Looft, T. (2020). Eggshell and environmental bacteria contribute to the intestinal microbiota of growing chickens. *J. Anim. Sci. Biotechnol.* 11, 1–17. doi: 10.1186/s40104-020-00459-w

McArthur, A. G., Waglechner, N., Nizam, F., Yan, A., Azad, M. A., Baylay, A. J., et al. (2013). The comprehensive antibiotic resistance database. *Antimicrob. Agents Chemother.* 57, 3348–3357. doi: 10.1128/AAC.00419-13

Navon-Venezia, S., Kondratyeva, K., and Carattoli, A. (2017). *Klebsiella pneumoniae*: a major worldwide source and shuttle for antibiotic resistance. *FEMS Microbiol. Rev.* 41, 252–275. doi: 10.1093/femsre/fux013

Power, M. L., Samuel, A., Smith, J. J., Stark, J. S., Gillings, M. R., and Gordon, D. M. (2016). *Escherichia coli* out in the cold: dissemination of human-derived bacteria into the Antarctic microbiome. *Environ. Pollut.* 215, 58–65. doi: 10.1016/j.envpol.2016.04.013

Pu, Q., Zhao, L.-X., Li, Y.-T., and Su, J.-Q. (2020). Manure fertilization increase antibiotic resistance in soils from typical greenhouse vegetable production bases. *J. Hazard Mater* 391:122267. doi: 10.1016/j.jhazmat.2020.122267

Seidlerova, Z., Kubasova, T., Faldynova, M., Crhanova, M., Karasova, D., Babak, V., et al. (2020). Environmental impact on differential composition of gut microbiota in indoor chickens in commercial production and outdoor, backyard chickens. *Microorganisms* 8:767. doi: 10.3390/microorganisms8050767

Tahamtani, F. M., Kittelsen, K., and Vasdal, G. (2022). Environmental enrichment in commercial flocks of aviary housed laying hens: relationship with plumage condition and fearfulness. *Poult. Sci.* 101:101754. doi: 10.1016/j.psj.2022.101754

Tamburini, S., Shen, N., Wu, H. C., and Clemente, J. C. (2016). The microbiome in early life: implications for health outcomes. *Nat. Med.* 22, 713–722. doi: 10.1038/nm.4142

Van Boeckel, T. P., Pires, J., Silvester, R., Zhao, C., Song, J., Criscuolo, N. G., et al. (2019). Global trends in antimicrobial resistance in animals in low-and middle-income countries. *Science* 365:eaaw1944. doi: 10.1126/science.aaw1944

Videnska, P., Sedlar, K., Lukac, M., Faldynova, M., Gerzova, L., Cejkova, D., et al. (2014). Succession and replacement of bacterial populations in the Caecum of egg laying hens over their whole life. *PLoS One* 9:e115142. doi: 10.1371/journal.pone.0115142

Volf, J., Crhanova, M., Karasova, D., Faldynova, M., Kubasova, T., Seidlerova, Z., et al. (2021). Eggshell and feed microbiota do not represent major sources of gut anaerobes for chickens in commercial production. *Microorganisms* 9:1480. doi: 10.3390/microorganisms9071480

Wang, Z., Han, M., Li, E., Liu, X., and Ning, K. (2020). Distribution of antibiotic resistance genes in an agriculturally disturbed lake in China: their links with microbial communities, antibiotics, and water quality. *J. Hazard. Mater.* 393:122426. doi: 10.1016/j.jhazmat.2020.122426

Wang, Y., Hu, Y., Cao, J., Bi, Y., Lv, N., Liu, F., et al. (2019). Antibiotic resistance gene reservoir in live poultry markets. *J. Infect.* 78, 445–453. doi: 10.1016/j.jinf.2019.03.012

Wang, L., Mike, L., and Yu, Z. (2016). Intestinal microbiota of broiler chickens as affected by litter management regimens. *Front. Microbiol.* 7:593. doi: 10.3389/fmicb.2016.00593

Xiao, S., Mi, J., Mei, L., Liang, J., Feng, K., Wu, Y., et al. (2021). Microbial diversity and community variation in the intestines of layer chickens. *Animals* 11:840. doi: 10.3390/ani11030840

Yang, Y., Chen, N., Sun, L., Zhang, Y., Wu, Y., Wang, Y., et al. (2021). Short-term cold stress can reduce the abundance of antibiotic resistance genes in the cecum and feces in a pig model. *J. Hazard. Mater.* 416:125868. doi: 10.1016/j.jhazmat.2021.125868

Yang, Y., Jiang, X., Chai, B., Ma, L., Li, B., Zhang, A., et al. (2016). ARGs-OAP: online analysis pipeline for antibiotic resistance genes detection from metagenomic data using an integrated structured ARG-database. *Bioinformatics* 32, 2346–2351. doi: 10.1093/bioinformatics/btw136

Yin, X., Jiang, X., Chai, B., Li, L., Yang, Y., Cole, J., et al. (2018). ARGs-OAP v2.0 with an expanded SARG database and hidden Markov models for enhancement characterization and quantification of antibiotic resistance genes in environmental metagenomes. *Bioinformatics* 34, 2263–2270. doi: 10.1093/bioinformatics/bty053

Zhang, S., Abbas, M., Rehman, M., Huang, Y., Zhou, R., Gong, S., et al. (2020). Dissemination of antibiotic resistance genes (ARGs) via integrons in *Escherichia coli*: a risk to human health. *Environ. Pollut.* 266:115260. doi: 10.1016/j.envpol.2020.115260

Zhao, Y., Su, J.-Q., An, X.-L., Huang, F.-Y., Rensing, C., Brandt, K. K., et al. (2018). Feed additives shift gut microbiota and enrich antibiotic resistance in swine gut. *Sci. Total Environ.* 621, 1224–1232. doi: 10.1016/j.scitotenv.2017.10.106

Zhou, Y., Li, Y., Zhang, L., Wu, Z., and Wang, H. H. (2020). Antibiotic administration routes and Oral exposure to antibiotic resistant Bacteria as key drivers for gut microbiota disruption and Resistome in poultry. *Front. Microbiol.* 11:1319. doi: 10.3389/fmicb.2020.01319

Zhu, T., Chen, T., Cao, Z., Zhong, S., Wen, X., Mi, J., et al. (2021). Antibiotic resistance genes in layer farms and their correlation with environmental samples. *Poult. Sci.* 100:101485. doi: 10.1016/j.psj.2021.101485

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The emergence of multi-drug resistant and virulence gene carrying *Escherichia coli* strains in the dairy environment: a rising threat to the environment, animal, and public health

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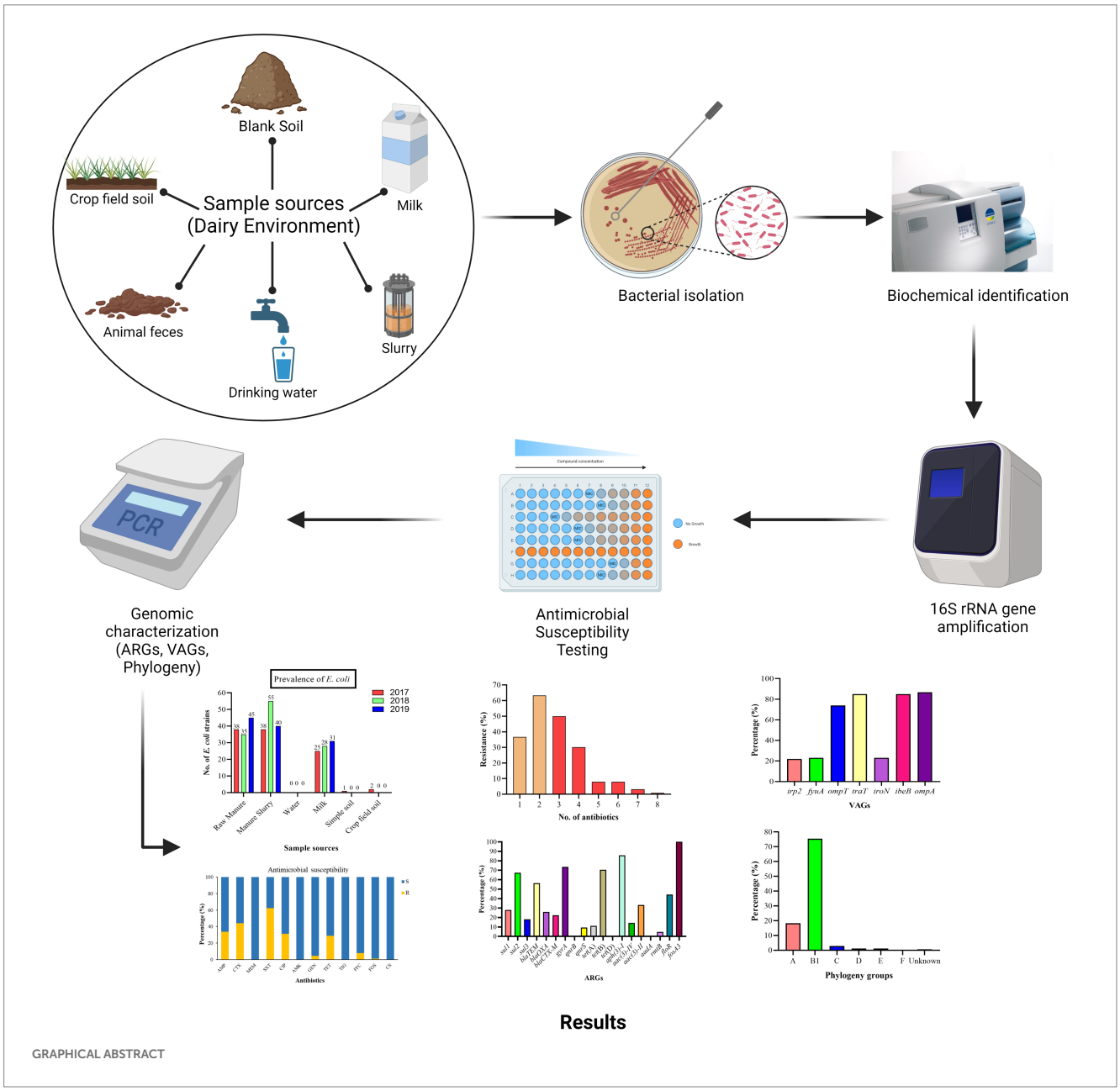
Escherichia coli is a common inhabitant of the intestinal microbiota and is responsible for udder infection in dairy cattle and gastro-urinary tract infections in humans. We isolated *E. coli* strains from a dairy farm environment in Xinjiang, China, and investigated their epidemiological characteristics, phenotypic and genotypic resistance to antimicrobials, virulence-associated genes, and phylogenetic relationship. A total of 209 samples were collected from different sources (feces, slurry, water, milk, soil) and cultured on differential and selective agar media (MAC and EMB). The presumptive identification was done by the VITEK2 system and confirmed by 16S rRNA gene amplification by PCR. Antimicrobial susceptibility testing was done by micro-dilution assay, and genomic characterization was done by simple and multiplex polymerase chain reaction (PCR). A total of 338 *E. coli* strains were identified from 141/209 (67.5%) of the samples. Most of the *E. coli* strains were resistant to sulfamethoxazole/trimethoprim (62.43%), followed by cefotaxime (44.08%), ampicillin (33.73%), ciprofloxacin (31.36%), tetracycline (28.99%), and a lesser extent to florfenicol (7.99%), gentamicin (4.44%), amikacin (1.77%), and fosfomycin (1.18%). All of the strains were susceptible to meropenem, tigecycline, and colistin sulfate. Among the resistant strains, 44.4% were identified as multi-drug resistant (MDR) showing resistance to at least one antibiotic from ≥3 classes of antibiotics. Eighteen out of 20 antibiotic-resistance genes (ARGs) were detected with *sul2* (67.3%), bla_{TEM} (56.3%), *gyrA* (73.6%), *tet*(B) (70.4%), *aph(3)-I* (85.7%), *floR* (44.4%), and *fosA3* (100%, 1/1) being the predominant genes among different classes of antibiotics. Among the virulence-associated genes (VAGs), *ompA* was the most prevalent (86.69%) followed by *ibeB* (85.0%), *traT* (84.91%), *ompT* (73.96%), *fyuA* (23.1%), *iroN* (23.1%), and *irp2* gene (21.9%). Most of the *E. coli* strains were classified under phylogenetic group B1 (75.45%), followed by A (18.34%), C (2.96%), D (1.18%), E (1.18%), and F (0.30%). The present study identified MDR *E. coli* strains carrying widely distributed ARGs and VAGs from the dairy environment. The findings suggested that the dairy farm environment may serve as a source of mastitis-causing pathogens in animals and horizontal transfer of antibiotic resistance and virulence genes carrying bacterial strains to humans via contaminated milk and meat, surface water and agricultural crops.

KEYWORDS

antibiotic resistance genes (ARGs), antimicrobial resistance, multi-drug resistance, virulence associated genes, *Escherichia coli*, dairy environment



GRAPHICAL ABSTRACT

1. Introduction

Escherichia coli (*E. coli*) is an opportunistic and common inhabitant of the intestinal microbiota of animals as well as humans (He et al., 2019). Moreover, *E. coli* is also the most common organism responsible for causing udder infection in animals (Cheng et al., 2019) and bloodstream infections in humans (Jara et al., 2021). The use of antibiotics to prevent disease and promote the health of growing animals remains an integral part of livestock farming. It has been 50 years since antibiotic-supplemented feeds were first approved for livestock to improve overall health and increase the productivity of animals (Afema et al., 2018). However, the emergence and spread of pathogens resistance to multiple antibiotics has become a growing problem for veterinary medicine and public health (Murray et al., 2022). It was estimated that antimicrobial resistance results in $55 billion annual economic loss in USA (Dadgostar, 2019). The China also rank high in consumption of antibiotics for food producing animals especially in dairy sector.

Transmission of antimicrobial resistance (AMR) may occur by multiple ways, but contact with human and animal feces is the most common pathway (Graham et al., 2019). Dairy cattle act as a potential source of spread of antibiotic-resistant and zoonotic bacterial strains, especially Shiga-toxin producing *E. coli* (STEC) through the

contamination of the farm environment and food products such as milk and meat, and direct contact with animals (Amézquita-López et al., 2018; Sobur et al., 2019). Animal farming, especially intensive livestock farming, plays a major role in AMR transmission between humans, animals, and the environment (Manyi-Loh et al., 2018). Due to the widespread use of antimicrobials in livestock production, livestock manure is considered a hotspot for the spread and transmission of AMR genes. Genetically diverse *E. coli* strains exist in animal manures, and their ability to survive in various ecological niches (Beattie et al., 2020). *E. coli* strains carrying $bla_{CTX\text{-}M}$ and bla_{CMY} genes confer resistance to β-lactam antibiotics are frequently found in animal manure (Cookson et al., 2022). Therefore, animal manure is thought to be harming to animals *via* udder infections by environmental pathogens such as *E. coli*, humans *via* contaminated food products, and environment by using manure as fertilizer in soil or waste water (Sarowska et al., 2019). This increases the potential of antibiotic resistance genes (ARGs) to integrate into human intestinal microbiota by horizontal gene transfer mechanism (Lima et al., 2020). A better understanding of the transmission and spread of AMR, especially in areas with intensive livestock production, is important to understand. Therefore, the present study investigated the prevalence of *E. coli* in the dairy farm environment and their drug resistance characteristics. We also investigated the diversity of virulence associated genes (VAGs) responsible for pathogenicity and their distribution within phylogenetic groups.

2. Materials and methods

2.1. Sample sources and collection strategy

A total of 209 environment samples were collected from 2017–2019 from a large dairy farm (herd size = 25,000 animals) in Xinjiang province, China. The environmental samples included were fecal samples ($n = 50$), manure slurry from a storage tank ($n = 36$), raw milk ($n = 90$), water samples from the residential area ($n = 9$), soil samples ($n = 12$), and crop field soil ($n = 12$) based on random sampling technique (Figure 1). A 50 g of manure sample was collected from the animal living area and storage tank from five different sites using a five-point mixed sampling method (Sharp et al., 2012) and stored in sterile zipper bags. Raw milk samples (10 mL) were collected and transferred to sterile falcon tubes according to the guidelines of the National Mastitis Council (Hogan and Smith, 1992). The water samples (50 mL) were collected in sterile water bottles from the residential area by randomly selecting three different water outlets. The blank and crop field soil samples were collected from different sites on farm and fodder growing fields, respectively. All the collected samples were kept at 4°C and transferred to the laboratory within 24 h for further processing.

2.2. Isolation and identification of *Escherichia coli*

The 25 g of fecal, manure, and soil samples were first mixed in 225 mL of phosphate-buffered saline (PBS) to solubilize them. After mixing, 1 mL of the liquid was transferred to a 10 mL LB broth tube for bacterial enrichment by incubation at 37°C with continuous mixing at 160 rpm. From each tube, 100 μL of the enrichment culture was sub-cultured on MacConkey (MAC) agar under prior mentioned incubation conditions. However, the water and milk samples were swabbed directly onto MacConkey agar and incubated at 37°C overnight. Based on colony shape and color, large, smooth, and pink colonies were picked and further streaked onto Eosin Methylene Blue (EMB) agar and incubated overnight at 37°C. The appearance of a metallic green sheen with dark center colonies on EMB agar was indicative of *E. coli* growth (Peng et al., 2022). Further, presumptive identification was done by the VITEK2 system (BioMerieux, France) (Alfinete et al., 2022) and confirmation by 16S rRNA gene amplification by PCR using primers reported previously (Liu et al., 2021). The PCR-amplified product was visualized on 1% agarose gel under the GelDoc XR system (Supplementary Figure S1). The confirmed isolates were preserved in 20% glycerol at −80°C for further analysis.

2.3. Detection of virulence-associated genes

The 16S rRNA-confirmed *E. coli* isolates were subjected to the identification of seven VAGs by the previously described method (Hu et al., 2022). The genomic DNA was extracted using a DNA extraction kit (Tiangen Biotech Beijing, Co., Ltd.) following the manufacturer's guidelines. The virulence genes were identified by PCR amplification of target gene primers mentioned in Supplementary Table S1. The PCR reaction mixture (25 μL) consisted of 12.5 μL PreTaq Mix (Vazyme Biotech, China), 1 μL forward primer, 1 μL reverse primer, 1 μL genomic DNA, and 9.5 μL of deionized water under the following conditions; prior denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for the 30s, annealing at varying temperatures mentioned in Supplementary Table S1 for 30s, initial extension at 72°C for 30s followed by a final extension at 72°C for 5 min. After amplification, the PCR product was run on 1% agarose gel electrophoresis at 180 V/200 mA followed by ethidium bromide staining for visualization, and images were taken under the GelDoc XR system (Supplementary Figure S2).

2.4. Antimicrobial susceptibility testing

The AST was done by broth micro-dilution assay following the EUCAST guidelines.[1] Briefly, the preserved isolates were thawed at room temperature and re-suspended in LH broth by vigorous mixing (120 rpm) at 37°C for 12 h. The loopful enriched broth was streaked on MacConkey agar following the overnight incubation. The bacterial inoculum was prepared by adjusting the cell density at 5×10^5 CFU/mL. The 96-well round bottom plate was used for broth dilution assay and 100 μL of Mueller Hinton (MH) broth was added from the 1st well to the 12th well with a micropipette. Next, 50 μL of prepared bacterial inoculum was added from the 1st to 11th well by keeping the 12th well as a negative control. The antibiotics were selected based on medical and veterinary use which includes trimethoprim-sulfamethoxazole (SXT), ampicillin (AMP), cefotaxime (CTX), tetracycline (TET), ciprofloxacin (CIP), gentamicin (GEN), amikacin (AMK), colistin sulfate (CS), florfenicol (FFC), meropenem (MEM), and tigecycline (TIG) were added from 1st to 10th well by keeping 11th well as a positive control. The reference strain

1 https://www.eucast.org

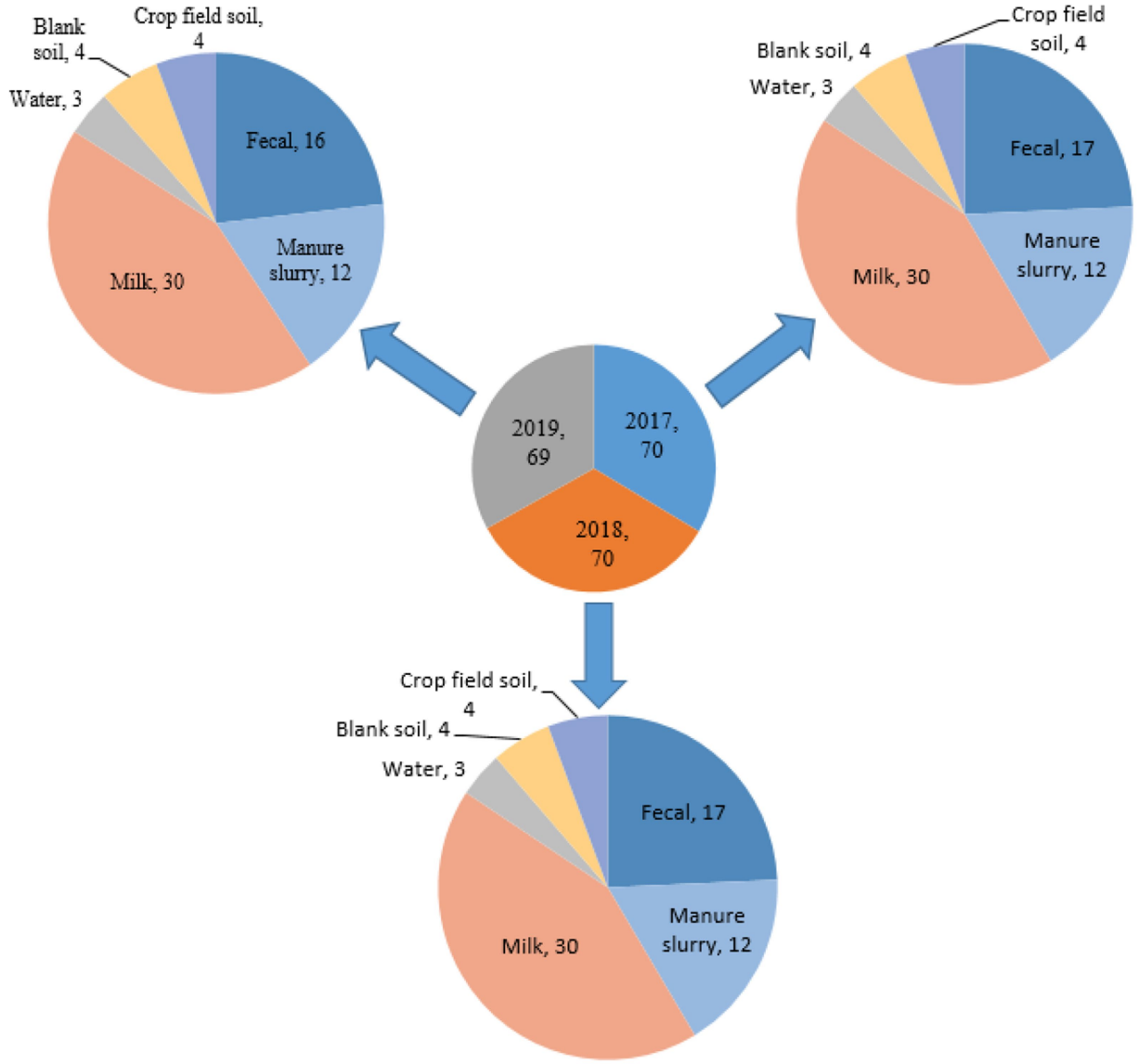


FIGURE 1
Distribution of samples collected from different sources.

E. coli ATCC 25922 was used as a quality control. The MIC (minimum concentration that inhibits visible growth of bacteria) of fosfomycin was calculated by the agar dilution method, recommended by EUCAST. The MIC of all antibiotics was evaluated by visualizing the growth in the bottom of the plate well as tinny buttons/turbidity. The MIC values were compared with standard EUCAST MIC breakpoints (Supplementary Table S2). The strains showing resistance to at least one antibiotic from ≥3 classes were classified as MDR.

2.5. Detection of antibiotic resistance genes

Phenotypically resistant *E. coli* strains were subjected to the detection of 20 ARGs from eight antibiotic classes (Supplementary Table S3) according to the method described previously (Yu et al., 2020). The bacterial DNA was extracted using a DNA extraction kit (Tiangen Biotech Beijing, Co., Ltd.) and used as a template for PCR amplification of 20 ARGs (listed in Supplementary Table S3). The PCR reaction mixture (25 μL) consisted of 12.5 μL PreTaq Mix (Vazyme Biotech, China), 1 μL of forward and reverse primer each, 1 μL of bacterial DNA, and 9.5 μL of deionized water. The reactions were performed under the following conditions: denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 30s, annealing at varying temperatures (see Supplementary Table S3) for 30s, and extension at 72°C for 30s, followed by a final extension at 72°C for 5 min. After amplification, the PCR product was separated on 1% agarose gel at 180 V/200 mA and stained with ethidium bromide for visualization using the GelDoc XR system.

2.6. Phylogenetic analysis

The phylogenetic grouping of *E. coli* strains was carried out by 2 sets of PCR using primers listed in Supplementary Table S4. The quadruple PCR reaction mixture (25 μL) consists of Premix Taq TM 12.5 μL, 1 μL of

each forward and reverse primers (*chuA*, *yjaA*, *tspE4C2*), 2 μL of each *arpA* forward and reverse primers, 1.5 μL DNA template, and 1 μL dd H_2O. PCR was carried out under the following conditions; pre-denaturation at 94°C for 4 min, 30 cycles of denaturation at 94°C for 5 s followed by annealing at 59°C for 20 s, and extension at 72°C for 5 min. The PCR reaction for group E and C identification consisted of Premix Taq TM 12.5 μL, 0.6 μL of trpBA forward and reverse primers each, 1 μL of each group-specific primer (Supplementary Table S4), 1.5 μL DNA template, and 2.8 μL dd H_2O. In the PCR reaction solution, trpBA primers were added as an internal control. The PCR amplifications conditions were pre-denaturation at 94°C for 4 min, 30 cycles of denaturation at 94°C for 5 s, annealing at 57°C (group E) or 59°C (group C) for 20 s, and final extension at 72°C for 5 min. After the PCR amplification, the PCR product was run on 1% agarose gel and visualized under the GelDoc XR system (Supplementary Figure S3), and the phylogenetic group was identified by comparing the results with Supplementary Table S4.

2.7. Data analysis

The prevalence was calculated using the formula described by Thrusfield (2018).

$$Prevalence\,(\%) = \frac{No.\ of\ positive\ isolates}{Total\ isolates} \times 100$$

The antimicrobial susceptibility data were analyzed by descriptive statistics using Microsoft Excel. Moreover, the data for various factors such as sampling source and sampling year affecting the prevalence, AMR and virulence rates were analyzed using the Pearson's Chi-Squared test keeping the level of significance, $\alpha = 5\%$ (Zhao et al., 2021; Ma et al., 2022). $p\text{-}value < 0.05$ was considered statistically significant and vice versa. The graphical representation of data was done by GraphPad Prism version 8.2.1 and Microsoft Excel.

3. Results

3.1. Isolation of *Escherichia coli* strain from different sources

A total of 209 samples were collected from different sites of dairy environment including fecal samples ($n = 50$), manure slurry from the storage tank ($n = 36$), raw milk ($n = 90$), water samples ($n = 9$), soil samples ($n = 12$), and crop field soil ($n = 12$) samples. In total, 534 suspected *E. coli* strains were isolated from 141/209 (67.5%) samples based on colony characteristics. Subsequently, 338 *E. coli* strains were confirmed by 16S rRNA gene amplification. The isolation rates were comparable over the years, with 30.8% (104/338) in 2017, 34.9% (118/338) in 2018, and 34.3% (116/338) in 2019 (Figure 2A). Overall, most of the *E. coli* strains were isolated from manure slurry (39.3%, 133/338), followed by fecal samples (34.9%, 118/338), raw milk (24.8%, 84/338), crop field soil (0.59%, 2/338), and least from blank soil (0.29%, 1/338). However, none of the *E. coli* strains was isolated from water samples (Figure 2B). In 2019, a higher number of *E. coli* strains were isolated from fecal and milk samples compared to other sampling years while more *E. coli* strains were isolated from slurry samples in 2018. Moreover, only 1 and 2 strains were isolated in 2017 from blank and crop field soil samples, respectively, while none in 2018 and 2019 (Figure 2B).

3.2. Antimicrobial susceptibility of *Escherichia coli* strains

The AST of 338 *E. coli* strains showed that 284/338 (84.0%) were resistant to at least one antibiotic and 54/338 (26.0%) were susceptible strains (Figure 3A). Most of the strains were resistant to trimethoprim/sulfamethoxazole (62.43%, 211/338), followed by cefotaxime (44.08%, 149/338), ampicillin (33.73%, 114/338), ciprofloxacin (31.36%, 106/338), tetracycline (28.99%, 98/338), and less to florfenicol (7.99, 27/338), gentamicin (4.44%, 15/338), amikacin (1.77%, 6/338), and fosfomycin (1.18%, 4/338). All of the *E. coli strains* were susceptible to meropenem, tigecycline, and colistin sulfate (Figure 3B). All *E. coli* strains from 2017–2019 were found 100% susceptible to meropenem, tigecycline, and colistin sulfate. Additionally, the AMR rate of AMP (44.23%), CIP (40.38%), TET (44.23%), and GEN (4.81%) was noted higher with a significant difference ($p < 0.05$) in 2017 than in other sampling years. Moreover, *E. coli* strains showed higher AMR to CTX (55.93%), SXT (68.64%), and AMK (3.39%) in 2018 with a significant difference ($p < 0.05$). However, none of the *E. coli* strain from 2017 and 2019 exhibited resistance to AMK and FOS (Figure 3C).

3.3. AMR characteristics of *Escherichia coli* strains isolated from different sources

Most of the *E. coli* strains from all samples were resistant to trimethoprim/sulfamethoxazole (SXT) and 100% susceptible to meropenem (MEM), tigecycline (TIG), and colistin sulfate (CS). Moreover, *E. coli* strains from fecal samples exhibited higher resistance to ampicillin (AMP), ciprofloxacin (CIP), and tetracycline (TET) in 2017 than other sampling years with a significant difference (Figure 4A). A similar trend was observed for *E. coli* strains isolated from milk and manure slurry (Figures 4B,C). Furthermore, *E. coli* strains isolated from blank and crop field soil in 2017 exhibited 100% resistant to CTX, CIP, TET, and SXT, while none of the strains isolated from 2018 and 2019 was resistant (Figures 4D,E). In addition, *E. coli* strains from crop field showed 50% resistance to AMP and florfenicol (FFC).

3.4. Drug resistance spectrum

Among the resistant strains, 126/284 (44.4%) were identified as multi-drug resistant (MDR) and 158/284 (55.6%) were recognized as non-MDR (Figure 5A). Most of the strains showed resistance to 2 antibiotics (63.38%), followed by 3 (50.0%), 1 (36.61%), 4 (30.28%), 5 and 6 (7.75% each), 7 (3.17%), and 8 (0.70%) antibiotics (Figure 5B). Moreover, diverse AMR patterns were recognized such as CTX + AMP, CTX + AMP + SXT, CTX + AMP + SXT + CIP, CTX + TET + SXT + CIP, CTX + AMP + SXT + CIP + GEN, CTX + AMP + SXT + CIP + TET + FFC, AMP + CTX + GEN + TET + SXT + FFC + FOS, and AMP + CTX + CIP + GEN + TET + SXT + FFC. Only 1 strain showed resistance to 8 antibiotics, AMP + CTX + CIP + AMK + GEN + TET + SXT + FFC (Table 1).

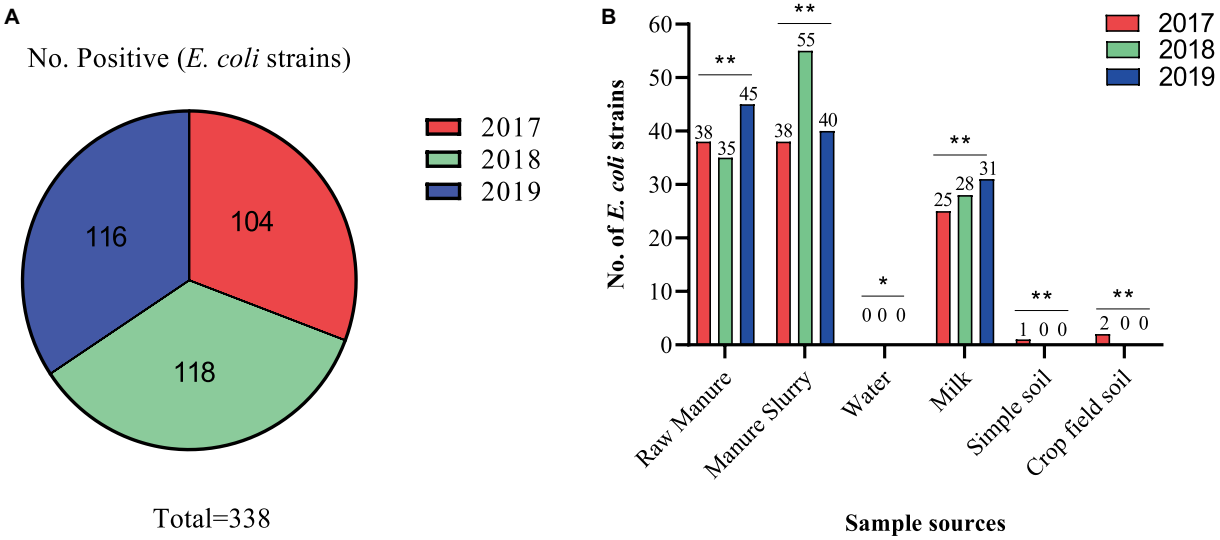


FIGURE 2
Isolation of *Escherichia coli* strains in different sampling years and sources. **(A)** Total number of *E. coli* strains isolated in different years. **(B)** Distribution of *E. coli* strains isolated from different sources in different years; **, indicate non-significant difference ($p > 0.05$); *, indicate not applicable; ***, indicate significant difference ($p < 0.05$).

3.5. Detection of ARGs and correlation with phenotypic resistance

The genotypic analysis was done by targeting 20 ARGs among 8 classes of antibiotics (mentioned in Table 1). Eighteen out of 20 ARGs were identified, and the prevalent genotypes included *sul2* (67.3%, sulfonamides), bla_{TEM} (56.3%, beta-lactams), *gyrA* (73.6%, quinolones), *tet*(B) (70.4%, tetracycline's), *aph*(3)-I (85.7%, aminoglycosides), *floR* (44.4%, amphenicol), and *fosA3* (100%, phosphonic). The percentage distribution of other ARGs identified was as follows: sulfonamides (*sul1*, 27.9%; *sul3*, 18.1%), β-lactams (bla_{OXA}, 25.8%; $bla_{CTX\text{-}M}$, 22.4%), aminoglycosides (*aac*(3)-IV, 14.3%; *aac*(3)-II, 33.3%; *aadA*, 0.00%; *rmtB*, 4.76%), quinolones (*qnrB*, 0.94%; *qnrS*, 9.43%), polymyxin (*pmrB*, 0.35%) and tetracycline's (*tet*(A), 11.2%; *tet*(D), 0.00%) (Figure 6).

The correlation between phenotypic resistance and genotypic detection of ARGs was noted variable. For example, no strains showed resistance to colistin sulfate phenotypically but one strain was carrying the ARG upon genotypic analysis. Moreover, the number of strains carrying ARGs was noted higher as compared to phenotypic resistance among sulfonamides, beta-lactams, and aminoglycosides-resistant strains while the inverse was noted among quinolone, tetracycline, and amphenicol-resistant strains. However, the phenotypic and genotypic expression was observed 100% correlated for fosfomycin-resistant strains (Table 2).

3.6. Virulome gene analysis

Among the VAGs, *ompA* was most prevalent (86.69%), followed by *ibeB* (85.0%), *traT* (84.91%), *ompT* (73.96%), *fyuA* (23.1%), *iroN* (23.1%), and *irp2* (21.9%) (Figure 7A). All of the *E. coli* strains carrying *the fyaA* gene were also carrying *the iroN* gene. Moreover, 93.59% of *E. coli* strains carrying *irp2* were also harboring *the fyuA* gene. VAGs such as *ompT*, *traT*, *iroN*, *ibeB*, and *ompA* were detected in *E. coli* strains from all sources while *irp2* and *fyuA* genes were not observed from manure slurry and fecal samples, respectively. However, both *irp2* and *fyuA* genes (36.9%, 31/84) were identified in strains of milk origin. Collectively, a higher percentage of VAGs was identified in strains of milk origin as compared to feces and slurry (Figure 7B).

3.7. Distribution of VAGs among phylogenetic groups

The phylogenetic analysis of 338 *E. coli* strains showed most of the strains belong to the B1 group (75.45%, 255/338), followed by A (18.34%, 62/338), C (2.96%, 10/338), D (1.18%, 4/338), E (1.18%, 4/338), and F (0.30%, 1/338). However, the phylogenetic group for 2 of the strains was not identified. The most prevalent VAGs among the various phylogenetic groups were as follows; B1 (*ompA*, 87.4%), A (*ibeB* and *ompA*, 88.7%), C (*traT* and *ompA*, 90.0%), D (*traT*, *ibeB*, and *ompA*, 100%), E (*traT*, 100%), and F (*traT*, 100%). Moreover, the percentage distribution of other VAGs among the phylogenetic groups is presented in Table 3.

4. Discussion

Antimicrobial resistance particularly in *the Enterobacteriaceae* family possesses a major threat to global public health. The present study isolated *E. coli* from the dairy environment, which serves as a reservoir of bacterial pathogens and ARGs and a source of spread of ARGs between the bacterial species *via* horizontal gene transfer and to humans *via* fecal contamination of drinking water and milk. The isolation rates of *E. coli* in this study were found similar to the findings of Sobur et al. (2019) who reported 75% prevalence of *E. coli* from dairy cattle and farm environment. Other studies conducted by Li et al. (2022) reported 84.6%

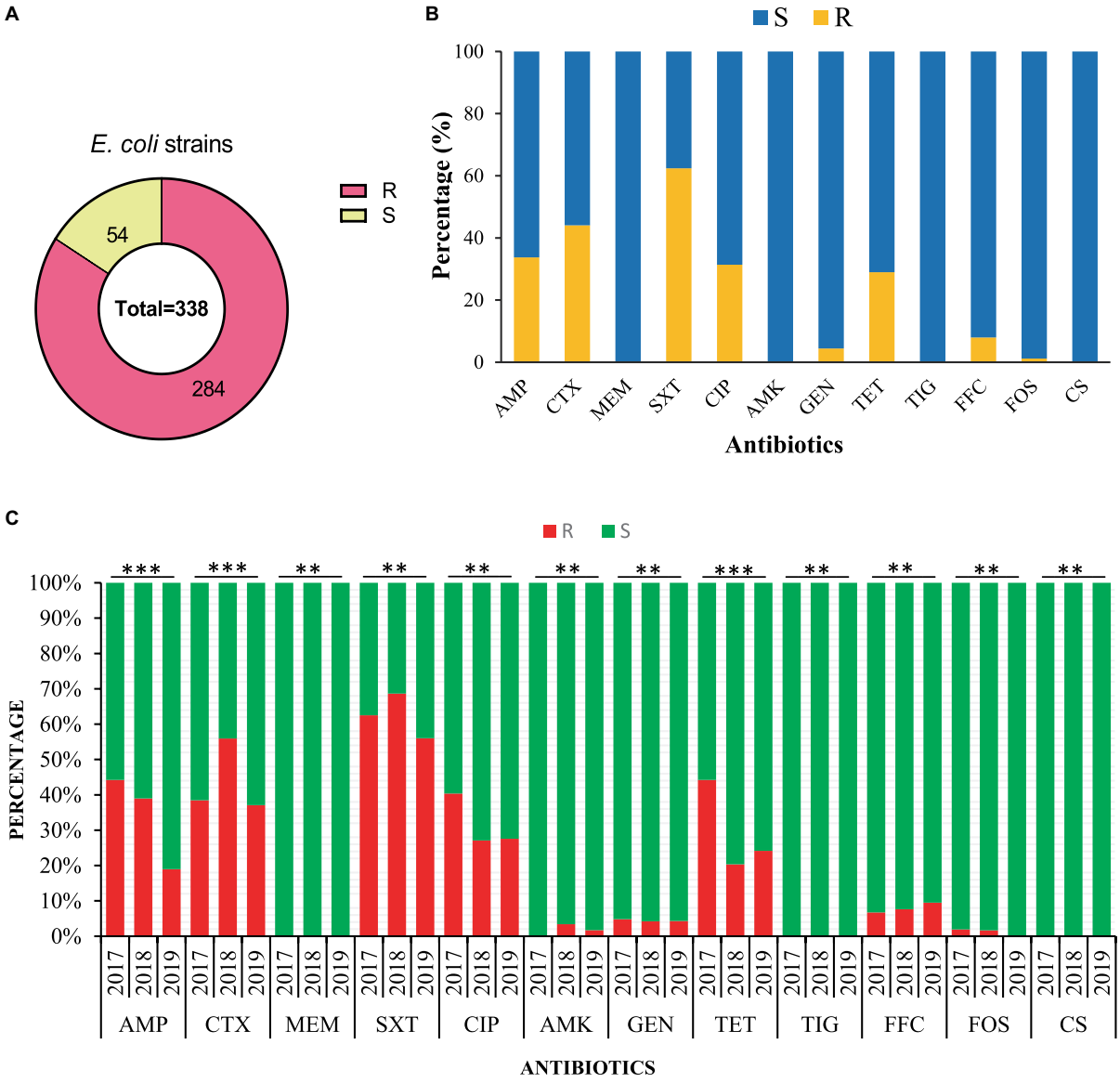


FIGURE 3
Antimicrobial susceptibility of the 338 *E. coli* strains isolated from the dairy environment. **(A)** The overall resistant (R) and susceptible (S) *E. coli* strains. **(B)** The overall antimicrobial susceptibility of 338 *E. coli* strains against individual antibiotic tested. **(C)** The comparison of antimicrobial susceptibility of *E. coli* strains from different sampling years. AMP, ampicillin; CTX, cefotaxime; MEM, meropenem; SXT, trimethoprim-sulfamethoxazole; CIP, ciprofloxacin; AMK, amikacin; GEN, gentamicin; TET, tetracycline; TIG, tigecycline; FFC, florfenicol; FOS, fosfomycin; CS, colistin sulfate; **, indicate non-significant difference ($p > 0.05$); *, indicate not applicable; ***, indicate significant difference ($p < 0.05$).

E. coli isolation from fecal samples of cattle, chicken, and pigs while 34.4% was noted by Liu et al. (2021) from raw milk samples and 81.1% from raw cheese (Imre et al., 2022). Beattie et al. (2020) also reported a similar isolation rate of *E. coli* from dairy manure in the USA. It is also reported that the presence of *E. coli* in the dairy environment may be the cause of clinical mastitis in dairy cows (Su et al., 2016).

The antimicrobial susceptibility results showed higher resistance to SXT, followed by CTX, AMP, CIP, TET, and the least resistance to FFC, GEN, AMK, and FOS. These results are consistent with the findings of Peng et al. (2022) who isolated *E. coli* strains from pigs that were highly resistant to SXT (80.38%), AMP (92.86%), and TET (96.26%). A similar study conducted by Beattie et al. (2020) reported that *E. coli* strains from manure isolates showed higher resistance to AMP and CTX. However, a lower resistance rate to AMK and GEN was also noted by Lu et al. (2022) and Liu et al. (2021) respectively. We noted the *E. coli* strains were susceptible to the "last resort" antimicrobials such as MEM, TIG, and CS, which is consistent with the findings of a study conducted by Hu et al. (2019) in the human setting. Moreover, other studies conducted by Wang et al. (2021), Zou et al. (2021), and Ma et al. (2022) in animal settings also reported 100% susceptibility of MEM and TIG against *E. coli* strains. We noted no resistance to CS, which is consistent with a previous report that colistin resistance is decreasing in animal and human settings because of the CS ban in China (Wang et al., 2020). The percentage of MDR *E. coli* was noted at 44.4% in the current study, which is comparable with what was previously

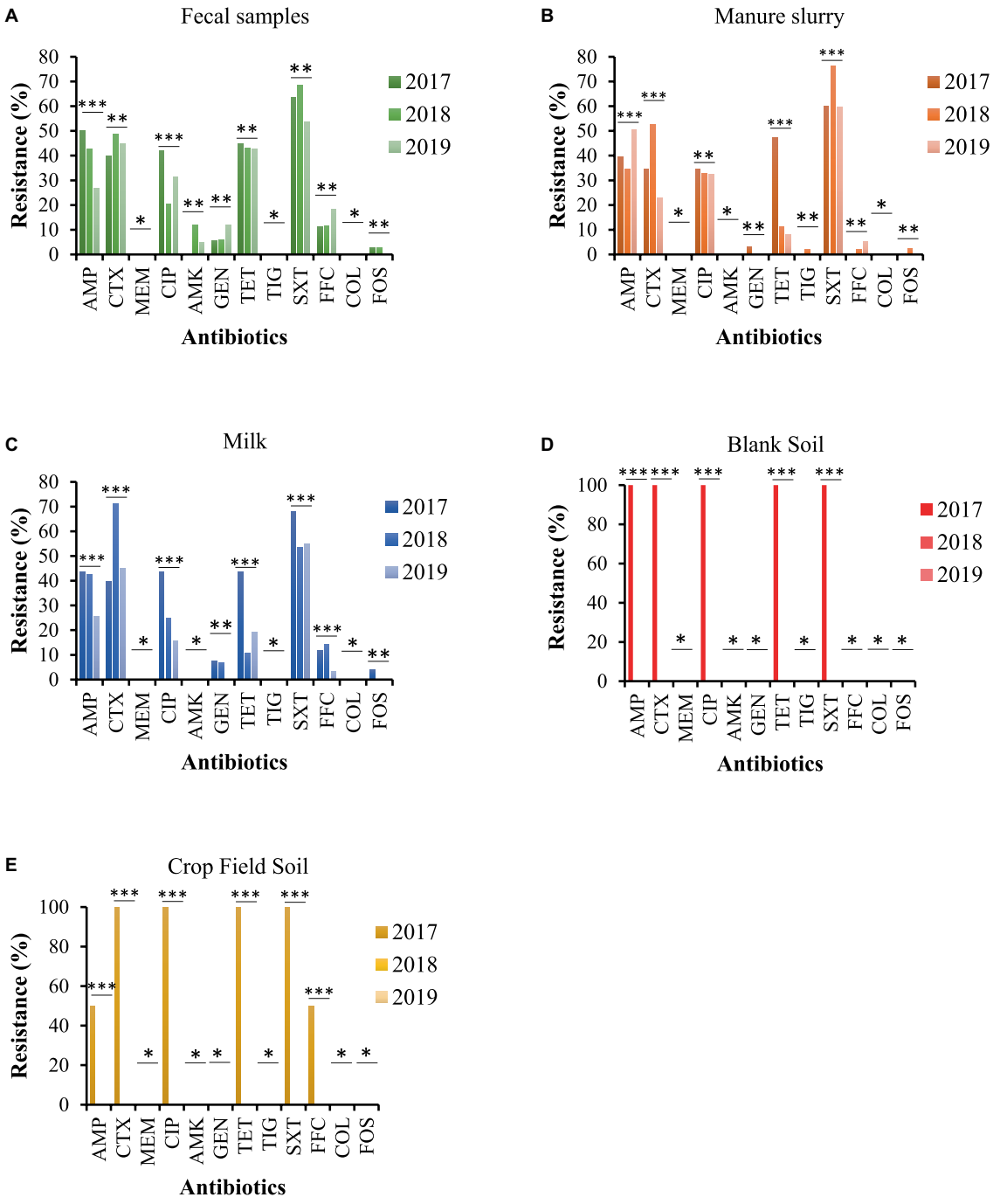


FIGURE 4
AMR rates of *E. coli* strains isolated from different sources. **(A)** fecal sample. **(B)** manure slurry from the storage tank. **(C)** raw milk. **(D)** blank soil. **(E)** crop field soil. AMP, ampicillin; CTX, cefotaxime; MEM, meropenem; CIP, ciprofloxacin; AMK, amikacin; GEN, gentamicin; TET, tetracycline; TIG, tigecycline; SXT, trimethoprim/sulfamethoxazole; FOS, fosfomycin; COL, colistin; FFC, florfenicol; **, indicate non-significant difference ($p > 0.05$); *, indicate not applicable; ***, indicate significant difference ($p < 0.05$).

reported (54.4%) by Su et al. (2016) and lower than what was reported by Yu et al. (2020) in dairy milk. Another study conducted by Salinas et al. (2019) reported higher resistance to SXT, CIP, AMP, CTX, and TET by *E. coli* isolated from child and domestic animal origin, which is also consistent with current findings.

We identified 18 ARGs out of 20 belonging to different classes of antibiotics. The most prevalent ARGs were *sul2* (67.3%, sulfonamides), bla_{TEM} (56.3%, beta-lactam), *gyrA* (73.6%, quinolones), *tet(B)* (70.4%, tetracycline's), *aph(3)-I* (85.7%, aminoglycosides), *floR* (44.4%, amphenicol), and *fosA3* (100%, fosfomycin). Previous studies reported AMR in humans is linked to food animals raised for milk and meat purposes because of environmental contamination and drug residues (Bacanlı and Başaran, 2019; Pormohammad et al., 2019; Ma et al., 2021). The use of antimicrobial drugs in food animals also enhances the percentage of MDR bacteria and ARGs in human microbiota (Ma et al., 2022). Moreover, *E. coli* is also known to serve

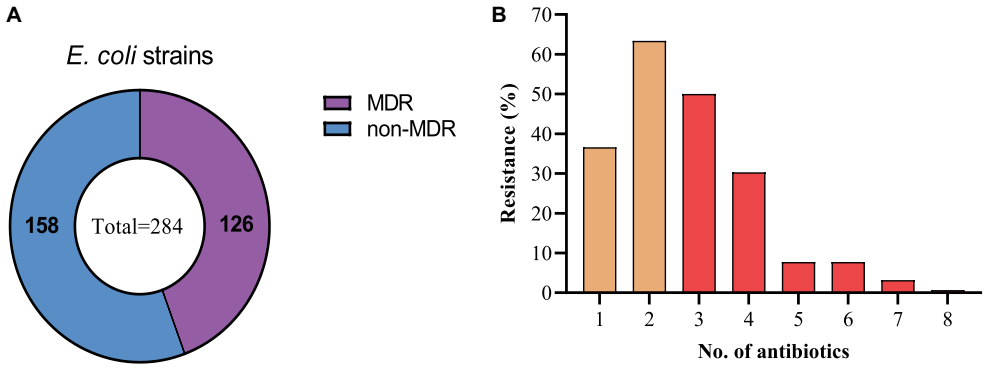


FIGURE 5
Drug-resistance spectrum of *E. coli* strains isolated from the dairy environment. **(A)** proportions of MDR and non-MDR strains. **(B)** Percentage resistance spectrum of 284 *E. coli* strains to 1 ~ 8 antibiotics.

TABLE 1 The phenotypic drug resistance spectrum of *Escherichia coli* strains.

Antibiotic classes	Phenotypic resistance spectrum	No. of antibiotics	No. of strains
Sulfonamides	SXT	1	104
Cephalosporin + Penicillin	CTX + AMP	2	180
Cephalosporin + Penicillin + Sulfonamides	CTX + AMP + SXT	3	142
Cephalosporin + Penicillin + Sulfonamides + Quinolones	CTX + AMP + SXT + CIP	4	46
Cephalosporin + Tetracycline + Sulfonamides + Quinolones	CTX + TET + SXT + CIP	4	40
Cephalosporin + Penicillin + Sulfonamides + Quinolones + Aminoglycosides	CTX + AMP + SXT + CIP + GEN	5	22
Cephalosporin + Penicillin + Sulfonamides + Quinolones + Tetracycline + Amphenicol	CTX + AMP + SXT + CIP + TET + FFC	6	22
Penicillin + Cephalosporin + Aminoglycoside + Tetracycline + Sulfonamides + Amphenicol + Phosphonic	AMP + CTX + GEN + TET + SXT + FFC + FOS	7	5
Penicillin + Cephalosporin + Quinolones + Aminoglycoside + Tetracycline + Sulfonamides + Amphenicol	AMP + CTX + CIP + GEN + TET + SXT + FFC	7	4
Penicillin + Cephalosporin + Quinolones + Aminoglycosides + Tetracycline + Sulfonamides + Amphenicol	AMP + CTX + CIP + AMK + GEN + TET + SXT + FFC	8	2

AMP, ampicillin; CTX, cefotaxime; CIP, ciprofloxacin; GEN, gentamicin; TET, tetracycline; TIG, tigecycline; SXT, trimethoprim/sulfamethoxazole; FFC, florfenicol; AMK, Amikacin.

as donor bacteria for horizontal gene transfer within and between species (Oladeinde et al., 2019). Lima et al. (2020) highlighted the importance of animal manure and manure-substituted agriculture lands as a major source of antibiotic residues, ARGs, and AMR bacteria in the environment posing a potential threat to public health *via* horizontal gene transfer mechanisms with the help of mobile genetic elements such as plasmids, transposons, and integrons. Qian et al. (2018) detected 109 ARGs from the fresh manure of chicken, cattle, and pigs responsible for AMR to a class of antibiotics widely used in human and animal settings.

We investigated multiple VAGs in the isolated *E. coli* and most of the investigated VAGs (*ompA, ibeB, traT, ompT, fyuA, iroN, irp2*) belong to ExPEC, which may cause urinary tract infections in humans. VAGs are responsible for the production of virulence factors which play an important role in the pathogenicity of bacteria through multiple mechanisms such as adhesion, invasion, toxin production, and immune evasion (Kudva et al., 2020). Virulence genes investigated in the present study have various functions. *ompA* encodes for outer membrane protein A, *ibeB* is an invasion protein gene, *traT* encodes for complement resistance protein, *ompT* encodes for outer membrane protease protein, *fyuA* encodes for yersiniabactin receptor, *iroN* encodes for aerobactin receptor, and *irp2* gene encodes for iron-responsive element binding protein 2. Zhang et al. (2021) investigated similar VAGs in *E. coli* strains isolated from healthy waterfowls in Hainan, China. A study carried out

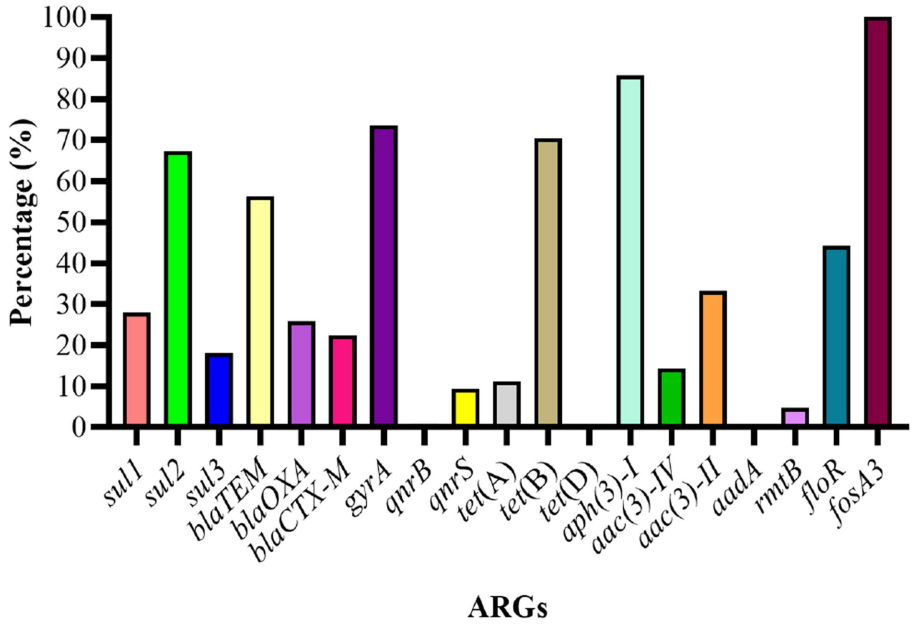


FIGURE 6
Percentage distribution of ARGs among *E. coli* strains.

TABLE 2 Percentage of resistant strains carrying ARGs and correlation with phenotypic resistance.

Antibiotic class	Resistance Phenotype	No. (%) of resistant strains (n = 338)	ARGs	Phenotypically resistant strains carrying ARGs		% difference in correlation
				No. (%)	Total/Class	
Sulfonamides	SXT	211 (62.4%)	*sul1*	59 (27.9)	239	13.3%
			sul2	142 (67.3)		
			sul3	38 (18.1)		
β-lactams	AMP + CTX	263 (77.8%)	bla_{TEM}	148 (56.3)	275	4.56%
			bla_{OXA}	68 (25.8)		
			$bla_{CTX\text{-}M}$	59 (22.4)		
Quinolone	CIP	106 (31.4%)	*gyrA*	78 (73.6)	89	16.0%
			qnrB	1 (0.94)		
			qnrS	10 (9.43)		
Tetracycline's	TET	98 (29.0%)	*tet*(A)	11 (11.2)	80	18.4%
			tet(B)	69 (70.4)		
			tet(D)	0 (0.00)		
Aminoglycosides	AMK + GEN	21 (6.21%)	*aph(3)-I*	18 (85.7)	29	38.1%
			aac(3)-IV	3 (14.3)		
			aac(3)-II	7 (33.3)		
			aadA	0 (0.00)		
			rmtB	1 (4.76)		
Amphenicol	FFC	27 (7.99%)	*floR*	12 (44.4)	12	55.5%
Phosphonic	FOS	4 (1.18%)	*fosA3*	4 (100)	4	0.00%
Polymyxin	CS	0 (0.00%)	*pmrB*	1	1	N/A

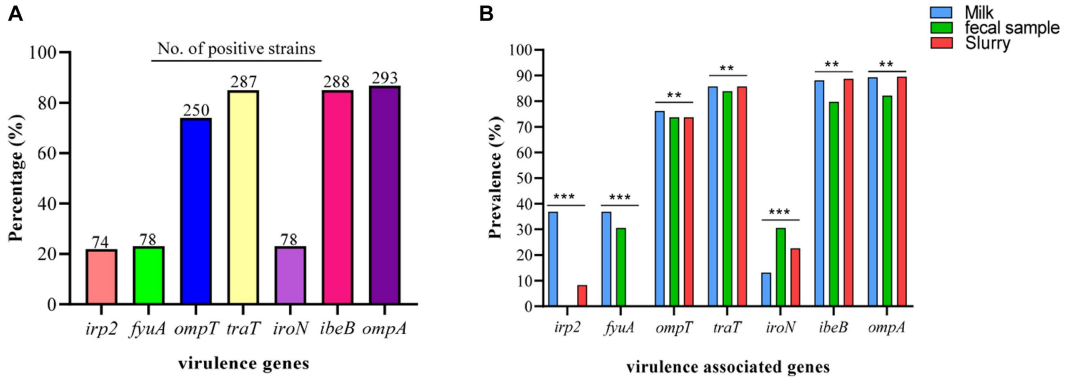


FIGURE 7
Escherichia coli strains carrying virulence-associated genes (VAGs). **(A)** Overall percentage and number of positive *E. coli* strains to carry VAGs. **(B)** Percentage distribution of VAGs among the *E. coli* strains isolated from different sources.

TABLE 3 Distribution of VAGs in different phylogeny groups.

VAGs	No. Positive (%)					
	A (*n* = 62)	B1 (*n* = 255)	C (*n* = 10)	D (*n* = 4)	E (*n* = 4)	F (*n* = 1)
fyuA	21 (33.9)	52 (20.4)	2 (20.0)	3 (75.0)	0 (0.00)	0 (0.00)
irp2	19 (30.6)	50 (19.6)	2 (20.0)	3 (75.0)	0 (0.00)	0 (0.00)
traT	47 (75.8)	220 (86.3)	9 (90.0)	4 (100)	4 (100)	1 (100)
ompT	39 (62.9)	202 (79.2)	2 (20.0)	4 (100)	2 (50.0)	0 (0.00)
iroN	15 (24.2)	61 (23.9)	2 (20.0)	0 (0.00)	0 (0.00)	0 (0.00)
ibeB	55 (88.7)	221 (86.7)	7 (70.0)	4 (100)	0 (0.00)	0 (0.00)
ompA	55 (88.7)	223 (87.4)	9 (90.0)	4 (100)	1 (25.0)	0 (0.00)

by Raimondi et al. (2019) also identified similar VAGs in *E. coli* isolated from the feces of healthy individuals in Italy. Another study conducted by Khalifeh and Obaidat (2022) identified *the iroN* gene in *E. coli* strains from milk and fecal origin similar to the present study. In the current study, most of the *E. coli* strains were classified under phylogenetic group B1, which is consistent with the findings of Raimondi et al. (2019). These results suggest that the occurrence of ARGs and VAGs may vary by antimicrobial use and other unknown factors. This study also suggests regular monitoring of antimicrobial usage on dairy farms and proper manure treatment before disposal be ensured.

5. Conclusion

The present study identified multi-drug resistant *E. coli* strains carrying various ARGs and VAGs in the dairy environment, which may pose a potential threat to human, animal, and environmental health. Moreover, all of the *E. coli* strains were susceptible to meropenem, tigecycline, and colistin sulfate, which may be considered as critical antibiotics for therapeutic purposes in human and animal settings. Given the widespread distribution of AMR in the dairy environment, it is a potential reservoir of transferring ARGs genes to humans *via* various direct and indirect gene transfer mechanisms. This prudent the use of antibiotics on dairy farms, proper manure treatment, and enhancement of sanitation, especially in milk processing and transportation, are necessary to reduce the risk to food safety, public health, and environmental health.

Data availability statement

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

Author contributions

MS: writing–original draft. ZH and XG: data curation and formal analysis. MT and RH: graphical representation of data. SW and RS: review and editing. XW and HZ: project administration. WP: conceptualization, supervision, project visualization, and funding acquisition. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1197579/full#supplementary-material

References

Afema, J. A., Ahmed, S., Besser, T. E., Jones, L. P., Sischo, W. M., and Davis, M. A. (2018). Molecular epidemiology of dairy cattle-associated *Escherichia coli* carrying Bla CTX-M genes in Washington state. *Appl. Environ. Microbiol.* 84, e02430–e02417. doi: 10.1128/AEM.02430-17

Alfinete, N. W., Bolukaoto, J. Y., Heine, L., Potgieter, N., and Barnard, T. G. (2022). Virulence and phylogenetic analysis of enteric pathogenic *Escherichia coli* isolated from children with diarrhoea in South Africa. *Int. J. Infect. Dis.* 114, 226–232. doi: 10.1016/j.ijid.2021.11.017

Amézquita-López, B. A., Soto-Beltrán, M., Lee, B. G., Yambao, J. C., and Quiñones, B. (2018). Isolation, genotyping and antimicrobial resistance of Shiga toxin-producing *Escherichia coli*. *J. Microbiol. Immunol. Infect.* 51, 425–434. doi: 10.1016/j.jmii.2017.07.004

Bacanlı, M., and Başaran, N. (2019). Importance of antibiotic residues in animal food. *Food Chem. Toxicol.* 125, 462–466. doi: 10.1016/j.fct.2019.01.033

Beattie, R. E., Bakke, E., Konopek, N., Thill, R., Munson, E., and Hristova, K. R. (2020). Antimicrobial resistance traits of *Escherichia coli* isolated from dairy manure and freshwater ecosystems are similar to one another but differ from associated clinical isolates. *Microorganisms* 8:747. doi: 10.3390/microorganisms8050747

Cheng, J., Qu, W., Barkema, H. W., Nobrega, D. B., Gao, J., Liu, G., et al. (2019). Antimicrobial resistance profiles of 5 common bovine mastitis pathogens in large Chinese dairy herds. *J. Dairy Sci.* 102, 2416–2426. doi: 10.3168/jds.2018-15135

Cookson, A. L., Marshall, J. C., Biggs, P. J., Rogers, L. E., Collis, R. M., Devane, M., et al. (2022). Whole-genome sequencing and virulome analysis of *Escherichia coli* isolated from New Zealand environments of contrasting observed land use. *Appl. Environ. Microbiol.* 88, e00277–e00222. doi: 10.1128/aem.00277-22

Dadgostar, P. (2019). Antimicrobial resistance: implications and costs. *Infect. Drug Resist.* 12, 3903–3910. doi: 10.2147/IDR.S234610

Graham, D. W., Bergeron, G., Bourassa, M. W., Dickson, J., Gomes, F., Howe, A., et al. (2019). Complexities in understanding antimicrobial resistance across domesticated animal, human, and environmental systems. *Ann. N. Y. Acad. Sci.* 1441, 17–30. doi: 10.1111/nyas.14036

He, T., Wang, R., Liu, D., Walsh, T. R., Zhang, R., Lv, Y., et al. (2019). Emergence of plasmid-mediated high-level tigecycline resistance genes in animals and humans. *Nat. Microbiol.* 4, 1450–1456. doi: 10.1038/s41564-019-0445-2

Hogan, J., and Smith, K. (1992). Using bulk tank milk cultures in a dairy practice. Workshop mastitis microbiology diagnostics, National Mastitis Council, Arlington.

Hu, F., Guo, Y., Yang, Y., Zheng, Y., Wu, S., Jiang, X., et al. (2019). Resistance reported from China antimicrobial surveillance network (CHINET) in 2018. *Eur. J. Clin. Microbiol. Infect. Dis.* 38, 2275–2281. doi: 10.1007/s10096-019-03673-1

Hu, B., Yang, X., Liu, Q., Zhang, Y., Jiang, D., Jiao, H., et al. (2022). High prevalence and pathogenic potential of Shiga toxin-producing *Escherichia coli* strains in raw mutton and beef in Shandong, China. *Curr. Res. Food Sci.* 5, 1596–1602. doi: 10.1016/j.crfs.2022.08.021

Imre, K., Ban-Cucerzan, A., Herman, V., Sallam, K. I., Cristina, R. T., Abd-Elghany, S. M., et al. (2022). Occurrence, pathogenic potential and antimicrobial resistance of *Escherichia coli* isolated from raw Milk cheese commercialized in Banat region, Romania. *Antibiotics* 11:721. doi: 10.3390/antibiotics11060721

Jara, M. C., Frediani, A. V., Zehetmeyer, F. K., Bruhn, F. R. P., Müller, M. R., Miller, R. G., et al. (2021). Multidrug-resistant hospital bacteria: epidemiological factors and susceptibility profile. *Microb. Drug Resist.* 27, 433–440. doi: 10.1089/mdr.2019.0209

Khalifeh, O. M., and Obaidat, M. M. (2022). Urinary tract virulence genes in extended-spectrum beta-lactamase *E. coli* from dairy cows, beef cattle, and small ruminants. *Acta Trop.* 234:106611. doi: 10.1016/j.actatropica.2022.106611

Kudva, I. T., Cornick, N. A., Plummer, P. J., Zhang, Q., Nicholson, T. L., Bannantine, J. P., et al. (2020). *Virulence mechanisms of bacterial pathogens*. Hoboken, NJ: John Wiley & Sons.

Li, F., Cheng, P., Li, X., Liu, R., Liu, H., and Zhang, X. (2022). Molecular epidemiology and colistin-resistant mechanism of *mcr*-positive and *mcr*-negative *Escherichia coli* isolated from animal in Sichuan province, China. *Front. Microbiol.* 13:8548. doi: 10.3389/fmicb.2022.818548

Lima, T., Domingues, S., and Da Silva, G. J. (2020). Manure as a potential hotspot for antibiotic resistance dissemination by horizontal gene transfer events. *Vet. Sci.* 7:110. doi: 10.3390/vetsci7030110

Liu, H., Meng, L., Dong, L., Zhang, Y., Wang, J., and Zheng, N. (2021). Prevalence, antimicrobial susceptibility, and molecular characterization of *Escherichia coli* isolated from raw milk in dairy herds in northern China. *Front. Microbiol.* 12:656. doi: 10.3389/fmicb.2021.730656

Lu, Q., Zhang, W., Luo, L., Wang, H., Shao, H., Zhang, T., et al. (2022). Genetic diversity and multidrug resistance of phylogenic groups B2 and D in InPEC and ExPEC isolated from chickens in Central China. *BMC Microbiol.* 22, 1–12. doi: 10.1186/s12866-022-02469-2

Ma, F., Xu, S., Tang, Z., Li, Z., and Zhang, L. (2021). Use of antimicrobials in food animals and impact of transmission of antimicrobial resistance on humans. *Biosafety Health* 3, 32–38. doi: 10.1016/j.bsheal.2020.09.004

Ma, J., Zhou, W., Wu, J., Liu, X., Lin, J., Ji, X., et al. (2022). Large-scale studies on antimicrobial resistance and molecular characterization of *Escherichia coli* from food animals in developed areas of eastern China. *Microbiol. Spectrum* 10, e02015–e02022. doi: 10.1128/spectrum.02015-22

Manyi-Loh, C., Mamphweli, S., Meyer, E., and Okoh, A. (2018). Antibiotic use in agriculture and its consequential resistance in environmental sources: potential public health implications. *Molecules* 23:795. doi: 10.3390/molecules23040795

Murray, C. J., Ikuta, K. S., Sharara, F., Swetschinski, L., Aguilar, G. R., Gray, A., et al. (2022). Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. *Lancet* 399, 629–655. doi: 10.1016/S0140-6736(21)02724-0

Oladeinde, A., Cook, K., Lakin, S. M., Woyda, R., Abdo, Z., Looft, T., et al. (2019). Horizontal gene transfer and acquired antibiotic resistance in *Salmonella enterica* serovar Heidelberg following in vitro incubation in broiler ceca. *Appl. Environ. Microbiol.* 85, e01903–e01919. doi: 10.1128/AEM.01903-19

Peng, Z., Hu, Z., Li, Z., Zhang, X., Jia, C., Li, T., et al. (2022). Antimicrobial resistance and population genomics of multidrug-resistant *Escherichia coli* in pig farms in mainland China. *Nat. Commun.* 13:1116. doi: 10.1038/s41467-022-28750-6

Pormohammad, A., Nasiri, M. J., and Azimi, T. (2019). Prevalence of antibiotic resistance in *Escherichia coli* strains simultaneously isolated from humans, animals, food, and the environment: a systematic review and meta-analysis. *Infect. Drug Resist.* 12, 1181–1197. doi: 10.2147/IDR.S201324

Qian, X., Gu, J., Sun, W., Wang, X.-J., Su, J.-Q., and Stedfeld, R. (2018). Diversity, abundance, and persistence of antibiotic resistance genes in various types of animal manure following industrial composting. *J. Hazard. Mater.* 344, 716–722. doi: 10.1016/j.jhazmat.2017.11.020

Raimondi, S., Righini, L., Candeliere, F., Musmeci, E., Bonvicini, F., Gentilomi, G., et al. (2019). Antibiotic resistance, virulence factors, phenotyping, and genotyping of *E. coli* isolated from the feces of healthy subjects. *Microorganisms* 7:251. doi: 10.3390/microorganisms7080251

Salinas, L., Cárdenas, P., Johnson, T. J., Vasco, K., Graham, J., and Trueba, G. (2019). Diverse commensal *Escherichia coli* clones and plasmids disseminate antimicrobial resistance genes in domestic animals and children in a semirural community in Ecuador. *Msphere* 4, e00316–e00319. doi: 10.1128/mSphere.00316-19

Sarowska, J., Futoma-Koloch, B., Jama-Kmiecik, A., Frej-Madrzak, M., Ksiazczyk, M., Bugla-Ploskonska, G., et al. (2019). Virulence factors, prevalence and potential transmission of extraintestinal pathogenic *Escherichia coli* isolated from different sources: recent reports. *Gut Pathogens* 11, 1–16. doi: 10.1186/s13099-019-0290-0

Sharp, J. L., Mobley, C., Hammond, C., Withington, C., Drew, S., Stringfield, S., et al. (2012). A mixed methods sampling methodology for a multisite case study. *J. Mixed Methods Res.* 6, 34–54. doi: 10.1177/1558689811417133

Sobur, M. A., Sabuj, A. A. M., Sarker, R., Rahman, A. T., Kabir, S. L., and Rahman, M. T. (2019). Antibiotic-resistant *Escherichia coli* and *Salmonella* spp. associated with dairy cattle and farm environment having public health significance. *Veterinary world* 12, 984–993. doi: 10.14202/vetworld.2019.984-993

Su, Y., Yu, C.-Y., Tsai, Y., Wang, S.-H., Lee, C., and Chu, C. (2016). Fluoroquinolone-resistant and extended-spectrum β-lactamase-producing *Escherichia coli* from the milk of cows with clinical mastitis in southern Taiwan. *J. Microbiol. Immunol. Infect.* 49, 892–901. doi: 10.1016/j.jmii.2014.10.003

Thrusfield, M. (2018). *Veterinary epidemiology*. Hoboken, NJ: John Wiley & Sons.

Wang, Y., Xu, C., Zhang, R., Chen, Y., Shen, Y., Hu, F., et al. (2020). Changes in colistin resistance and mcr-1 abundance in *Escherichia coli* of animal and human origins following the ban of colistin-positive additives in China: an epidemiological comparative study. *Lancet Infect. Dis.* 20, 1161–1171. doi: 10.1016/S1473-3099(20)30149-3

Wang, W., Yu, L., Hao, W., Zhang, F., Jiang, M., Zhao, S., et al. (2021). Multi-locus sequence typing and drug resistance analysis of swine origin *Escherichia coli* in Shandong of China and its potential risk on public health. *Front. Public Health* 9:780700. doi: 10.3389/fpubh.2021.780700

Yu, Z., Wang, J., Ho, H., Wang, Y., Huang, S., and Han, R. (2020). Prevalence and antimicrobial-resistance phenotypes and genotypes of *Escherichia coli* isolated from raw milk samples from mastitis cases in four regions of China. *J. Global Antimicrobial Resist.* 22, 94–101. doi: 10.1016/j.jgar.2019.12.016

Zhang, S., Chen, S., Rehman, M. U., Yang, H., Yang, Z., Wang, M., et al. (2021). Distribution and association of antimicrobial resistance and virulence traits in *Escherichia coli* isolates from healthy waterfowls in Hainan, China. *Ecotoxicol. Environ. Safety* 220:112317. doi: 10.1016/j.ecoenv.2021.112317

Zhao, X., Lv, Y., Adam, F. E. A., Xie, Q., Wang, B., Bai, X., et al. (2021). Comparison of antimicrobial resistance, virulence genes, phylogroups, and biofilm formation of *Escherichia coli* isolated from intensive farming and free-range sheep. *Front. Microbiol.* 12:699927. doi: 10.3389/fmicb.2021.699927

Zou, M., Ma, P.-P., Liu, W.-S., Liang, X., Li, X.-Y., Li, Y.-Z., et al. (2021). Prevalence and antibiotic resistance characteristics of extraintestinal pathogenic *Escherichia coli* among healthy chickens from farms and live poultry markets in China. *Animals* 11:1112. doi: 10.3390/ani11041112

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Protective effects of yeast extract against alcohol-induced liver injury in rats

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Oxidative stress, inflammatory response, and gut-liver axis dysbiosis have been suggested as the primarily involved in the pathogenesis of alcoholic liver injury. Previous research established that yeast extract (YE) has antioxidant, immune-boosting or microbiota-regulating properties. However, there is currently lack of information regarding the efficacy of YE on alcoholic liver injury. This study seeks to obtain data that will help to address this research gap using a Wistar male rat experimental model. Histologic and biochemical analysis results showed that the groups treated with both low-dose yeast extract (YEL) and high-dose yeast extract (YEH) had lower degrees of alcohol-induced liver injury. The abundance of *Peptococcus* and *Ruminococcus* reduced in the low-dose yeast extract (YEL) group, while that of *Peptococcus*, *Romboutsia, Parasutterella*, and *Faecalibaculum* reduced in the high-dose (YEH) group. Furthermore, Spearman analysis showed that the gut microbes were significantly associated with several liver-related indicators. For the analysis of differential metabolites and enriched pathways in the YEL group, the abundance of lysophosphatidylcholine (16:0/0:0) significantly increased, and then the levels of histamine, adenosine and 5′ -adenine nucleotide were remarkedly elevated in the YEH group. These findings suggest that both high and low doses of YE can have different protective effects on liver injury in alcoholic liver disease (ALD) rats, in addition to improving gut microbiota disorder. Besides, high-dose YE has been found to be more effective than low-dose YE in metabolic regulation, as well as in dealing with oxidative stress and inflammatory responses.

KEYWORDS

yeast extract, alcoholic liver injury, gut microbiota, metabolomics, rat

1. Introduction

Alcoholic liver disease (ALD) is a widespread worldwide condition and is one of the main causes of chronic liver damage (Singal et al., 2018). According to a systematic analysis of the Global Burden of Disease Study that was published by the World Health Organization (WHO), alcohol abuse was responsible for an estimated three million deaths in 2016, thereby accounting for nearly 6% of total global deaths (GBD 2016 Alcohol Collaborators, 2018). Alcoholic fatty liver (AFL), alcoholic hepatitis (AH), alcoholic cirrhosis (AHF), and alcoholic hepatocellular carcinoma (AHC) are the main pathological changes of alcoholic liver disease, and they can exist alone, partially overlap, or even coexist (Magdaleno et al., 2017). Alcohol abuse can cause damage to multiple organs, including the intestines, brain, and the liver, which is mainly involved in alcohol metabolism. In China, alcohol has become the second leading cause of liver

injury, after hepatitis virus (Crabb et al., 2020). Mild liver damage caused by alcohol can be alleviated by long-term alcohol abstinence. However, when the situation develops to a severe stage in the later stage, the damage to the liver is extreme, irreversible, and even life-threatening (Joshi et al., 2016). Therefore, early intervention against and protection from ALD is of paramount importance.

Ethanol is mainly metabolized in the liver to form acetaldehyde, a reaction that is catalyzed by alcohol dehydrogenase (Setshedi et al., 2010). The acetaldehyde is then converted to acetic acid by acetaldehyde dehydrogenase (Kong et al., 2019). When excessive alcohol is consumed, acetaldehyde accumulates in the liver, and this, in turn, activates cytochrome P450 2E1 (CYP2E1) (Hyun et al., 2021). The activated CYP2E1 promotes acetaldehyde production through the formation of reactive oxygen species (ROS) (Cederbaum et al., 2009; Leung and Nieto, 2013). Meanwhile, the cumulative acetaldehyde reacts with antioxidants such as glutathione, reduces the level of antioxidants in the body, leads to an imbalance between oxidation and antioxidants in the body, and aggravates liver damage (Peixoto et al., 2004). This imbalance will increase neutrophil inflammation and protease secretion to generate ROS, eventually leading to oxidative stress (Lee et al., 2017; Albillos et al., 2020). The ROS also impair intestinal integrity (Seok et al., 2013). Additionally, research has found that alcohol consumption can increase the expression of miR-212, which down-regulates the expression of Zonula occludens 1 (ZO-1), ultimately leading to increased intestinal permeability (Tang et al., 2008). Therefore, lipopolysaccharide (LPS) produced by intestinal Gram-negative bacteria can be transported into the liver through the portal vein, thereby triggering the secretion of Kupffer cells such as interleukin-6 (IL-6), leukocytes-1β (IL-1β) (Aldred and Nagy, 1999; Wang et al., 2012), and tumor necrosis factor-α (TNF-α) (Wei et al., 2022). All these factors contribute to the development of ALD.

Yeast extract (YE) is a valuable source of amino acids, proteins, vitamin B-complex, peptides, and free nucleotides (Chae et al., 2001; Takalloo et al., 2020). It has been widely used as a nutritional resource, food flavoring, additive, and vitamin supplement (Eom et al., 2021). By adding YE to the feed, it can significantly improve the growth performance and antioxidant capacity of juvenile pacific white shrimp, along with the relative abundance of beneficial bacteria in the gut microbiota, while reducing the relative numbers of the conditional pathogenic bacteria (Zheng et al., 2021). A daily supplement of 100 mg/kg YE has been shown to reduce blood triglycerides and total cholesterol in rats (Waszkiewicz-Robak, 2013; Tao et al., 2023). YE also exerted anti-inflammatory properties by down-regulating the gene expression of inflammatory factors such as TNF-α and IL-1β in the body (Yuan et al., 2017; Jin et al., 2018). In terms of disease prevention, research has shown that yeast cell wall extract has a certain alleviating effect on necrotic enteritis in broiler chickens (M'Sadeq et al., 2015). Therefore, the YE has great potential for disease prevention and control across various fields. However, to date, there is a lack of research reports on the application of YE in the study of alcoholic liver injury. In this study, a rat model was established to explore the protective effects of different doses of the YE on alcoholic liver injury. By combining 16S rRNA sequencing and TM widely targeted metabolomics analysis, the "gut-liver" axis mechanism of the YE's protective action on the alcoholic liver injury was investigated.

Abbreviations: ADH, Alcohol Dehydrogenase; AKP, Alkaline phosphatase; ALD, Alcohol liver disease; ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; GPX, Glutathione peroxidase; IL-1β, interleukin-1β; INF-γ, interferon-γ; iNOS, inducible nitric oxide synthase; LDH, Lactate dehydrogenase; MDA, Malondialdehyde; OH Scavenging Ability, Hydroxyl Radical Scavenging Ability; SOD, Superoxide dismutase; T-AOC, Total antioxidant capacity; TC, Total Cholesterol; TG, Triglyceride; TNF-α, tumor necrosis factor-α; TNOS, total nitric oxide synthase..

2. Materials and methods

2.1. Materials

YE (MF-4, purity≥98%, nutrient composition are shown in Supplementary Table S1)was supplied from Mufan Biotechnology Co., Ltd. (Henan, China). Silymarin (H20181067, 140 mg/capsule) was obtained from Plantextrakt GmbH & Co. KG. The liquid diet was purchased from Trophic Animal Feed High-Tech Co., Ltd. (Nantong, China). Detection kits for alanine aminotransferase (ALT), aspartate aminotransferase (AST), total antioxidant capacity (T-AOC), glutathione peroxidase (GPX), malondialdehyde (MDA), superoxide dismutase (SOD), lactic dehydrogenase (LDH), and superoxide Anion (O2-) were purchased from Beijing Boxbio Science & Technology Co., Ltd. Detection kits for alkaline phosphatase (AKP), triglyceride (TG), total cholesterol (T-CHO), lipid peroxidation (LPO), alcohol dehydrogenase (ADH), Hydroxyl Radical Scavenging Ability (OH Scavenging Ability), total nitric oxide synthase (TNOS), and inducible nitric oxide synthase (iNOS) were purchased from Jiancheng Corp (Nanjing, China). ELISA kits for leukocytes-1β (IL-1β), tumor necrosis factor-α (TNF-α), interferon-γ (INF-γ) were supplied from Elabscience Biotechnology Co., Ltd. Fish oil was purchased from Shanghai Yien Chemical Technology Co., Ltd. (Shanghai, China). Leukocytes-1β (IL-1β), tumor necrosis factor-α (TNF-α) and interferon-γ (INF-γ) were supplied from Elabscience Biotechnology Co., Ltd. Fish oil was purchased from Shanghai Yien Chemical Technology Co., Ltd. (Shanghai, China).

2.2. Animals and experimental design

Four-week-old male Wistar rats were purchased from Lanzhou University Animal Experimental Center (Lanzhou, China). The rats were housed in a clean environment with a 12-h light–dark cycle at 20–22°C and 45 ± 5% humidity. All the rats were free to access food and water *ad libitum*. All experiments involving animals were approved by the Ethics Committee of School of Public Health, Lanzhou University (IRB22030601). After one-week of acclimatization, the rats were randomly divided into the following five groups (11 rats per group): liquid diet control group (LC), alcohol liquid diet group (AL), low-dosage yeast extract intervention group (YEL, 60 mg/kg/day), high-dosage yeast extract intervention group (YEH, 120 mg/kg/day), and silymarin intervention group (PC, 120 mg/kg/day). AL, PC, YEL, and YEH groups were all given liquid diet with an alcohol concentration of 8% (v/v). Both types of liquid diet (with and without alcohol) had calorie densities of 1 kcaL/mL. The source of fatty acids in the diet was fish oil. During the first 3 days of adaptive feeding, all groups were given a control liquid diet. From the fourth to the nineth day, all groups (except for the LC group) entered the adaptive feeding stage of an alcohol liquid diet. The

ratio of liquid control feed to liquid alcohol feed was 2:1, 1:1, and 1:2, and each ratio was fed for 2 days. After the adaptive feeding period, the rats were provided with a complete liquid alcohol diet. At the start of the alcohol feeding procedure, YE and silymarin were well-dissolved in ultrapure water prior to daily administration to the animals via gavage, over a period of 6 weeks. Food intake and body weight were monitored daily and weekly, respectively. At the end of the experiments, the rats were gavaged with 40% alcohol (15 mL/kg). After 12 h, the rats were anesthetized with an intraperitoneal injection of 3% sodium pentobarbital and then sacrificed. The liver of each rat was weighed to calculate the liver organ coefficient (wet liver weight, mg/ body weight, g). The livers were removed and rinsed with cold PBS. Portions of the liver were used for histological evaluation, and others were stored at −80°C for further analysis. Fecal samples were immediately frozen in liquid nitrogen and stored at −80°C until analysis was done.

2.3. Hepatic histological analysis

Samples of the right liver lobe were fixed in 4% paraformaldehyde for 24 h, embedded in paraffin wax, and then cut into 5 μm-thick sections. The sections were blurred by Hematoxylin–eosin (H&E) staining and Masson's trichrome. The pathological changes on the liver were observed using the BX53 system microscope with an attached camera (Olympus, Japan) at 400× magnification.

2.4. Analysis of liver function index

The liver samples (about 100–150 mg) were mixed with 0.9% normal saline at a ratio of 1:9 to make homogenates. Centrifugation was then done at 3000 rpm for 15 min, at 4°C. The supernatant was collected for subsequent analyzes. The levels of ALT, AST, AKP, ADH, and LDH were assessed according to the instructions on the commercial assay kits.

2.5. Detection of oxidative stress indicators

To evaluate the oxidative stress indicators, homogenates were prepared using liver samples weighing 200 mg combined with normal saline, at a ratio of 1:9. The levels of MDA, T-AOC, LPO, SOD activity, and GPX activity were detected, following the manufacturer's instructions.

2.6. Inflammatory parameters analysis

The liver tissues (about 50–100 mg) were mixed with normal saline at a ratio of 1:9 to prepare homogenates. Supernatants were collected after centrifugation. The levels of TNOS and iNOS in the liver tissues were analyzed by assay kits (Nanjing, China), according to the manufacturers' instructions. The levels of IL-1β, TNF-α, and INF-γ in liver tissue were measured using ELISA kits (Elabscience Biotechnology, China) followed by the method described in the instructions.

2.7. 16S rRNA gene sequencing analysis

The analysis was conducted at Metware Biotechnology Co., Ltd. (Wuhan, China). Genomic DNA from the fecal samples (six samples were randomly selected from each group) was extracted by the Magnetic Soil and Stool DNA kit (TianGen Biotech Co., Ltd., Beijing, China). The bacterial sample was used to amplify the V4 hypervariable region of the 16S rRNA gene with primers 515F(GTGCCAG CMGCCGCGGTAA) and 806R(GGACTACHVGGGTWTCTAAT) (Itoh et al., 2014). Purified PCR products were quantified, using enzyme-labeled quantification, and the sequencing library preparation was performed based on the manufacturer's instructions (Illumina, San Diego, CA, United States). Quantification was performed by Qubit and Q-PCR;sequencing was done using NovaSeq6000 (Illumina, San Diego, CA, United States). For these data, the reads of each sample were assembled using FLASH (v1.2.11, http://ccb.jhu.edu/software/FLASH/) after truncating the barcode and primer sequences, resulting in the Raw Tags. The Raw Tags were then truncated at the first low-quality base site, with consecutive low-quality values (default quality threshold: ≤ 19) and a base number reaching the set length (default length value is 3). Subsequently, the truncated Tags were filtered to remove those with a continuous high-quality length below 75% of the Tags length. The effective Tags were sequenced using Novaseq 6,000 (Illumina, San Diego, CA, United States), and the Uparse algorithm (v7.0.1001, http://www.drive5.com/uparse/) was used to cluster all sequences into OTUs (Operational Taxonomic Units). For the species annotation analysis of OTUs sequences, the Mothur method and the SSUrRNA database of SILVA138.1[1] were used, setting the threshold between 0.8 ~ 1.

Furthermore, the Qiime software (version 1.9.1) was used to the "observed number," as well as the Shannon and Simpson indices. R software (version 4.1.2) was employed in creating the Rank abundance curve. The nonparametric test of the difference between groups in Alpha diversity index was done using the Kruskal-Wallis test. LEfSe analysis was performed using the LEfSe software (version 1.0, https://huttenhower.sph.harvard.edu/lefse/), and the default LDA score filtering value was set to 4. The Mothur software (University of Michigan, Michigan, United States) was used in Metastats analysis at each classification level to obtain the p value ($p < 0.05$). The Benjamini and Hochberg False Discovery Rate method was then used to correct the p value to obtain the q value.

2.8. Metabolomics analysis

In this research, the metabolites were analyzed at Metware Biotechnology Co., Ltd. (Wuhan, China). Fecal samples (six samples were randomly selected from each group) stored at −80°C refrigerator were thawed. A 400 μL solution (methanol: water = 7:3, v/v) containing an internal standard was added into each sample (20 mg) and vortexed for 3 min. All samples were sonicated in an ice bath for 10 min and vortexed for 1 min before being placed in −20°C for 30 min. These samples were then centrifuged at 12000 rpm for 10 min at 4°C. Each supernatant was recentrifuged at 12000 rpm for 3 min at 4°C. 200 μL

1 http://www.arb-silva.de/

aliquots of each supernatant were transferred for ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS, Sciex, United States) analysis, which included both non-targeted and broad-spectrum targeted detection. The non-targeted metabolites were separated on a pre-column (ACQUITY HSS T3 C18,1.8 μm, 2.1 mm*100 mm), with the column temperature being set at 40°C. *mobile* phase A consisted of water with 0.1% formic acid, and mobile phase B was comprised of acetonitrile with 0.1% formic acid. The gradient program was as follows: 95:5 v/v at 0 min; 10:90 v/v at 10.0 and 11.0 min; 95:5 v/v at 11.1 min; 95:5 v/v at 14.0 min. The flow rate was 0.4 mL/min, with an injection volume of 5 μL. The Quadrupole-Time of Flight mass spectrometry analysis was performed with an electrospray ionization (ESI) temperature of 500°C, voltage of 5,500 V (positive) and −4,500 V (negative), ion source gas I (GS I) at 50 psi, gas II (GS II) at 50 psi, and Curtain gas (CUR) at 25 psi. The delustering potential collision energy was set at 80 psi, and the collision energy spread was at 15 psi. For Broad-spectrum targeted detection, a waters ACQUITY UPLC HSS T3 C18 chromatographic column (1.8 μm, 2.1 mm*100 mm) was employed, with an injection volume of 2 μL. Tandem mass spectrometry (QTRAP®) analysis was carried out using the same ESI temperature, voltage, ion source gas, and curtain gas condition as those in the Quadrupole-Time of Flight mass spectrometry.

All sample extracts were mixed in equal amounts to form a QC sample. Based on the database MWDB (including secondary spectrum, retention time RT), DB-all public database (including HMDB, KEGG and other databases), AI prediction library, and MetDNA, metabolites identification and extraction of multiple ion pairs, as well as retention time RT information were performed The MRM accurate quantification of the metabolites in the new library was carried out using the Q-Trap instrument platform.

The OPLS-DA function of the MetaboAnalyst R package in the R software was used for analysis. Based on the OPLS-DA results, the Variable Importance in Projection (VIP) of the OPLS-DA model was determined from the obtained multivariate analysis, and the differential metabolites were further screened by combining with the fold change. The selected data with different amounts of the metabolites were treated with UV (unit variance scaling). The R software Complex Heatmap package was used to draw the cluster heat map. Pearson correlation analysis was used to analyze the association between different metabolites that were identified according to the screening criteria.

Finally, the KEGG (Kanehisa and Goto, 2000)[2] and HMDB[3] database were used to annotate metabolites and identify the pathways of differential metabolite enrichment.

2.9. Statistical analysis

All statistical analysis was carried out using the SPSS statistical software (version 22.0, IBM, White Plains, New York, United States), and the measurement data were expressed as mean ± standard error of mean deviation. One-way ANOVA was used for comparison among multiple groups and the significance of difference between the two groups was determined by LSD test. When the variances are not equal, Dunnett's T3 was used to test the differences between the two groups. Values of $p < 0.05$ were considered significant. All images were generated by OriginPro (2019, OriginLab, Massachusetts, United States) and GraphPad Prism (version 9.5.1, Graphpad Software, California, United States).

3. Result

3.1. Effect of YE on alcohol-induced hepatoxicity

To explore the effects of YE on ALD, H&E and Masson staining were performed to check the histomorphological changes that were induced by alcohol. The hepatocytes arranged neatly without obvious pathological changes in the LC group, but there was irregular arrangement of hepatocytes, diffuse steatosis, and a large number of inflammatory cell infiltration in AL group (Figure 1B). According to Masson staining, there were more parts that were dyed blue in the AL group, suggesting a more serious case of fibrosis (Figure 1A). Compared with the AL group, the pathological changes observed in the liver were alleviated in the PC, YEL, and YEH groups, with YEH exhibiting the most significant improvement (Figures 1A,B). In comparison to the AL group, a remarkable decrease in the liver organ coefficient was observed in the YEH group ($p < 0.05$, Figure 1C), whereas no significant reduction was noted in the PC and YEL groups. Biochemical analysis demonstrated that the levels of hepatic ALT, AST, and AKP, which are diagnostic markers of liver injury, remarkably increased in the AL group compared to the LC group. However, the administration of YE and Silymarin effectively inhibited the increase (Figures 1D–F). The administration of YE also reduced the accumulation of TC and TG ($p < 0.01$, Figures 1G,H). Additionally, high doses of YE significantly decreased LDH levels ($p < 0.01$, Figure 1J). Furthermore, YE administration effectively prevented the decrease in ADH levels ($p < 0.001$, Figure 1I).

3.2. YE inhibited alcohol-induced oxidative stress

The levels of T-AOC, GPX, SOD and·OH scavenging ability remarkably decreased in the AL group than in the LC group (Figures 2A,B,D,F). On the other hand, the levels of MDA and O_2^- exhibited opposing trends ($p < 0.05$, Figures 2C,E). It is noteworthy that the groups with the YE intervention exhibited strong antioxidant activity by resisting alcohol-induced liver damage, which showed good protective effects ($p < 0.05$, Figures 2A–F). However, there were no significant changes in the levels of T-AOC and O_2^- after treatment with Silymarin ($p > 0.05$, Figures 2D,E). The highest level of ·OH scavenging ability was observed in the YEL group ($p < 0.05$, Figure 2F).

3.3. YE reversed alcohol-induced inflammatory response

Some studies have reported that excessive or chronic alcohol use can trigger an inflammatory response in the liver (Xiao et al., 2020; Hyun et al., 2021). As shown in Figure 3, alcohol ingestion significantly

2 https://www.kegg.jp
3 http://www.hmdb.ca/

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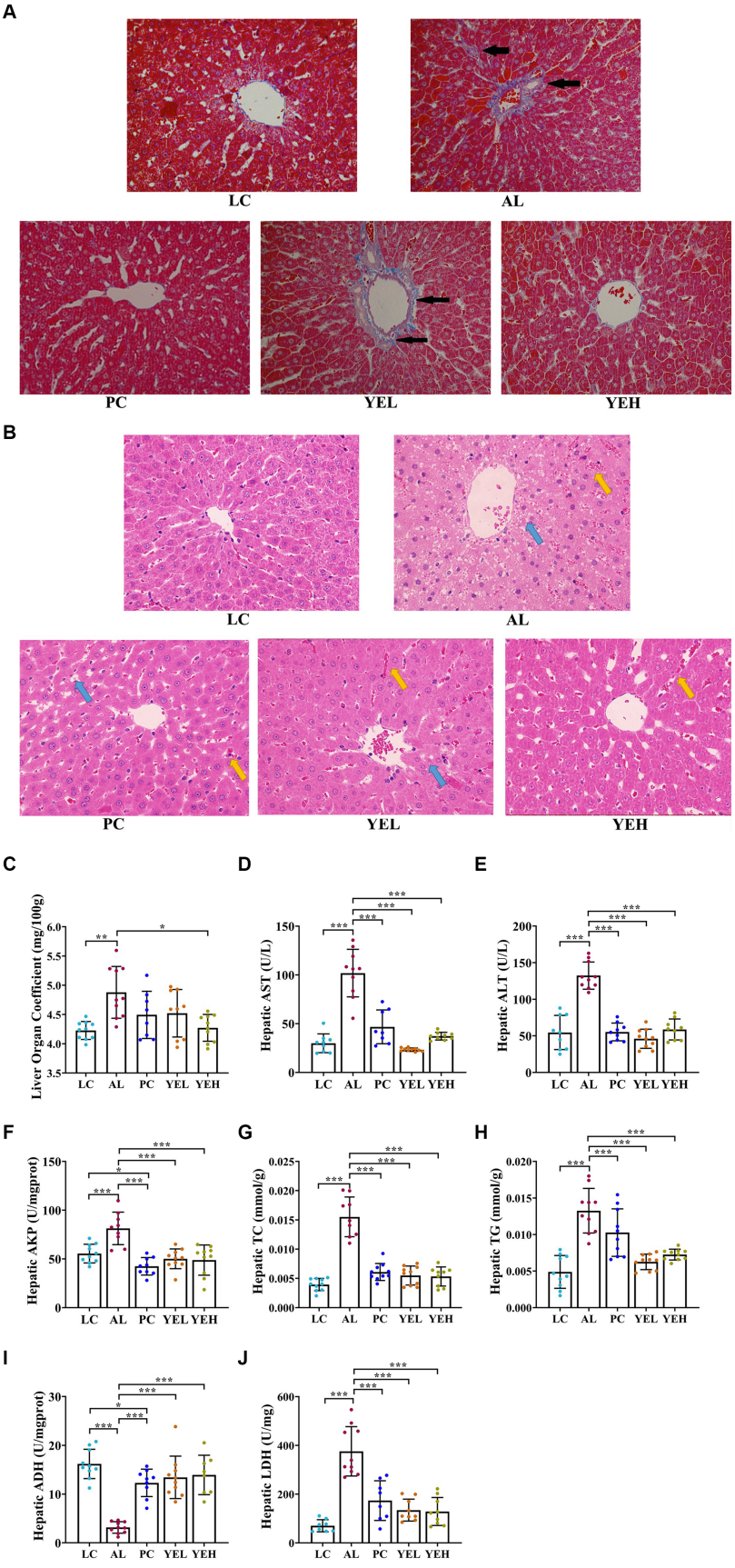

FIGURE 1

(Continued)

FIGURE 1 (Continued)

Effect of yeast extract on alcohol-induced liver injury. **(A)** Histopathological changes of liver sections stained with Masson (400× magnification), black arrow: liver tissue fibrosis. **(B)** Histopathological changes of liver sections stained with H&E (400× magnification), yellow arrow: inflammatory exudation; blue arrow, steatosis. **(C)** The levels of liver organ coefficient. **(D)** The hepatic AST levels. **(E)** The hepatic ALT levels. **(F)** The hepatic AKP levels. **(G)** The hepatic TC levels. **(H)** The hepatic TG levels. **(I)** The hepatic ADH contents. **(J)** The hepatic LDH contents. All data were expressed as mean ± SD ($n = 8–10$), $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

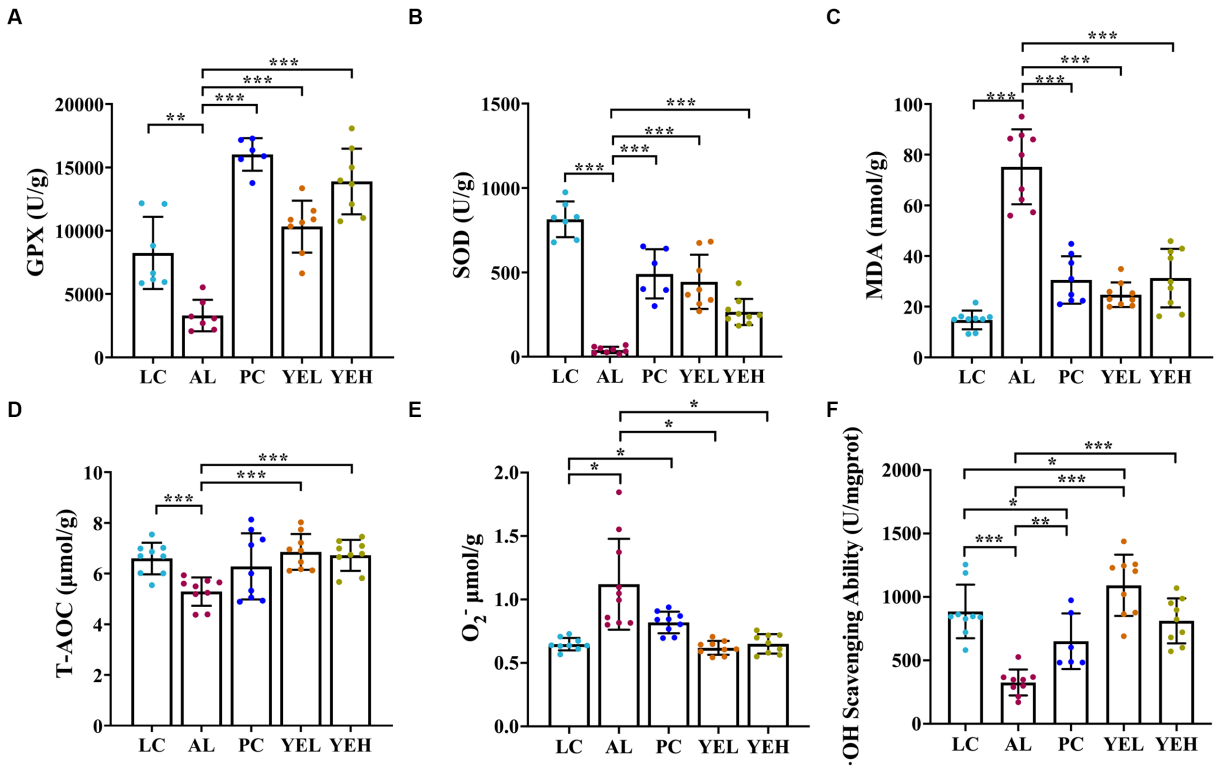

FIGURE 2

Effect of yeast extract on oxidative stress in alcohol-induced liver injury in rats. **(A)** The levels of GPX. **(B)** The levels of SOD. **(C)** The levels of MDA. **(D)** The levels of T-AOC. **(E)** The levels of O_2^-. **(F)**·OH scavenging ability. Data were expressed as mean ± SD ($n = 6–9$), $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

increased the levels of IL-1β, TNF-α, IFN-γ, iNOS, and TNOS in the liver of rats ($p < 0.05$, Figures 3A–E). Compared with the AL group, administration with high-dosage YE inhibited alcohol-induced inflammatory response, and this was consistent with LC group ($p < 0.05$, Figures 3A–E). However, the levels of IL-1β, TNF-α, and IFN-γ in the YEL group and those of IFN-γ in the Silymarin group did not exhibit obvious changes ($p > 0.05$, Figures 3A–C). These findings suggested that YE intervention can reduce the levels of inflammatory factors and alleviate alcohol-induced liver damage. High dosage of YE appear to provide superior protective effects.

3.4. Effects of YE intervention on gut microbiota

ALD is closely associated with the gut microbiota. Long-term consumption of alcohol can substantially alter the structure of the gut microbiota in ALD patients (Bishehsari et al., 2017; Wang et al., 2021). To characterize alterations in gut microbiota, fecal samples were subjected to 16S rRNA gene sequencing. The analysis revealed specific and shared OTUs among the five groups of mice (Supplementary Figure S1A). In comparison to the LC group, all experimental groups exhibited an increase in both the Shannon and Simpson diversity index (Supplementary Figures S1B,C). The Weighted Unifrac analysis demonstrated that the β-diversity of gut microbiota in the PC, YEL, and YEH groups was significantly distinct from that of the AL group, but closer to that of the LC group (Supplementary Figures S1D,E). These results suggest that YE intervention could reverse alcohol-induced dysbiosis of gut microbiota in rats.

The structure of the gut microbiome was investigated at both phylum and genus levels (Figures 4A,B). Bacteroidetes and Firmicute were the most abundant at the phylum level in all groups. However, when compared with the AL group, the ratio of Firmicutes to Bacteroidetes (F/B) was reduced in the other groups, though the change was not significant (Figure 4C). On the other hand, the population of Proteobacteria was significantly elevated in both the YEH and PC groups ($p < 0.05$), compared to the AL group. At the genus level, intervention with YEL and YEH resulted in a decrease in the relative abundance of *Peptococcus* and *Tyzzerella*, while an increase in the relative abundance of *Gemella* (Figures 4D–F), compared to the AL group was observed. In the YEL group, the relative abundance of

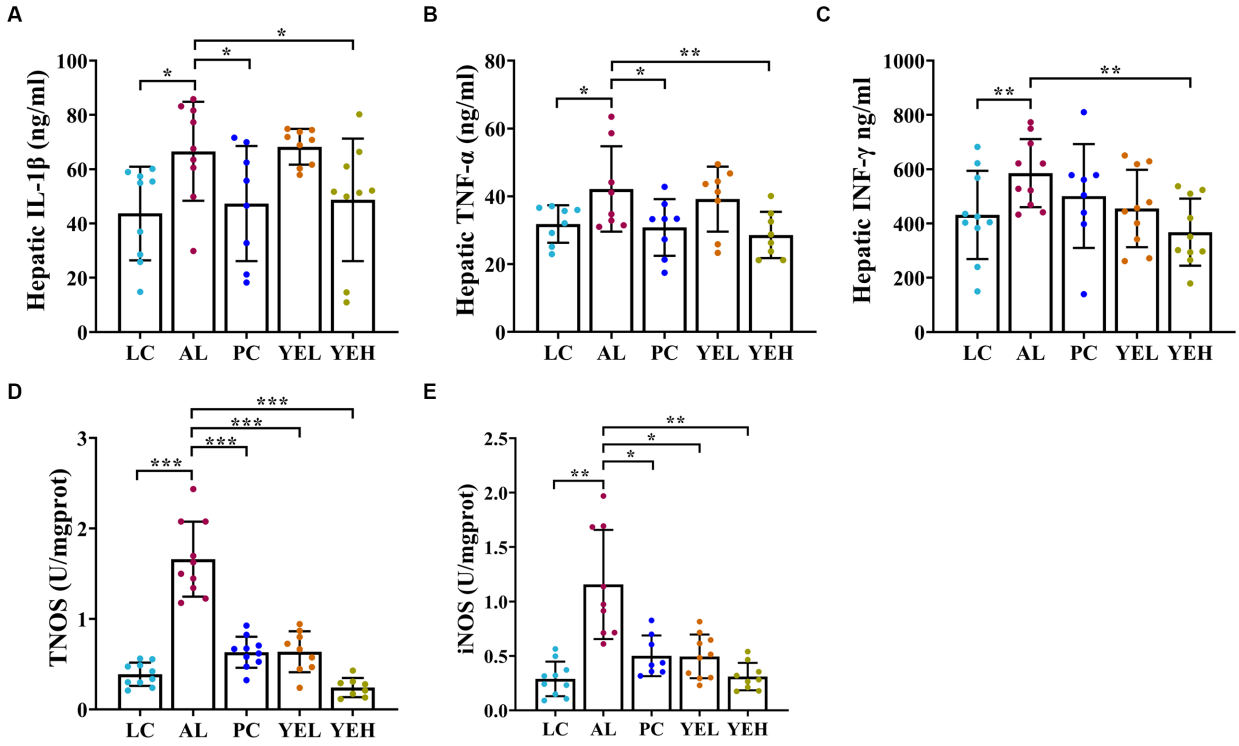

FIGURE 3
Effect of yeast extract on inflammatory response in alcohol-induced liver injury in rats. **(A)** The levels of IL-1β. **(B)** The levels of TNF-α. **(C)** The levels of IFN-γ. **(D)** The levels of TNOS. **(E)** The levels of iNOS. Data were expressed as mean ± SD ($n = 8–10$), $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Ruminococcus and *Faecalibacterium* was significantly decreased (Figures 4G,H). It was also noted that the administration of YEH resulted in a significantly reduced relative abundance of *Faecalibaculum*, *Helicobacter, Parasutterella* and *Romboutsia* compared to the AL group, while the *Corynebacterium* remarkably increased (Figures 4I–M).

Linear discriminant analysis (LDA) and LEfSe analysis were used to identify biomarkers with significant differences. Based on LDA scores, the relative abundance of *Ruminococcus* was significantly higher in the AL group compared to the YEL group (Figure 5A). In addition, *Romboutsia, Faecalibaculum*, and *Parasutterella* were found to be more abundant in the AL group than in the YEH group (Figure 5B). The alterations in gut microbiota have a certain degree of correlation with some physicochemical indicators that are related to liver injury. As shown in Figure 5C, the genera such as *Tyzzerella* and *Peptococcus* decreased after YE intervention, and these positively correlated with ALT, AST, and MDA. In addition, positive correlation was observed between *Peptococcus* and AKP. However, negative correlation was noted between *Peptococcus* and ADH, SOD, GSH, and T-AOC. After YE intervention, *Gemella* increased, while *Corynebacterium* levels only increased in the YEH group. Both genera showed significant positive correlations with hydroxyl radical scavenging ability, but significant negative correlations with TNOS were observed (Figure 5C). In the YEL group, *Faecalibacterium* and *Ruminococcus* significantly decreased. *Faecalibacterium* showed a positive correlation with MDA and TC, but a negatively correlation with SOD. On the other hand, the abundance of *Ruminococcus* positively correlated with ALT, AST, AKP, TC, TG, and MDA, while it was negatively associated with levels of ADH, SOD, GPX, and hydroxyl radical scavenging ability (Figure 5C). The genera *Romboutsia* and *Faecalibaculum* decreased in the YEH intervention group. *Romboutsia* exhibited a significant positive correlation with TNOS and iNOS, while *Faecalibaculum* showed a positive correlation with TC and iNOS, but a negative association with GPX (Figure 5C).

3.5. Yeast extract altered metabolic profiles

To further explore and understand the effects of altered gut microbiota on metabolite changes, a total of 2029 metabolites were detected through the extensive targeted metabolomics analysis (Figure 6A). According to the orthogonal partial least-squares discriminant analysis (OPLS-DA), metabolites in the YEH group could be well separated from those in the AL group ($Q^2 > 0.5$) (Figure 6C). Based on the threshold of fold change ≥2 or fold change ≤0.5 and VIP > 1, 201 differential metabolites were identified between the AL and YEL groups, while 470 differential metabolites were found between AL and YEH groups. By combining pathways with significant enrichment in KEGG and HMDB databases, it was discovered that among the samples of AL and YEH, 14 metabolites were significantly different (two up-regulated metabolites and 12 down-regulated metabolites). Additionally, there were six remarkedly different metabolites (five up-regulated metabolites and one down-regulated metabolite) among the samples of AL and YEL groups. The heatmaps of specific metabolic difference substances are shown (Figures 7A,B).

To explore the relationship between the co-regulation of gut microbes and metabolites, the Spearman correlation analysis was performed (Figure 7C). *Gemella* levels were found to exhibit notable

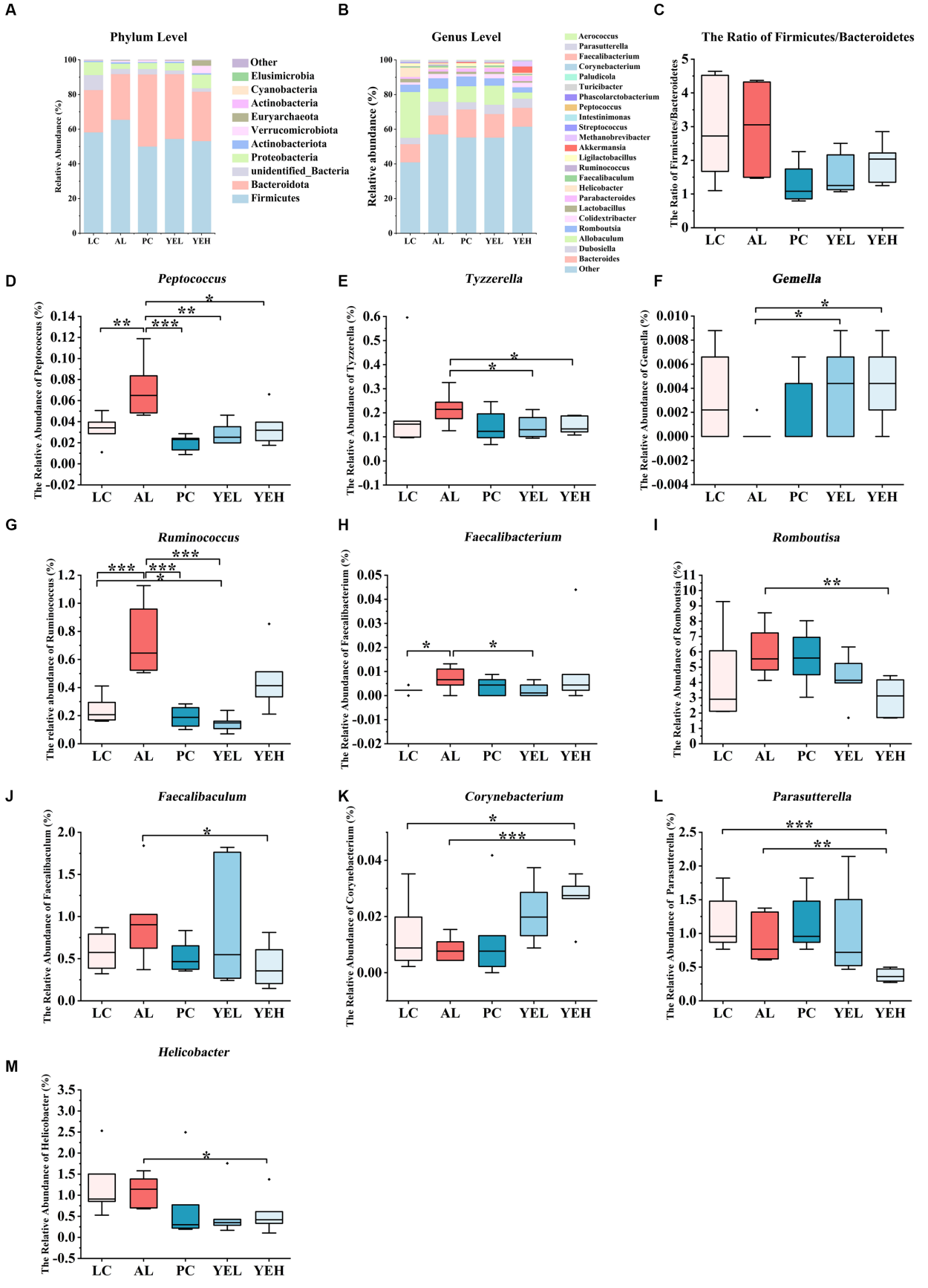

FIGURE 4

(Continued)

FIGURE 4 (Continued)

Yeast extract intervention altered the gut microbiota structure of alcohol-induced liver injury in rats. **(A)** Bacterial taxonomic composition at phylum level. **(B)** Bacterial taxonomic composition at genus level. **(C)** The ratio of Firmicutes to Bacteroidetes (F/B). **(D)** The relative abundance of *Peptococcus*. **(E)** The relative abundance of *Tyzzerella*. **(F)** The relative abundance of *Gemella*. **(G)** The relative abundance of *Ruminococcus*. **(H)** The relative abundance of *Faecalibacterium*. **(I)** The relative abundance of *Romboutsia*. **(J)** The relative abundance of *Faecalibaculum*. **(K)** The relative abundance of *Corynebacterium*. **(L)** The relative abundance of *Parasutterella*. **(M)** The relative abundance of *Helicobacter*, $n = 6$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

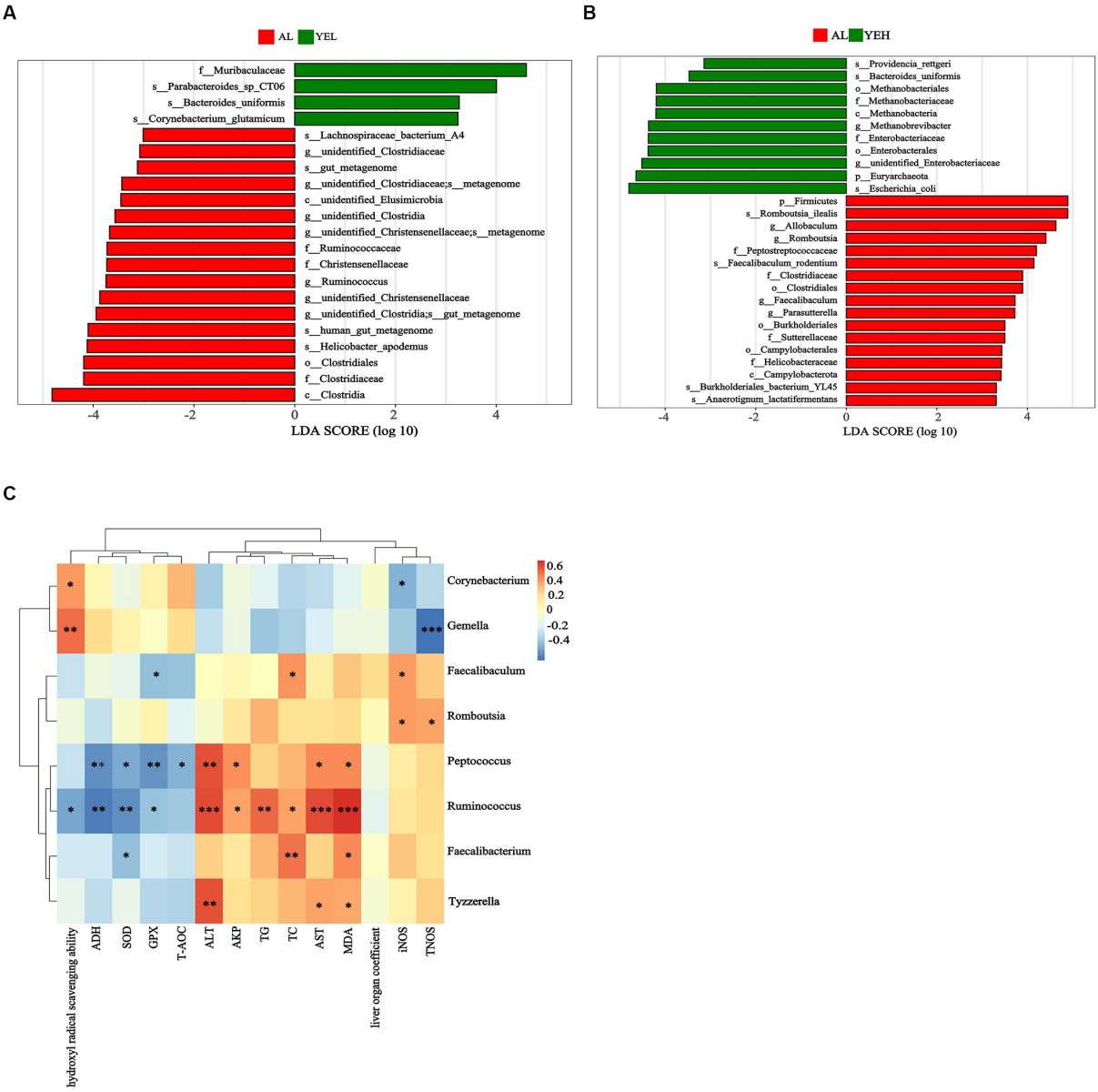

FIGURE 5

Linear discriminant analysis (LDA) and the correlation between genera and liver related indicators. **(A)** Linear discriminant analysis between AL group and YEL group. **(B)** Linear discriminant analysis between AL group and YEH group. **(C)** Spearman's correlation between the liver related indicators and the relative abundance of significantly different genera, $n = 6$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Red squares represent positive correlation and blue squares represent negative correlation. The darker the red, the greater the correlation coefficient, and the darker the blue, the smaller the correlation coefficient.

negative correlation with 17β-estradiol 3-(β-D-glucuronide) concentrations. *Parasutterella* positively correlated with histamine and adenosine, while *Corynebacterium* inversely correlated with histamine.

According to the KEGG database, the YEL notably affected insulin resistance (Figure 8A), while the HMDB database indicated that the intervention of the YEL significantly affects two major metabolic pathways: taurine and hypotaurine metabolism, as well as phospholipid biosynthesis. According to the KEGG database, there are significant differences in steroid hormone biosynthesis, morphine addiction, cGMP-PKG signaling pathway, and gastric acid secretion

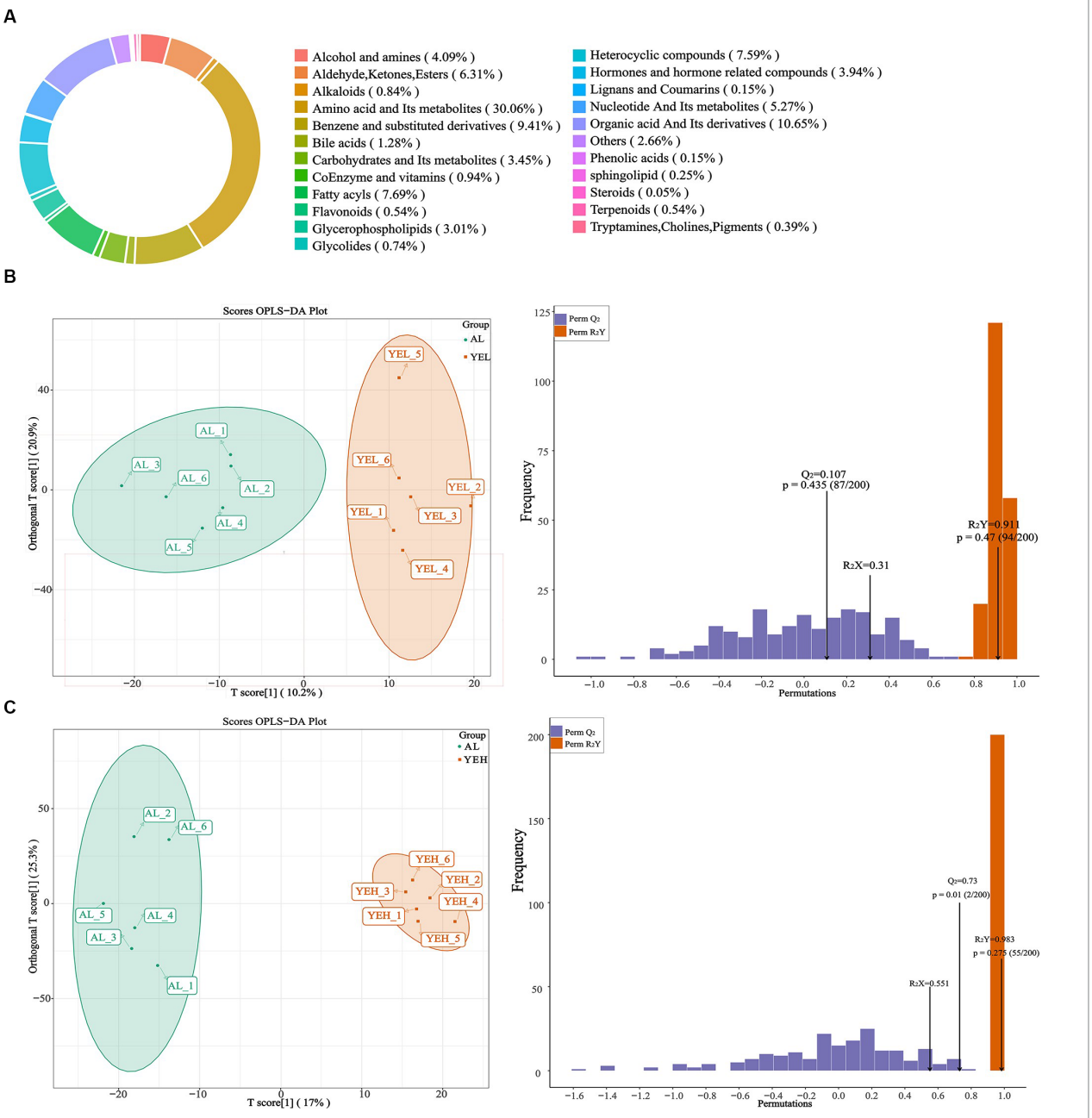

FIGURE 6
Effects of yeast extract on metabolic profiling. **(A)** Composition of all metabolites. **(B)** The orthogonal partial least-squares discriminant analysis (OPLS-DA) of AL and YEH groups. **(C)** OPLS-DA of AL and YEH groups.

between the AL and YEH groups (Figure 8C). Based on the HMDB database, YEH significantly impacted three metabolic pathways: intracellular signaling via adenosine receptor A2a and adenosine, intracellular signaling through adenosine receptor A2b and adenosine, and intracellular signaling mediated histamine receptor H2 and histamine (Figure 8D).

4. Discussion

YE has strong antioxidant and anti-inflammatory properties. Oxidative stress and inflammation are important mechanisms that greatly contribute to alcoholic liver injury. Yeast extract primarily consists of yeast cell wall, yeast protein, and yeast nucleotide. The presence of β-glucan in the yeast cell wall has been scientifically demonstrated to enhance the activity of immune cells, thereby boosting immunity (Zhang et al., 2018). Additionally, yeast nucleotide has been found to mitigate the adverse effects of *Eimeria* on the intestinal tract (Leung et al., 2019). In this study, we hypothesized that the YE could exert protective effects against liver injury. We established an animal model for alcoholic liver injury. Related physicochemical indicators, as well as the response of the gut microbiota and metabolites were analyzed to verify our hypothesis.

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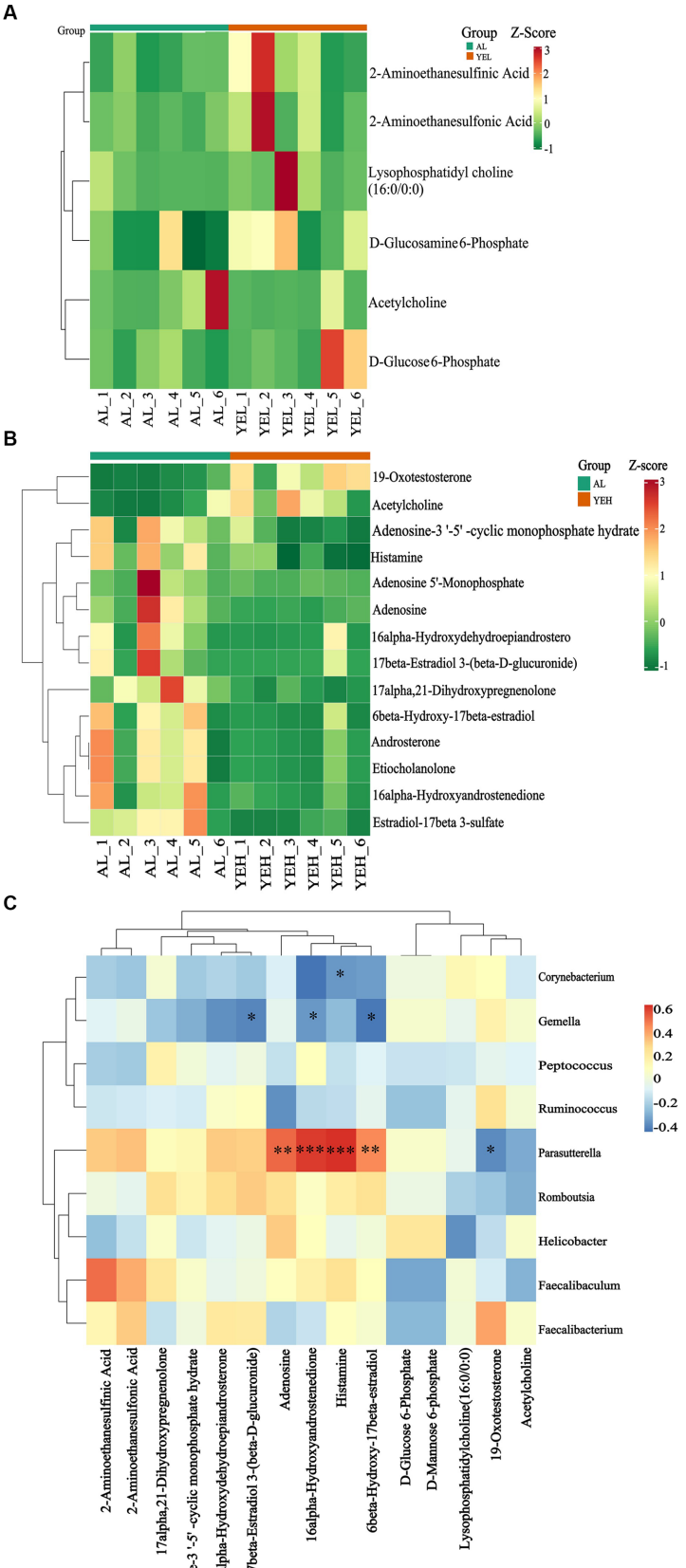

FIGURE 7

(Continued)

FIGURE 7 (Continued)
Effects of yeast extract on metabolic profiles. **(A)** Heatmap of differential metabolites in AL and YEL groups. **(B)** Heatmap of differential metabolites in AL and YEH groups. **(C)** Spearman's correlation between the relative abundance of significantly different genera and differential metabolites. Red squares represent positive correlation while blue squares represent negative correlation. The darker the red, the greater the correlation coefficient, and the darker the blue, the smaller the correlation coefficient, $n = 6$.

As expected, the levels of AST, ALT, AKP (Figures 1D–F), TC and TG (Figures 1G,H) in the rats of the AL group significantly increased. Hepatocyte damage can be assessed by the rise in ALT and AST levels (Giannini et al., 2005; Ozer et al., 2008), while the increase of AKP might suggest that cholestasis could occur in the liver (Ramaiah, 2007). Chronic alcohol consumption can lead to hepatic lipid metabolism disorders, resulting in a rise of TG and total TC levels (Xu et al., 2018). After the YE intervention, the levels of the above-mentioned indicators were significantly reduced in both the YEL and the YEH groups. This indicated that YE can effectively alleviate alcohol-induced liver cell dysfunction and promote lipid metabolism.

Oxidative stress is an imbalance between antioxidants and oxidation in the body, with the former being significantly lower. Ethanol consumption depletes a substantial amount of antioxidant enzymes (such as SOD or GPX) and generates an abundance of ROS, which ultimately causes damage. The intervention of the YE can markedly increase GPX, SOD and T-AOC (Figures 2A,B,D), while significantly decreasing MDA (Figure 2C), thereby effectively reducing oxidative stress (Yuan et al., 2018; Gao et al., 2022).

After the YE and silymarin interventions, the levels of TNOS and iNOS were significantly reduced (Figures 3D,E). TNOS and iNOS are crucial indicators of inflammatory injury so reducing the expression levels of their related genes can lower the inflammatory response (Lee et al., 2016). Continuous intake of ethanol induces the production of iNOS (Davis and Syapin, 2004), an isoform of nitric oxide synthase which oxidizes L-arginine to L-citrulline and generates nitric oxide (Chen et al., 2015). The nitric oxide combines with the superoxide anion to form an oxidant that causes lipid peroxidation, which damages the body (Zhu et al., 2023). In addition, the intervention of YEH significantly reduced the levels of IL-1β, TNF-α, and IFN-γ (Figures 3A–C), with effects that were even better than those that were observed in the silymarin intervention group. LPS that are produced by intestinal bacteria can stimulate Kupffer cells to secrete TNF-α. In addition, rats with lower IL-1β levels exhibited a significant reduction in the transition from steatosis to steatohepatitis and liver fibrosis, suggesting that IL-1β also plays a key role in liver injury (Tilg and Moschen, 2011). The results demonstrate that the intervention of the YE can effectively improve the alcohol-induced liver oxidative stress and inflammatory response, with the YEH exhibiting better anti-inflammation efficacy.

The 16S rRNA sequencing results showed that the β-diversity of LC, YEL, and YEH was significantly higher than that of the AL group (Supplementary Figures S1D,E), with no significant difference among the three groups. Studies indicated that ethanol can significantly decrease the level of β-diversity, which is closely related to alterations in the gut microbiota (Piacentino et al., 2021; Xia et al., 2021). In terms of gut microbiota composition, the ratio of Firmicutes to Bacteroidetes in the YE group was lower than that in the alcohol-induced model group (Figure 4C). This ratio positively correlated with the extent of gut microbiota disturbance (Shao et al., 2018; Belancic, 2020; Jasirwan et al., 2021). This indicated that the YE has certain positive regulatory effects on the gut microbiota.

At the genus level, the intervention of the YEL could significantly reduce the relative abundance of *Ruminococcus* and *Faecalibacterium* (Figures 4G,H). Studies have shown that the relative abundance of *Ruminococcus* in the intestinal tract of obese and non-alcoholic fatty liver children is significantly higher than that of healthier children (Del Chierico et al., 2017). In addition, *Ruminococcus* has also been confirmed to be positively associated with liver function indicators like alanine aminotransferase (AST) and aspartate aminotransferase (ALT) (Jiao et al., 2022; Quan et al., 2022). The relative abundance of *Faecalibacterium* in patients with alcoholic hepatitis significantly increased (Philips et al., 2019).

In the YEH intervention group, the relative abundances of *Faecalibaculum*, *Helicobacter, Romboutsia,* and *Parasutterella* were significantly lower than those in the alcohol-induced model group (Figures 4I,J,L,M). According to the analysis of the correlation between intestinal microbes and liver injury factors, *Romboutsia* and *Faecalibaculum* positively correlated with TNOS and iNOS. Previous studies have shown that alcohol intake can significantly increase the relative abundance of *Romboutsia*, accompanied by an increase in the mRNA of fibrosis-related markers (Yu et al., 2020). It was also reported that the relative abundance of *Helicobacter* increases in mouce with alcohol feeding (Philips et al., 2022) and positively correlate with the extent of the oxidative stress that is caused by chronic inflammation (Hardbower et al., 2013). By comparing the differences in gut microbiota between nonalcoholic fatty liver rats induced by high-fat diet and normal diet rats (Zhuge et al., 2022), it was found that *Helicobacter*, *Romboutsia* and *Faecalibaculum* were enriched in the high-fat diet group, further characterized by the presence of hepatic oxidative stress, fibrosis, and intestinal inflammation. Additionally, *Parasutterella* was positively associated with intestinal inflammation and irritable bowel disorder (Huang et al., 2017; Chen et al., 2018). In this study, the intervention of YEH significantly reduced the relative abundance of the above-mentioned microorganisms, indicating that it can alleviate the liver inflammatory response and lipid metabolism disorder that is caused by alcohol. This happens through the downregulation of the levels of *Helicobactere*, *Romboutsia, Faecalibaculum,* and *Parasutterella*, thereby exerting a protective effect on the liver.

The intervention of YEH can also significantly increase the relative abundance of *Corynebacterium* (Figure 4K). Both YEL and YEH interventions can reduce the relative abundance of *Peptococcus* and *Tyzzerella* (Figures 4D,E). Studies have reported that the relative abundance of *Corynebacterium* and *Tyzzerella* is significantly decreased in people with non-alcoholic fatty liver disease (Kordy et al., 2021; Zhang et al., 2021). Besides, a mouse experiment targeting liver fat accumulation showed a significant increase in the relative content of *Tyzzerella*. The contents of TC, TNF- α, IFN- γ, and IL-1β are positively correlated with *Tyzzerella*. This further indicates that YE can enhance antioxidant ability in the liver, which reduces alcohol-induced liver damage.

According to KEGG and HMDB databases, there were enriched pathways between the AL and YEL groups and between the AL and

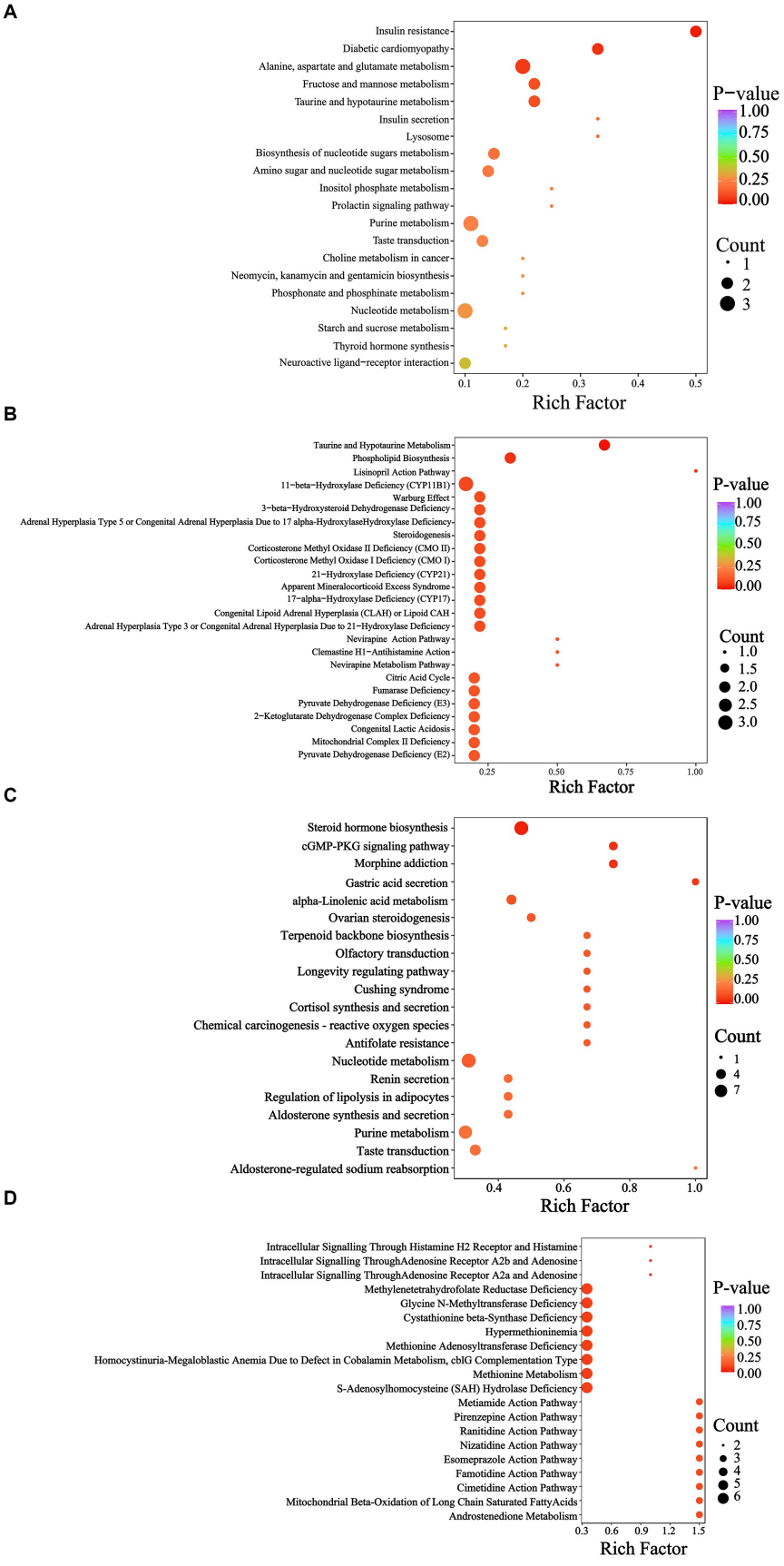

FIGURE 8

(Continued)

FIGURE 8 (Continued)
Metabolic pathway analysis of differential metabolites. **(A)** KEGG enrichment pathways based on the differential metabolites (AL and YEL). **(B)** HMDB enrichment pathways based on the differential metabolites (AL and YEL). **(C)** KEGG enrichment pathways based on the differential metabolites (AL and YEH). **(D)** HMDB enrichment pathways based on the differential metabolites (AL and YEH).

YEH groups. Compared with the AL group, YEL intervention significantly up-regulated the levels of D-mannose-6-phosphate and D-glucose-6-phosphate (Figure 7A). D-glucose-6-phosphate is a product from the phosphorylation of glucose by glucokinase. If it accumulates in the liver for a long time, it can promote triglyceride synthesis and lead to lipid accumulation in the liver (Rajas et al., 2019). In addition, low doses of YE can also affect the metabolism of taurine and hypo taurine (Figure 8B), resulting in a significant increase in the content of taurine (2-aminoethane sulfonic acid). This substance is widely used as a dietary supplement, which can reduce the expression of TNF-α, IL-6, and IL-1β mRNA and reduce the inflammatory response (Shi et al., 2021). Taurine can also increase the activity of SOD and MDA, thereby reducing oxidative stress that is caused by alcohol (Murakami et al., 2018).

Lysophosphatidylcholine (16:0/0:0) (Figure 7A), which is present in the phospholipid biosynthesis pathway, was also significantly increased. It has been confirmed to alleviate lung injury of acute respiratory distress syndrome by down-regulating the mRNA expression of inflammatory factors such as TNF-α, IL-6, and IL-1β, as well as by reducing neutrophil infiltration (Du et al., 2022). This suggests that lysophosphatidylcholine (16:0/0:0) has certain anti-inflammatory effects. However, in the YEL group, the content of acetylcholine (Figures 7A,B) in the pathway was significantly lower than that in the AL group, while the levels of this substance in the YEH group were significantly higher (Figure 7B). Acetylcholine can alleviate colonic inflammation by promoting the secretion of Interleukin 10 (IL-10) and down-regulating the mRNA expression of TNF-α, IFN-γ, and IL-1β. Thus, the YEH group has better anti-inflammatory properties.

Observations from this study showed that the YEH intervention tends to downregulate the secretion of gastric acid secretion (Figure 8C). Some studies have reported that long-term alcohol consumption can damage the gastric mucosal barrier defense system and reduce gastric mucosa's ability to resist the invasion of gastric acid, bile, and various digestive enzymes. Excessive gastric acid can cause mucosal damage (Ning et al., 2012; Wu et al., 2021). At the same time, gastric acid inhibitors (Zhang et al., 2016) have been confirmed to reduce the damage of alcohol to the stomach. Therefore, high doses of yeast extract have a certain protective effect on the stomach. In addition, YEH was able to reduce the content of histamine in this pathway, which mediates GI tract damage that is induced by alcohol (Zimatkin and Anichtchik, 1999). Studies in mouse models have found that the oral administration of histamine can cause gut microbiota disorder, oxidative stress, inflammatory response (Luo et al., 2022), and cause liver fibrosis (Kennedy et al., 2020). In morphine addiction and cGMP-PKG signaling pathways, high doses of yeast extract modulate both pathways by reducing the levels of adenosine and 5′ -adenine nucleotide (Figure 7B). The acetaldehyde that is produced by alcohol metabolism can inhibit adenosine reabsorption (Nagy et al., 1990) and is associated with the level of adenosine and cGMP-PKG signaling. ATP- binding produces adenosine (Pardo et al., 2013; Purnell et al., 2023), which, in excess, inhibits respiration (Dunwiddie and Masino, 2001) and promotes liver fibrosis (Fausther, 2018). Also, when 5 '-monophosphate was injected, the expression of genes related to fatty acid metabolism in the liver was significantly inhibited (Kondo et al., 2020). However, histamine and adenosine levels positively correlated with *Parasutterella*, while *Corynebacterium* inversely correlated with histamine (Figure 7C). While *Corynebacterium* numbers showed a significant positive correlation with ·OH clearance, those of *Parasutterella* were shown to be positively correlated with intestinal inflammation and irritability (Huang et al., 2017; Chen et al., 2018). Therefore, the antioxidant activity of YE is closely related to the gut microbiota and their metabolites.

5. Conclusion

In this study, the administration of different concentrations of yeast extract to ALD rats could alleviate the alcohol-induced liver hepatoxicity, oxidative stress, and inflammatory response. Compared with the YEL, the YEH inhibited the inflammatory response to a greater extent exhibiting even better results than the positive drug group. Yeast extract can also improve gut microbiota disorders and regulate their metabolic products in ALD rats, thus alleviating liver oxidative stress and inflammatory damage through the "liver-intestine" axis. It is important to note that high-dose yeast extract has a greater effect on metabolic products. However, there are some limitations associate with study that can be addressed in future research. First, although we have shown that yeast extract has a protective effect on alcohol-induced liver damage, it is a complex mixture whose functional components need to be further explored. Second, we observed that yeast extract intervention had impacts on some gut microbiota and their metabolites. However, further exploration of the functional validation of both is still required to better explain the mechanism through which yeast extract affects alcohol-induced liver damage.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI – PRJNA975355.

Ethics statement

The animal study was reviewed and approved by Medical Ethics Committee of School of Public Health, Lanzhou University.

Author contributions

DL, ZL, and YL designed this study. ZL, MW, YW, LF, and SW performed experimental work. DL and YL revised methodology and

provided resources. ZL analyzed data and wrote the first draft of the manuscript. DL, YL, HL, XL, YZ and DG supervised and interpreted the experimental data, and critically revised the manuscript. All authors contributed to the article and approved the submitted version.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1217449/full#supplementary-material

References

Albillos, A., de Gottardi, A., and Rescigno, M. (2020). The gut-liver axis in liver disease: pathophysiological basis for therapy. *J. Hepatol.* 72, 558–577. doi: 10.1016/j.jhep.2019.10.003

Aldred, A., and Nagy, L. E. (1999). Ethanol dissociates hormone-stimulated cAMP production from inhibition of TNF-alpha production in rat Kupffer cells. American journal of physiology-gastrointestinal and liver. *Physiology* 276, G98–G106. doi: 10.1152/ajpgi.1999.276.1.G98

Belancic, A. (2020). Gut microbiome dysbiosis and endotoxemia - additional pathophysiological explanation for increased COVID-19 severity in obesity. *Obes. Med.* 20, 1–3. doi: 10.1016/j.obmed.2020.100302

Bishehsari, F., Magno, E., Swanson, G., Desai, V., Voigt, R. M., Forsyth, C. B., et al. (2017). Alcohol and gut-derived inflammation. *Alcohol. Res.* 38, 163–171.

Cederbaum, A. I., Lu, Y., and Wu, D. (2009). Role of oxidative stress in alcohol-induced liver injury. *Arch. Toxicol.* 83, 519–548. doi: 10.1007/s00204-009-0432-0

Chae, H. J., Joo, H., and In, M. J. (2001). Utilization of brewer's yeast cells for the production of food-grade yeast extract. Part 1: effects of different enzymatic treatments on solid and protein recovery and flavor characteristics. *Bioresour. Technol.* 76, 253–258. doi: 10.1016/s0960-8524(00)00102-4

Chen, Y. J., Wu, H., Wu, S. D., Lu, N., Wang, Y. T., Liu, H. N., et al. (2018). Parasutterella, in association with irritable bowel syndrome and intestinal chronic inflammation. *J. Gastroenterol. Hepatol.* 33, 1844–1852. doi: 10.1111/jgh.14281

Chen, H., Xu, Y., Wang, J., Zhao, W., and Ruan, H. (2015). Baicalin ameliorates isoproterenol-induced acute myocardial infarction through iNOS, inflammation and oxidative stress in rat. *Int. J. Clin. Exp. Pathol.* 8, 10139–10147.

Crabb, D. W., Im, G. Y., Szabo, G., Mellinger, J. L., and Lucey, M. R. (2020). Diagnosis and treatment of alcohol-associated liver diseases: 2019 practice guidance from the American Association for the Study of Liver Diseases. *Hepatology* 71, 306–333. doi: 10.1002/hep.30866

Davis, R. L., and Syapin, P. J. (2004). Acute ethanol exposure modulates expression of inducible nitric-oxide synthase in human astroglia: evidence for a transcriptional mechanism. *Alcohol* 32, 195–202. doi: 10.1016/j.alcohol.2004.01.006

Del Chierico, F., Nobili, V., Vernocchi, P., Russo, A., De Stefanis, C., Gnani, D., et al. (2017). Gut microbiota profiling of pediatric nonalcoholic fatty liver disease and obese patients unveiled by an integrated meta-omics-based approach. *Hepatology* 65, 451–464. doi: 10.1002/hep.28572

Du, X., Xu, L., Ma, Y., Lu, S., Tang, K., Qiao, X., et al. (2022). Herbal inhibitors of SARS-CoV-2 M(pro) effectively ameliorate acute lung injury in mice. *IUBMB Life* 74, 532–542. doi: 10.1002/iub.2616

Dunwiddie, T. V., and Masino, S. A. (2001). The role and regulation of adenosine in the central nervous system. *Annu. Rev. Neurosci.* 24, 31–55. doi: 10.1146/annurev.neuro.24.1.31

Eom, S. J., Lim, T. G., Jhun, H., Lee, N. H., Kang, M. C., and Song, K. M. (2021). Inhibitory effect of *Saccharomyces cerevisiae* extract obtained through ultrasound-assisted extraction on melanoma cells. *Ultrason. Sonochem.* 76, 1–6. doi: 10.1016/j.ultsonch.2021.105620

Fausther, M. (2018). Extracellular adenosine: a critical signal in liver fibrosis. American journal of physiology-gastrointestinal and liver. *Physiology* 315, G12–G19. doi: 10.1152/ajpgi.00006

Gao, S., Shi, J., Wang, K., Tan, Y., Hong, H., and Luo, Y. (2022). Protective effects of oyster protein hydrolysates on alcohol-induced liver disease (ALD) in mice: based on the mechanism of anti-oxidative metabolism. *Food Funct.* 13, 8411–8424. doi: 10.1039/d2fo00660j

GBD 2016 Alcohol Collaborators (2018). Alcohol use and burden for 195 countries and territories, 1990-2016: a systematic analysis for the global burden of disease study 2016. *Lancet* 392, 1015–1035. doi: 10.1016/S0140-6736(18)31310-2

Giannini, E. G., Testa, R., and Savarino, V. (2005). Liver enzyme alteration: a guide for clinicians. *Can. Med. Assoc. J.* 172, 367–379. doi: 10.1503/cmaj.1040752

Hardbower, D. M., de Sablet, T., Chaturvedi, R., and Wilson, K. T. (2013). Chronic inflammation and oxidative stress: the smoking gun for *Helicobacter pylori*-induced gastric cancer? *Gut Microbes* 4, 475–481. doi: 10.4161/gmic.25583

Huang, C., Chen, J., Wang, J., Zhou, H., Lu, Y., Lou, L., et al. (2017). Dysbiosis of intestinal microbiota and decreased antimicrobial peptide level in Paneth cells during hypertriglyceridemia-related acute necrotizing pancreatitis in rats. *Front. Microbiol.* 8, 1–13. doi: 10.3389/fmicb.2017.00776

Hyun, J., Han, J., Lee, C., Yoon, M., and Jung, Y. (2021). Pathophysiological aspects of alcohol metabolism in the liver. *Int. J. Mol. Sci.* 22, 1–16. doi: 10.3390/ijms22115717

Itoh, H., Aita, M., Nagayama, A., Meng, X. Y., Kamagata, Y., Navarro, R., et al. (2014). Evidence of environmental and vertical transmission of Burkholderia symbionts in the oriental chinch bug, Cavelerius saccharivorus (Heteroptera: Blissidae). *Appl. Environ. Microbiol.* 80, 5974–5983. doi: 10.1128/AEM.01087-14

Jasirwan, C. O. M., Muradi, A., Hasan, I., Simadibrata, M., and Rinaldi, I. (2021). Correlation of gut Firmicutes/Bacteroidetes ratio with fibrosis and steatosis stratified by body mass index in patients with non-alcoholic fatty liver disease. *Biosci. Microbiota. Food Health* 40, 50–58. doi: 10.12938/bmfh.2020-046

Jiao, M., Yan, S., Shi, Q., Liu, Y., Li, Y., Lv, J., et al. (2022). Alcohol-related elevation of liver transaminase is associated with gut microbiota in male. *Front. Med.* 9, 1–12. doi: 10.3389/fmed.2022.823898

Jin, M., Xiong, J., Zhou, Q. C., Yuan, Y., Wang, X. X., and Sun, P. (2018). Dietary yeast hydrolysate and brewer's yeast supplementation could enhance growth performance, innate immunity capacity and ammonia nitrogen stress resistance ability of Pacific white shrimp (*Litopenaeus vannamei*). *Fish Shellfish Immunol.* 82, 121–129. doi: 10.1016/j.fsi.2018.08.020

Joshi, K., Kohli, A., Manch, R., and Gish, R. (2016). Alcoholic liver disease: high risk or low risk for developing hepatocellular carcinoma? *Clin. Liver Dis.* 20, 563–580. doi: 10.1016/j.cld.2016.02.012

Kanehisa, M., and Goto, S. (2000). KEGG: Kyoto encyclopedia of genes and genomes. *Nucleic Acids Res.* 28, 27–30. doi: 10.1093/nar/28.1.27

Kennedy, L., Meadows, V., Demieville, J., Hargrove, L., Virani, S., Glaser, S., et al. (2020). Biliary damage and liver fibrosis are ameliorated in a novel mouse model lacking l-histidine decarboxylase/histamine signaling. *Lab. Investig.* 100, 837–848. doi: 10.1038/s41374-020-0405-8

Kondo, Y., Sueyoshi, K., Zhang, J., Bao, Y., Li, X., Fakhari, M., et al. (2020). Adenosine 5'-monophosphate protects from hypoxia by lowering mitochondrial metabolism and oxygen demand. *Shock* 54, 237–244. doi: 10.3390/ijms20112712

Kong, L. Z., Chandimali, N., Han, Y. H., Lee, D. H., Kim, J. S., Kim, S. U., et al. (2019). Pathogenesis, early diagnosis, and therapeutic Management of Alcoholic Liver Disease. *Int. J. Mol. Sci.* 20, 1–27. doi: 10.3390/ijms20112712

Kordy, K., Li, F., Lee, D. J., Kinchen, J. M., Jew, M. H., La Rocque, M. E., et al. (2021). Metabolomic predictors of non-alcoholic steatohepatitis and advanced fibrosis in children. *Front. Microbiol.* 12, 1–13. doi: 10.3389/fmicb.2021.713234

Lee, Y. H., Kim, J. H., Kim, S. H., Oh, J. Y., Seo, W. D., Kim, K. M., et al. (2016). Barley sprouts extract attenuates alcoholic fatty liver injury in mice by reducing inflammatory response. *Nutrients* 8, 1–13. doi: 10.3390/nu8070440

Lee, S. J., Lee, D. E., Kang, J. H., Nam, M. J., Park, J. W., Kang, B. S., et al. (2017). New potential biomarker proteins for alcoholic liver disease identified by a comparative proteomics approach. *J. Cell. Biochem.* 118, 1189–1200. doi: 10.1002/jcb.25770

Leung, T. M., and Nieto, N. (2013). CYP2E1 and oxidant stress in alcoholic and non-alcoholic fatty liver disease. *J. Hepatol.* 58, 395–398. doi: 10.1016/j.jhep.2012.08.018

Leung, H., Yitbarek, A., Snyder, R., Patterson, R., Barta, J. R., Karrow, N., et al. (2019). Responses of broiler chickens to Eimeria challenge when fed a nucleotide-rich yeast extract. *Poult. Sci.* 98, 1622–1633. doi: 10.3382/ps/pey533

Luo, Q., Shi, R., Liu, Y., Huang, L., Chen, W., and Wang, C. (2022). Histamine causes Pyroptosis of liver by regulating gut-liver Axis in mice. *Int. J. Mol. Med.* 23, 1–13. doi: 10.3390/ijms23073710

Magdaleno, F., Blajszczak, C. C., and Nieto, N. (2017). Key events participating in the pathogenesis of alcoholic liver disease. *Biomol. Ther.* 7, 1–14. doi: 10.3390/biom7010009

M'Sadeq, S. A., Wu, S. B., Choct, M., Forder, R., and Swick, R. A. (2015). Use of yeast cell wall extract as a tool to reduce the impact of necrotic enteritis in broilers. *Poult. Sci.* 94, 898–905. doi: 10.3382/ps/pev035

Murakami, S., Ono, A., Kawasaki, A., Takenaga, T., and Ito, T. (2018). Taurine attenuates the development of hepatic steatosis through the inhibition of oxidative stress in a model of nonalcoholic fatty liver disease in vivo and in vitro. *Amino Acids* 50, 1279–1288. doi: 10.1093/nar/28.1.27

Nagy, L. E., Diamond, I., Casso, D. J., Franklin, C., and Gordon, A. S. (1990). Ethanol increases extracellular adenosine by inhibiting adenosine uptake via the nucleoside transporter. *J. Biol. Chem.* 265, 1946–1951. doi: 10.1016/S0021-9258(19)39923-5

Ning, J. W., Lin, G. B., Ji, F., Xu, J., and Sharify, N. (2012). Preventive effects of geranylgeranylacetone on rat ethanol-induced gastritis. *World J. Gastroenterol.* 18, 2262–2269. doi: 10.3748/wjg.v18.i18.2262

Ozer, J., Ratner, M., Shaw, M., Bailey, W., and Schomaker, S. (2008). The current state of serum biomarkers of hepatotoxicity. *Toxicology* 245, 194–205. doi: 10.1016/j.tox.2007.11.021

Pardo, M., Betz, A. J., San Miguel, N., López-Cruz, L., Salamone, J. D., and Correa, M. (2013). Acetate as an active metabolite of ethanol: studies of locomotion, loss of righting reflex, and anxiety in rodents. *Front. Mol. Neurosci.* 7, 1–10. doi: 10.3389/fnbeh.2013.00081

Peixoto, F., Vicente, J., and Madeira, V. M. A. (2004). A comparative study of plant and animal mitochondria exposed to paraquat reveals that hydrogen peroxide is not related to the observed toxicity. *Toxicol. In Vitro* 18, 733–739. doi: 10.1016/j.tiv.2004.02.009

Philips, C. A., Phadke, N., Ganesan, K., Rajesh, S., Padsalgi, G., Ahamed, R., et al. (2019). Gut microbiota in alcoholic hepatitis is disparate from those in acute alcoholic pancreatitis and biliary disease. *J. Clin. Exp. Hepatol.* 9, 690–698. doi: 10.1016/j.jceh.2019.04.001

Philips, C. A., Schnabl, B., and Bajaj, J. S. (2022). Gut microbiome and alcohol-associated liver disease. *J. Clin. Exp. Hepatol.* 12, 1349–1359. doi: 10.1016/j.jceh.2021.12.016

Piacentino, D., Grant-Beurmann, S., Vizioli, C., Li, X., Moore, C. F., Ruiz-Rodado, V., et al. (2021). Gut microbiome and metabolome in a non-human primate model of chronic excessive alcohol drinking. *Transl. Psychiatry* 11, 1–15. doi: 10.1038/s41398-021-01728-6

Purnell, B. S., Thompson, S., Bowman, T., Bhasin, J., George, S., Rust, B., et al. (2023). The role of adenosine in alcohol-induced respiratory suppression. *Neuropharmacology* 222, 1–10. doi: 10.1016/j.neuropharm.2022.109296

Quan, T., Zhou, F., Chen, H., Jian, L., Yang, Y., Xia, F., et al. (2022). Ficus hirta Vahl. Ameliorates nonalcoholic fatty liver disease through regulating lipid metabolism and gut microbiota. *Oxidative Med. Cell. Longev.* 2022, 1–31. doi: 10.1155/2022/3474723

Rajas, F., Gautier-Stein, A., and Mithieux, G. (2019). Glucose-6 phosphate, a central hub for liver carbohydrate metabolism. *Meta* 9, 1–14. doi: 10.3390/metabo9120282

Ramaiah, S. K. (2007). A toxicologist guide to the diagnostic interpretation of hepatic biochemical parameters. *Food Chem. Toxicol.* 45, 1551–1557. doi: 10.1016/j.fct.2007.06.007

Seok, J., Warren, H. S., Cuenca, A. G., Mindrinos, M. N., Baker, H. V., Xu, W., et al. (2013). Genomic responses in mouse models poorly mimic human inflammatory diseases. *Proc. Natl. Acad. Sci. U. S. A.* 110, 3507–3512. doi: 10.1073/pnas.1222878110

Setshedi, M., Wands, J. R., and Monte, S. M. (2010). Acetaldehyde adducts in alcoholic liver disease. *Oxidative Med. Cell. Longev.* 3, 178–185. doi: 10.4161/oxim.3.3.12288

Shao, T., Zhao, C., Li, F., Gu, Z., Liu, L., Zhang, L., et al. (2018). Intestinal HIF-1alpha deletion exacerbates alcoholic liver disease by inducing intestinal dysbiosis and barrier dysfunction. *J. Hepatol.* 69, 886–895. doi: 10.1016/j.jhep.2018.05.021

Shi, Y., Hu, Y., Wang, Z., Zhou, J., Zhang, J., Zhong, H., et al. (2021). The protective effect of taurine on oxidized fish-oil-induced liver oxidative stress and intestinal barrier-function impairment in juvenile *Ictalurus punctatus*. *Antioxidants* 10, 1–19. doi: 10.3390/antiox10111690

Singal, A. K., Bataller, R., Ahn, J., Kamath, P. S., and Shah, V. H. (2018). ACG clinical guideline: alcoholic liver disease. *Am. J. Gastroenterol.* 113, 175–194. doi: 10.1038/ajg.2017.469

Takalloo, Z., Nikkhah, M., Nemati, R., Jalilian, N., and Sajedi, R. H. (2020). Autolysis, plasmolysis and enzymatic hydrolysis of baker's yeast (*Saccharomyces cerevisiae*): a comparative study. *World J. Microbiol. Biotechnol.* 36, 1–14. doi: 10.1007/s11274-020-02840-3

Tang, Y., Banan, A., Forsyth, C. B., Fields, J. Z., Lau, C. K., Zhang, L. J., et al. (2008). Effect of alcohol on miR-212 expression in intestinal epithelial cells and its potential role in alcoholic liver disease. *Alcohol Clin. Exp. Res.* 32, 355–364. doi: 10.1111/j.1530-0277.2007.00584.x

Tao, Z., Yuan, H., Liu, M., Liu, Q., Zhang, S., Liu, H., et al. (2023). Yeast extract: characteristics, production, applications and future perspectives. *J. Microbiol. Biotechnol.* 33, 151–166. doi: 10.4014/jmb.2207.07057

Tilg, H., and Moschen, A. R. (2011). IL-1 cytokine family members and NAFLD: neglected in metabolic liver inflammation. *J. Hepatol.* 55, 960–962. doi: 10.1016/j.jhep.2011.04.007

Wang, H. J., Gao, B., Zakhari, S., and Nagy, L. E. (2012). Inflammation in alcoholic liver disease. *Annu. Rev. Nutr.* 32, 343–368. doi: 10.1146/annurev-nutr-072610-145138

Wang, R., Tang, R., Li, B., Ma, X., Schnabl, B., and Tilg, H. (2021). Gut microbiome, liver immunology, and liver diseases. *Cell. Mol. Immunol.* 18, 4–17. doi: 10.1038/s41423-020-00592-6

Waszkiewicz-Robak, B. E. (2013). "Spent Brewer's yeast and Beta-glucans isolated from them as diet components modifying blood lipid metabolism disturbed by an Atherogenic diet" in *Lipid Metabolism*. ed. R. V. Baez (London: IntechOpen), 262–290.

Wei, Y., Li, M., Feng, Z., Zhang, D., Sun, M., Wang, Y., et al. (2022). The protective effects of corn oligopeptides on acute alcoholic liver disease by inhibiting the activation of Kupffer cells NF-kappaB/AMPK signal pathway. *Nutrients* 14, 1–13. doi: 10.3390/nu14194194

Wu, Y., Hu, J., Long, X., Pan, Y., Mu, J., Park, K. Y., et al. (2021). *Lactobacillus plantarum* ZS62 alleviates alcohol-induced gastric injury in mice via an anti-oxidative mechanism. *Drug Des. Devel. Ther.* 15, 1667–1676. doi: 10.2147/DDDT.S292243

Xia, T., Duan, W., Zhang, Z., Li, S., Zhao, Y., Geng, B., et al. (2021). Polyphenol-rich vinegar extract regulates intestinal microbiota and immunity and prevents alcohol-induced inflammation in mice. *Food Res. Int.* 140, 1–11. doi: 10.1016/j.foodres.2020.110064

Xiao, J., Zhang, R., Wu, Y., Wu, C., Jia, X., Dong, L., et al. (2020). Rice bran phenolic extract protects against alcoholic liver injury in mice by alleviating intestinal microbiota Dysbiosis, barrier dysfunction, and liver inflammation mediated by the endotoxin-TLR4-NF-kappaB pathway. *J. Agric. Food Chem.* 68, 1237–1247. doi: 10.1021/acs.jafc.9b04961

Xu, L., Yu, Y., Sang, R., Li, J., Ge, B., and Zhang, X. (2018). Protective effects of Taraxasterol against ethanol-induced liver injury by regulating CYP2E1/Nrf2/HO-1 and NF-kappaB signaling pathways in mice. *Oxidative Med. Cell. Longev.* 2018, 1–11. doi: 10.1155/2018/8284107

Yu, L., Wang, L., Yi, H., and Wu, X. (2020). Beneficial effects of LRP6-CRISPR on prevention of alcohol-related liver injury surpassed fecal microbiota transplant in a rat model. *Gut Microbes* 11, 1015–1029. doi: 10.1080/19490976.2020.1736457

Yuan, X. Y., Liu, W. B., Liang, C., Sun, C. X., Xue, Y. F., Wan, Z. D., et al. (2017). Effects of partial replacement of fish meal by yeast hydrolysate on complement system and stress resistance in juvenile Jian carp (*Cyprinus carpio* var. Jian). *Fish Shellfish Immunol.* 67, 312–321. doi: 10.1016/j.fsi.2017.06.028

Yuan, R., Tao, X., Liang, S., Pan, Y., He, L., Sun, J., et al. (2018). Protective effect of acidic polysaccharide from Schisandra chinensis on acute ethanol-induced liver injury through reducing CYP2E1-dependent oxidative stress. *Biomed. Pharmacother.* 99, 537–542. doi: 10.1016/j.fsi.2017.06.028

Zhang, P., Cao, S., Zou, T., Han, D., Liu, H., Jin, J., et al. (2018). Effects of dietary yeast culture on growth performance, immune response and disease resistance of gibel carp (*Carassius auratus* gibelio CAS III). *Fish and Shellfish Immunolo.* 82, 400–407. doi: 10.1016/j.fsi.2018.08.044

Zhang, Q., Fan, X. Y., Cao, Y. J., Zheng, T. T., Cheng, W. J., Chen, L. J., et al. (2021). The beneficial effects of *Lactobacillus brevis* FZU0713-fermented Laminaria japonica on lipid metabolism and intestinal microbiota in hyperlipidemic rats fed with a high-fat diet. *Food Funct.* 12, 7145–7160. doi: 10.1039/d1fo00218j

Zhang, Y., Xiu, M., Jiang, J., He, J., Li, D., Liang, S., et al. (2016). Novokinin inhibits gastric acid secretion and protects against alcohol-induced gastric injury in rats. *Alcohol* 56, 1–8. doi: 10.1016/j.alcohol.2016.08.003

Zheng, L., Shiwei, X., Zhenxiao, Z., Yongjian, L., Lixia, T., and Niu, J. (2021). Effects of yeast and yeast extract on growth performance, antioxidant ability and intestinal microbiota of juvenile Pacific white shrimp (*Litopenaeus vannamei*). *Aquaculture* 530, 1–11. doi: 10.1016/j.aquaculture.2020.735941

Zhu, M., Wang, X., Wang, K., Zhao, Z., Dang, Y., Ji, G., et al. (2023). Lingguizhugan decoction improves non-alcoholic steatohepatitis partially by modulating gut microbiota and correlated metabolites. *Front. Cell. Infect. Microbiol.* 13, 1–17. doi: 10.3389/fcimb.2023.1066053

Zhuge, A., Li, S., Lou, P., Wu, W., Wang, K., Yuan, Y., et al. (2022). Longitudinal 16S rRNA sequencing reveals relationships among alterations of gut microbiota and nonalcoholic fatty liver disease progression in mice. *Microbiol. Spectr.* 10, 1–18. doi: 10.1128/spectrum.00047-22

Zimatkin, S. M., and Anichtchik, O. V. (1999). Alcohol-histamine interactions. *Alcohol Alcohol.* 34, 141–147. doi: 10.1093/alcalc/34.2.141

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Identification of the gut microbiota affecting *Salmonella pullorum* and their relationship with reproductive performance in hens

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Introduction: Pullorum disease is one of the common bacterial infectious diseases caused by *Salmonella pullorum* (*S. pullorum*), which can result in a decrease in the reproductive performance of laying hens, thus causing considerable economic losses. However, studies about the characteristics of intestinal microbiota with pullorum and their potential association with reproductive performance in hens are still limited. This study was to identify the gut microbiota associated with *S. pullorum* in poultry.

Methods: A total of 30 hens with *S. pullorum*-negative (PN) and 30 hens with *S. pullorum*-positive (PP) were analyzed for hatching eggs laid in 2 weeks (HEL), fertilization eggs (FE), chick number (CN), and microbial structure.

Results: There were significant differences in HEL ($p < 0.01$), FE ($p < 0.01$), and CN ($p < 0.01$) between PP and PN. Histomorphological observations showed abnormal morphology of the ovaries and fallopian tubes and low integrity of epithelial tissue in the ileum and cecum in PP. 16S rRNA gene sequencing revealed that beneficial cecal microbes, such as *Bacteroides*, *Desulfovibrio*, and *Megamonas*, were positively correlated with reproductive performance and had lower abundance in PP ($p = 0.001$). Furthermore, diminished phosphotransferase system (PTS) and pentose phosphate pathway, butanoate metabolism and oxidative phosphorylation were also found in PP.

Discussion: Taken together, this study clarified the morphological characteristics of the reproductive tract and intestines of chickens infected with *S. pullorum* and preliminarily explored the potential association between cecal microbiota and reproductive performance in hens. Our data may provide a reference for revealing the intestinal microbial characteristics of hens in resisting pullorum and exploring novel approaches to infection control in future studies.

KEYWORDS

gut microbiota, poultry, pullorum, microbial structure, prediction functions

Introduction

Pullorum disease, caused by *Salmonella pullorum* (*S. pullorum*), is transmitted both vertically and horizontally in chickens (Berchieri et al., 2001; Li et al., 2018; Zhou et al., 2022). It is an acute systemic disease and is more common in young birds (Soria et al., 2012; Wang et al., 2020), and some of the infected adult birds are asymptomatic carriers that transmit the bacteria to the offspring and other chickens in the flock, some of which show the symptoms

of diarrhea, decreased fertility and laying, reproductive tract abnormalities, inappetence, and weight loss (Shivaprasad, 2000; Wigley et al., 2001; Setta et al., 2012; Ding et al., 2021; Shen et al., 2022). Every year, *Salmonella* infection leads to serious economic losses in the poultry industry, especially in developing countries (Wigley et al., 2002; Barrow and Freitas Neto, 2011; Li et al., 2018, 2019). Previous studies suggested that in mainland China, PD was highly prevalent in the autumn, followed by the winter. Their findings also demonstrated that PD still posed a major threat to the poultry industry and that comprehensive and stringent strategies should be used to prevent and control this disease (Lv et al., 2022; Jiang et al., 2023). There is conclusive evidence that increased stocking density and larger farms result in an increased occurrence, persistence, and spread of *Salmonella* in laying hen flocks (Hazards et al., 2019). It causes decreased production performance and even the death of poultry, as well as being a threat to public health. Although eradication programs have been carried out as a prevention and control measure, the agglutination test results are erratic, including false-negative reactions and a lack of sensitivity, so *Salmonella* infection is still one of the most important problems worldwide (Barrow et al., 2012; Wang et al., 2020).

The use of antibiotics to prevent and treat bacterial diseases, such as *Salmonella,* leads to an increase in multiple drug-resistant bacteria worldwide (Zhou et al., 2020). Sulfonamides have been used in the treatment of pullorum disease, including sulfadiazine, sulfamerazine, sulfathiaole, sulfamethazine, and sulfaquinoxaline. However, most studies have indicated that no drug or combination of drugs has been found to be capable of eliminating infection from treated flocks (Shivaprasad, 2000). Globally, the animal industries are moving toward restricting and eventually a total ban on the usage of antibiotic growth promoters (Liao and Nyachoti, 2017). Meanwhile, the Chinese government is promoting a reduction in antibiotic use currently (Zhang et al., 2015). This trend prompts people to actively seek an ideal alternative to antibiotics.

The inclusion of alternative feed additives in lieu of antibiotics in animal diets is definitely required to support a profitable and sustainable poultry industry (Liao and Nyachoti, 2017). As is known, the gut microbiota can be manipulated by feed additives such as exclusion products, probiotics, prebiotics, organic acids, plant extracts, essential oils, and feed enzymes (Shin et al., 2008; De Lange et al., 2010; Le Bon et al., 2010; Heo et al., 2013). However, there is no report on preventing and controlling pullorum disease in the Chinese local chicken breeds. An animal's character can be affected by changes in the microbiota. As it is an opportunistic pathogen, the occurrence of pullorum disease is mainly caused by an imbalance of the intestinal microbiota (Shivaprasad, 2000). Probiotics are defined as a microbial feed supplement that beneficially affects the host animal by improving its intestinal balance (Fuller, 1989). They are a category of feed additives that can be used to replenish the gut microbial population while recuperating the host immune system (Liao and Nyachoti, 2017). Many recent studies have shown that humans and animals fed probiotics have altered intestinal microbiota, increased intestinal immunity, improved resistance to disease, reduced shedding of pathogens and disease symptoms, and improved health status (Zhao et al., 2013; Chiu et al., 2014; Upadhaya et al., 2015; Gao et al., 2017; Liao and Nyachoti, 2017; Li et al., 2017; Chen et al., 2020; Wang et al., 2021). The gut microbiota affecting the pullorum and its functions in chickens need further study.

Due to the large diversity of bacterial species, the gut microbiome contains circa 9 million unique protein-coding genes, but they have not been studied extensively (Rosenberg and Zilber-Rosenberg, 2018). The availability of high-throughput sequencing will shortly enable the sequencing of whole bacterial populations, enabling a more comprehensive view of bacterial evolution among related bacterial species (Barrow and Freitas Neto, 2011). This study aimed to analyze the intestinal microbial characteristics of hens resisting pullorum and their relationships with hatching eggs laid in 2 weeks (HEL), fertilization eggs (FE), and chick number (CN) in hens.

Materials and methods

Ethics statement

All procedures and the use of animals were carried out in accordance with the Guidelines for the Ethics and Animal Welfare Committee of the Shanghai Academy of Agricultural Sciences (No. SAASPZ0522051).

Animal experiment and sample collection

S. pullorum infections in chickens were diagnosed by *S. pullorum* and *S. gallinarum* polyvalent antigen rapid slide agglutination test reagents (Beijing Zhonghai Biotech Co., Ltd., China). On a clean glass slide, 50 μl of polyvalent antigen and 50 μl of venous blood were placed. The samples were deemed positive if 50% or more agglutination occurred in the mixture within 2 min, and samples without agglutination were considered negative. In this study, two chicken groups were obtained after three times of the slide agglutination tests, and the results of the *S. pullorum* infection texts were positive or negative all three times. A total of 60 New Pudong chickens [samples from 30 hens with *S. pullorum*-negative (PN) and 30 hens with *S. pullorum*-positive (PP)] were selected from the experimental farm of the Shanghai Academy of Agricultural Sciences, Shanghai, China. These 60 hens were artificially inseminated at the age of 47 weeks. Recording the number of eggs laid, fertilized, and hatched chicks per hen and the performance, including HEL, FE, and CN, were analyzed during chick hatching processes. All eggs received disinfection before hatching. All hens were in the same poultry house (PN hens were distributed in the east of the poultry house and PP hens were distributed in the west of the poultry house), selected according to a unified breed standard, and fed antibiotic-free corn-soybean diets (Supplementary Table S1). Antibiotics in the feed or for any therapeutic purposes were not provided for hens after the age of 1 week. These 60 hens were slaughtered at the age of 49 weeks. After slaughter, tissue samples of the ovaries, fallopian tubes, ileum, and cecum with the size of about 2 × 3 cm were cut with a sterile scalpel and quickly stored in 4% paraformaldehyde for separate morphological observation. Cecal content was individually collected in 2 ml centrifuge tubes for 16S rRNA gene sequencing. All samples were kept in an ice box for

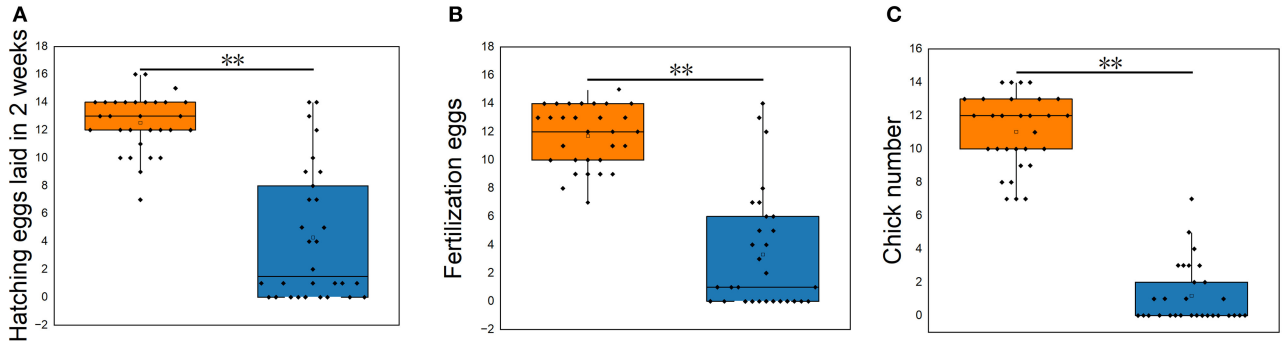

FIGURE 1
(A–C) Comparisons of reproductive performances in *S. pullorum*-negative hens and *S. pullorum*-positive hens. PN, *S. pullorum*-negative group; PP, *S. pullorum*-positive group.

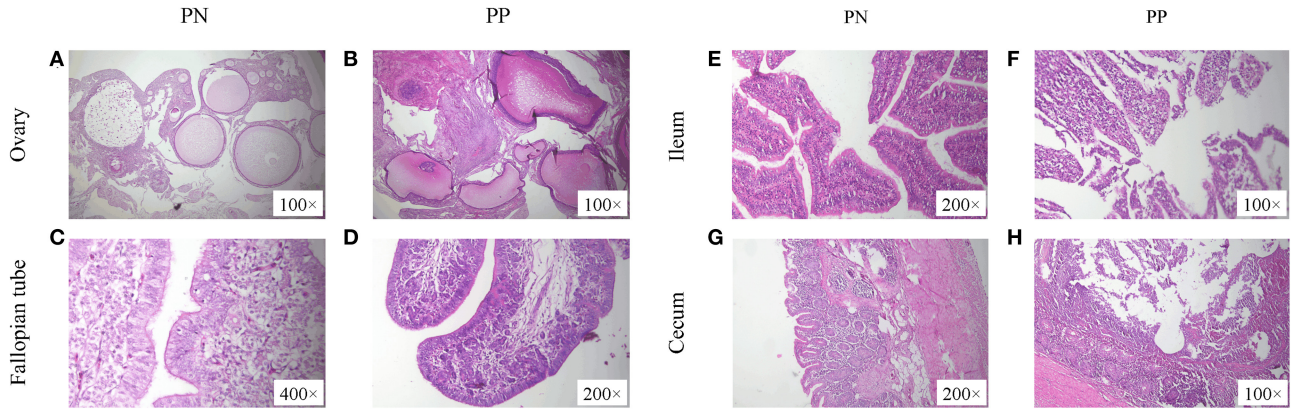

FIGURE 2
(A–H) Morphological observation of ovaries, fallopian tubes, ileum, and cecum of PN and PP. PN, *S. pullorum*-negative group; PP, *S. pullorum*-positive group.

preservation and transportation and then stored at −80°C in the laboratory (Janssen and Kersten, 2015).

Morphological observation of the ovaries, fallopian tubes, ileum, and cecum

Tissues were routinely embedded in paraffin wax blocks, sectioned at 5 μm thickness, mounted on glass slides, and stained with hematoxylin & eosin (H&E). Morphological observations were conducted by a Nikon ECLIPSE 80i light microscope with a computer-assisted morphometric system (Nikon Corporation, Tokyo, Japan).

16S rRNA sequencing and bioinformatics analysis

The gut microbiota population in the hens with *S. pullorum*-negative ($n = 30$) and *S. pullorum*-positive ($n = 30$) was analyzed by 16S rRNA gene sequencing, respectively. Microbial community genomic DNA was extracted from cecum samples using the E.Z.N.A.® soil DNA Kit (Omega Bio-tek, Norcross, GA, United States) according to the manufacturer's instructions. The DNA extract was checked on 1% agarose gel, and the DNA concentration and purity were determined using NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, United States). The hypervariable V3-V4 region of the 16S rRNA gene with a length of ~468 bp was targeted for sequencing. PCR amplification was performed with gene-specific primers 338 F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806 R (5′-GGACTACHVGGGTWTCTAAT-3′) under the following conditions: initial denaturation at 95°C for 3 min, followed by 27 cycles of denaturing at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 45 s, single extension at 72°C for 10 min, and end at 4°C.

The PCR mixtures contain 5×TransStartFastPfu buffer 4 μl, 2.5 mM dNTPs 2 μl, forward primer (5 μM) 0.8 μl, reverse primer (5 μM) 0.8 μl, TransStartFastPfu DNA Polymerase 0.4 μl, template DNA 10 ng, and finally ddH_2O up to 20 μl. PCR reactions were performed in triplicate. The PCR product was extracted from a 2% agarose gel, purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, United States) according to the manufacturer's instructions, and quantified using

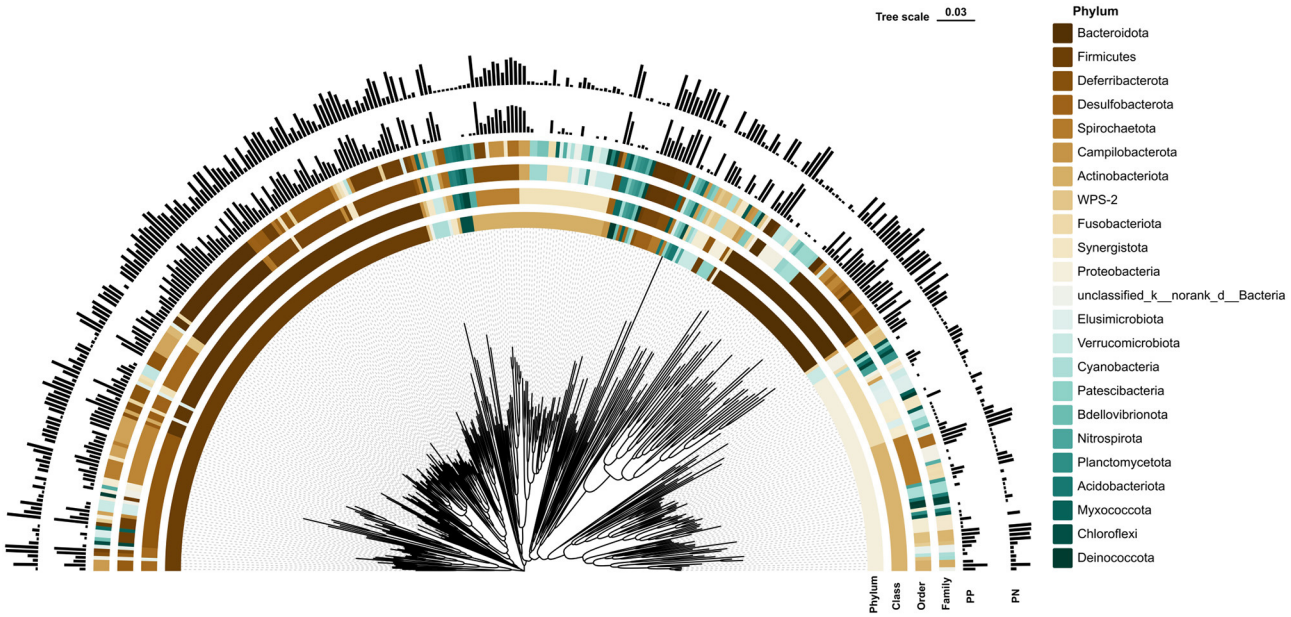

FIGURE 3
Phylogenetic tree of cecal microbiota and its microbial community composition in PN and PP. PN, *S. pullorum*-negative group; PP, *S. pullorum*-positive group.

the Quantus™ Fluorometer (Promega, United States). Purified amplicons were pooled in equimolar amounts and paired-end sequenced on an Illumina MiSeq PE300 platform (Illumina, San Diego, United States) according to the standard protocols by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). All obtained raw sequence datasets have been deposited into the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) with the accession number PRJNA799073.

The raw 16S rRNA gene sequencing reads were demultiplexed, quality-filtered by the FastP version 0.20.0 (Chen et al., 2018) and merged by the FLASH version 1.2.7 (Magoc and Salzberg, 2011) with the following criteria: the 300 bp reads were truncated at any site receiving an average quality score of <20 over a 50 bp sliding window, and the truncated reads shorter than 50 bp were discarded; reads containing ambiguous characters were also discarded; only overlapping sequences longer than 10 bp were assembled according to their overlapped sequence. The maximum mismatch ratio of the overlap region is 0.2. Reads that could not be assembled were discarded; samples were distinguished according to the barcode and the primers, and the sequence direction was adjusted for exact barcode matching and a 2-nucleotide mismatch in primer matching. Operational taxonomic units (OTUs) with a 97% similarity cutoff (Schloss et al., 2009) were clustered using the UPARSE version 7.1, and chimeric sequences were identified and removed. The taxonomy of each OTU representative sequence was analyzed by RDP Classifier version 2.2 (Wang et al., 2007) against the 16S rRNA database (Silva version 138) using a confidence threshold of 0.7.

Statistical analysis

Reproductive performance, including HEL, FE, and CN, was calculated using the SAS 9.4 software (Shen et al., 2014). Alpha diversity was calculated using the Mothur (Schloss et al., 2009). Venn diagrams and rank abundance distribution curves were performed using Mothur. Linear discriminate analysis effect size (LEfSe) was used to identify the bacteria enriched (Segata et al., 2011). The pair-wise phylogenetic distance was measured by weighted UniFrac (Lozupone et al., 2006) to compare community compositions across samples. Principal component analysis (PCA) was used to compress dimensionality into 2D principal coordinate analysis plots (Vazquez-Baeza et al., 2013), enabling visualization of sample relationships. PICRUSt was used to explore the functional composition of that bacterial community that the data might convey (Langille et al., 2013). The visualization of conventional results was achieved by Origin 2023. The co-occurrence network is implemented through the Gephi 0.10 software.

Results

Comparisons of reproductive performances in *S. pullorum*-negative hens and *S. pullorum*-positive hens

The reproductive performance showed that the average hatching eggs laid in 2 weeks (HEL), average fertilization eggs (FE), and average chick number (CN) of *S. pullorum*-negative hens were

higher than that of *S. pullorum*-positive hens ($p < 0.01$, Figure 1). The average HEL of *S. pullorum*-negative hens was 12.5, which was 0.5 more than that of *S. pullorum*-positive hens. The average FE of *S. pullorum*-negative hens was 11.7, which was 8.4 times more than that of *S. pullorum*-positive hens. The average CN of *S. pullorum*-negative hens was 11.0, which was 9.8 times more than that of *S. pullorum*-positive hens.

Morphological observation of the reproductive tract and intestinal tract

Morphological observations showed that the presence of deformed follicles, follicular dysplasia, or even necrosis occurred in the *S. pullorum*-positive hens (Figures 2A, B). Moreover, in the *S. pullorum*-positive hens, epithelial cells of the oviductal mucosa consisted mostly of ciliated cells, with few secretory cells and an underdeveloped lamina propria (Figures 2C, D). Histomorphological observations of the ileum and cecum in PP revealed that the intestinal epithelial integrity was low and there was severe intestinal epithelial damage. Severe detachment of mucosal epithelial cells and exposure of the lamina propria in the intestinal lumen were mainly observed (Figures 2E, H).

Bacterial community structure and prediction functions of *S. pullorum*-negative hens and *S. pullorum*-positive hens

More than 4 million sequences were obtained from all samples, and there were 16,888 high-quality sequences per sample. The average sequence length was 417 bp. Microbial composition analysis showed that, at the phylum level, the two most dominant phyla were *Firmicutes* and *Bacteroidetes*, which comprised 72.6% of the total sequences in PN and 71.6% of the total sequences in PP, respectively (Figure 3). At the genus level, a total of 357 genera were identified from all samples, and the enrichment of these genera in the PN and PP had a large variation (Figure 3). The two most dominant genera were *Bacteroides* and *Rikenellaceae_RC9_gut_group* belonging to the phylum *Bacteroidota* and comprised 25.70% of the total sequences in PN and 24.42% of the total sequences in PP, respectively (Figure 3). A total of 1,880 OTUs were identified from all samples, and the VENN diagram showed the unique OTUs and genera of the two groups and the shared OTUs and genera (Supplementary Figure S1). In addition, principal component analysis (PCA) at the genus level revealed a significant separation between the samples of PN and PP, indicating a large difference in the cecum microbiota of these two groups (Figure 4). The diversity and richness of the cecum microbiota were significantly lower in the PP group than in the PN group (Figures 5A, E).

A Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) analysis was performed to investigate the functional properties of microbiota. Using the KEGG pathway annotation information, we found that among the major microbial functions (top 100), the PP cecum microbial functions were generally weaker than the PN group (Figure 6A), where the phosphotransferase system (PTS), pentose phosphate pathway, butanoate metabolism, and oxidative phosphorylation were significantly less abundant in PP than PN (Figures 6B–E).

Correlations of reproductive performances with the gut microbiota

It is noteworthy that the alpha diversity of the cecum microbiota was positively correlated with the reproductive performance (HEL, FE, and CN) of hens ($p < 0.01$, Figures 5B–D, F–H). To further investigate the microbial differences between the PP and PN groups, the Wilcoxon rank-sum test was used, and it was found that there were 68 significantly different genera between these two groups ($p < 0.05$, Supplementary Table S2). LEfSe was used to further determine the taxa that most likely explain the differences between PN and PP samples. A total of 65 genera were found to be potential biomarkers between PN and PP. There was a significant enrichment of 50 genera (including *Bacteroides*, *Desulfovibrio*, and *Megamonas*) in PN (Figure 7 A). Additionally, enrichment of 15 genera, including *Salmonella*, was found in PP (Figure 7A). Notably, we found significant positive correlations between *Bacteroides*, *Desulfovibrio*, and *Megamonas*, with reproductive performance (HEL, FE, and CN, Figures 7B–D).

Analysis of the co-occurrence network of microorganisms between *S. pullorum*-negative hens and *S. pullorum*-positive hens

Analysis of the co-occurrence network of microorganisms at the genus level found that in the PP and PN groups, the genus *Firmicutes* is the core microbe in the co-occurrence network and has extensive connections with microbes from other phyla (Figure 8). In addition, compared with the control group, the association between members of the *Firmicutes* phylum in the PP group seems to be closer (Figure 8B).

Discussion

Pullorum is a disease caused by *S. pullorum* through both horizontal and vertical transmission. It is known that a percentage of birds that survive clinical disease when they are infected as young chicks may show few signs of infection but may become carriers. Intestinal carriage of *S. pullorum* in the poultry host does not cause substantive gastrointestinal disease and is asymptomatic. Therefore, it is of great significance to identify gut microbiota related to pullorum disease in chickens. This will lead to the development of novel prevention and control strategies for *S. pullorum* infection in poultry production.

Among the different diseases occurring in poultry, those caused by the genus *Salmonella* are the most common, leading to serious

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economic losses to the poultry industry in terms of mortality, reduced growth, and loss of egg production (Shivaprasad, 2000; Wigley et al., 2001). One aim of the current study was to investigate and characterize the effect that *S. pullorum* infection had on reproductive performances. The results illustrated that *S. pullorum* infection in chickens reduced their reproductive performances and altered the gut microbial composition, resulting in variations in the microbial metabolic pathways and functions. Previous studies have reached the same conclusion (Ding et al., 2021). This study illustrated that pullorum disease reduced reproductive performance including HEL, FE, and CN. Pullorum disease is manifested by decreased egg production, fertility, and hatchability in hens. Shivaprasad HL showed that regressing ovarian follicles can be found in the ovaries of chickens and microscopic lesions in adults include fibrinosuppurative to pyogranulomatous inflammation of ovarian follicles characterized by necrosis. Caeca may contain necrotic caseous debris within the lumen and necrosis of the mucosa with the infiltration of heterophils into the lamina propria (Shivaprasad, 2000). The other aim of this study was to investigate the different bacterial communities between *S. pullorum*-negative hens and *S. pullorum*-positive hens and their microbial functions. The availability of high-throughput sequencing will shortly enable the sequencing of whole bacterial populations, giving us a more comprehensive view of bacterial evolution among related bacterial species (Barrow and Freitas Neto, 2011). Shen et al. studied the dissemination pattern of *S. pullorum* in different organs and at different time points. It showed that the cecum carried *S. pullorum* throughout the experiment duration, while the small intestine did not carry *S. pullorum* during the last few days of the experiment (Shen et al., 2022). Therefore, this study examined DNA sequence data and the bacterial community structure of *S. pullorum*-negative hens and *S. pullorum*-positive hens in the cecum. It obtained a large number of effective sequences. The two most dominant phyla were *Firmicutes* and Bacteroidota. The similar results were obtained in previous studies (Wei et al., 2013; Khan et al., 2020; Rychlik, 2020). The two most dominant genera were *Bacteroides* and *Rikenellaceae_RC9_gut_group*, which belong to the phylum *Bacteroidota* comprised 34.1% of the total sequences in PN and

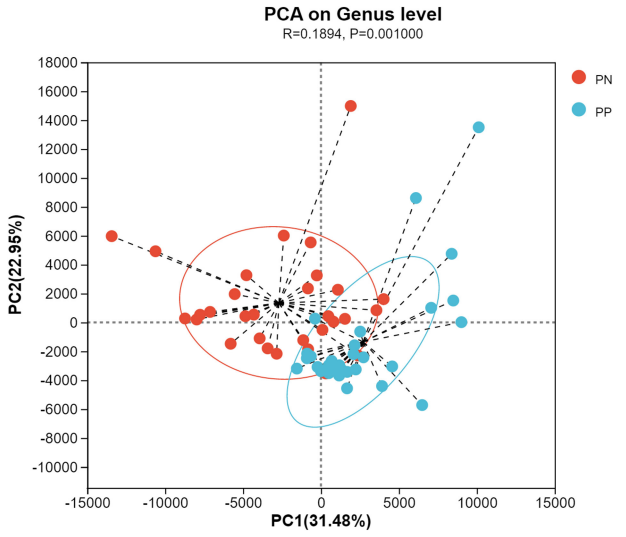

FIGURE 4
Principal component analysis (PCA) at genus level between PN and PP. PN, *S. pullorum*-negative group; PP, *S. pullorum*-positive group.

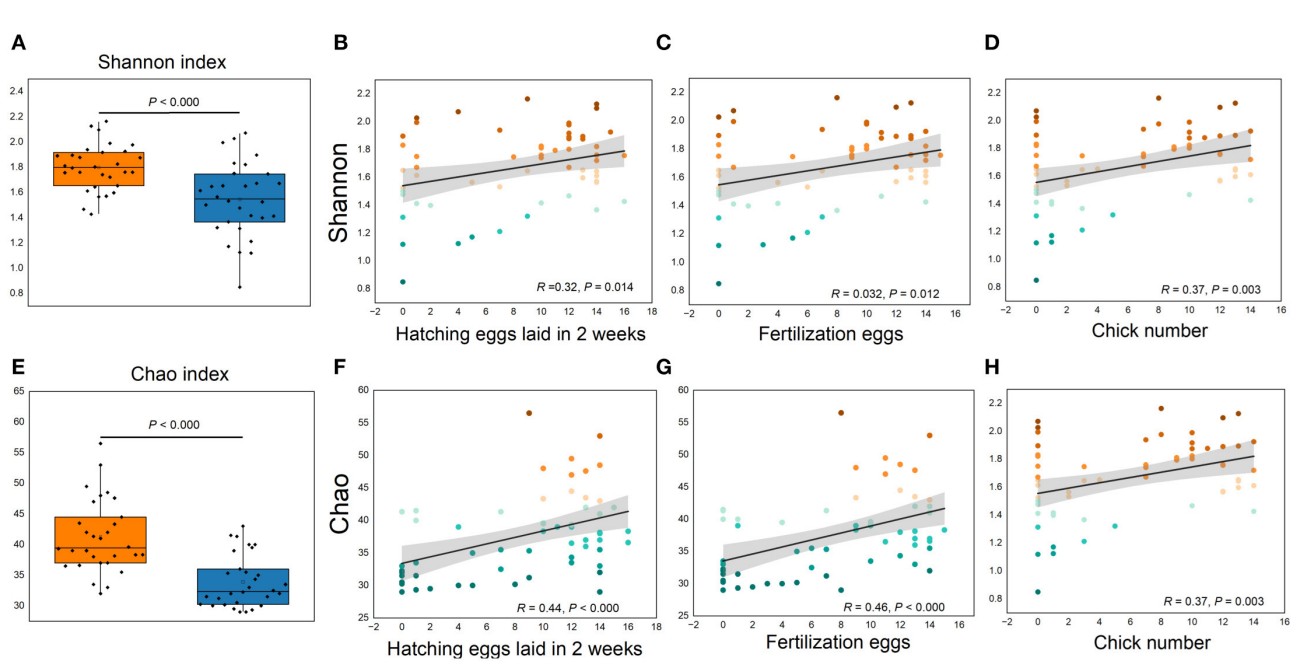

FIGURE 5
(A–H) Cecum microbial alpha diversity and its relationships with reproductive performance. **(A, E)**, Shannon and Chao indexes, respectively. **(B, D, F, H)** Correlations between reproductive performance with Shannon and Chao indexes, respectively.

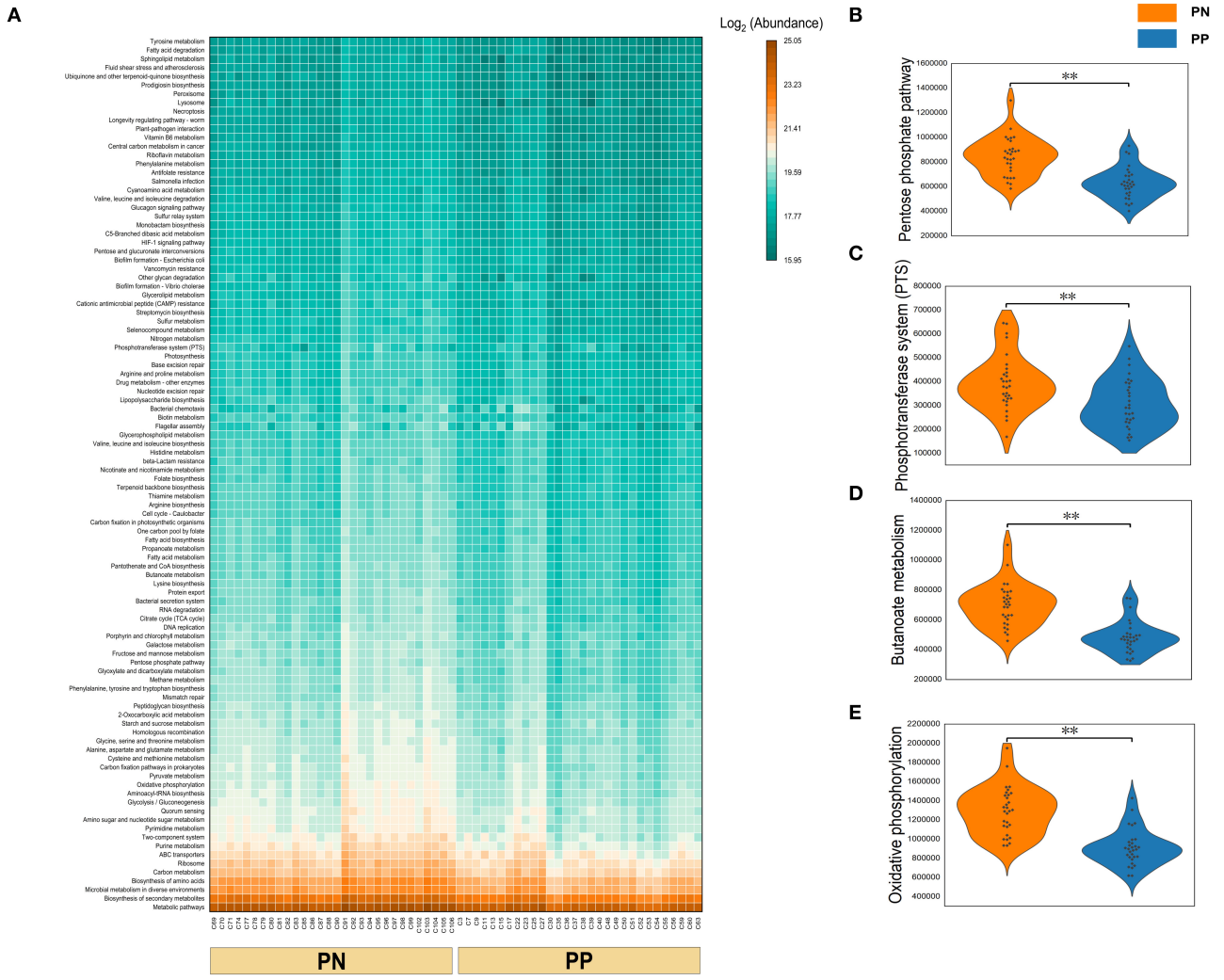

FIGURE 6
(A–E) Functional composition of microbiota and their differences between two groups. Microbial functional composition. **(A)** Heatmap of the functional composition (top 100) of microbiota. **(B–E)** Comparison of abundance of phosphotransferase system (PTS) and pentose phosphate pathway, butanoate metabolism, and oxidative phosphorylation.

16.2% of the total sequences in PP, respectively. Ding *et al.* investigated that the dominant phyla were *Firmicutes* (65.5% in group N and 62.1% in group P), *Fusobacteria* (16.3% in group N and 18.7% in group P), and *Proteobacteria* (9.37% in group N and 9.95% in group P); the preponderant genera were *Lactobacillus*, *Fusobacterium*, *Peptoclostridium*, and *Gallibacterium* (Ding et al., 2021). The reasons that lead to different results in similar studies are complicated. Many factors can cause different microbiome compositions (e.g., breeds, age, gender, nutritional level, and sample selection). It is interesting that although significant enrichment of *Salmonella* was found in PN, there was a low abundance of *Salmonella* in PP. The basis of host specificity in salmonellosis continues to elude scientific explanation (Barrow et al., 2012). The outcome of infection is the combined effect of the microbial gene set and the host's genetic background. After an intestinal infection, where are the sites of *Salmonella* serovar Pullorum persistence in convalescent birds? Wigley *et al.* showed that *Salmonella* serovar Pullorum evades the immune response by surviving intracellularly within macrophages, which would be required by *Salmonella* serovar Pullorum to persist in both the spleen and the reproductive tract (Wigley et al., 2001). *Salmonella* serovar Pullorum localizes in the reproductive tract of chickens and, as a consequence, may be transmitted vertically to chicks by transovarian transmission of the bacteria into developing hatching eggs (Wigley et al., 2001). The correlation analysis between reproductive performances (HEL, FEN, and CN) and gut microbiota was therefore significant.

After a systemic disease, there may be negative effects on immunity, such as the balance of gut microbes, which could be upset, and the ability of harmful bacteria to secrete toxins, which could be increased. In this study, the ileum and cecum in PP revealed low intestinal epithelial integrity, and this should cause negative effects in the absorption and digestion of feed nutrients. Intestinal integrity is essential to prevent animal bacterial diseases

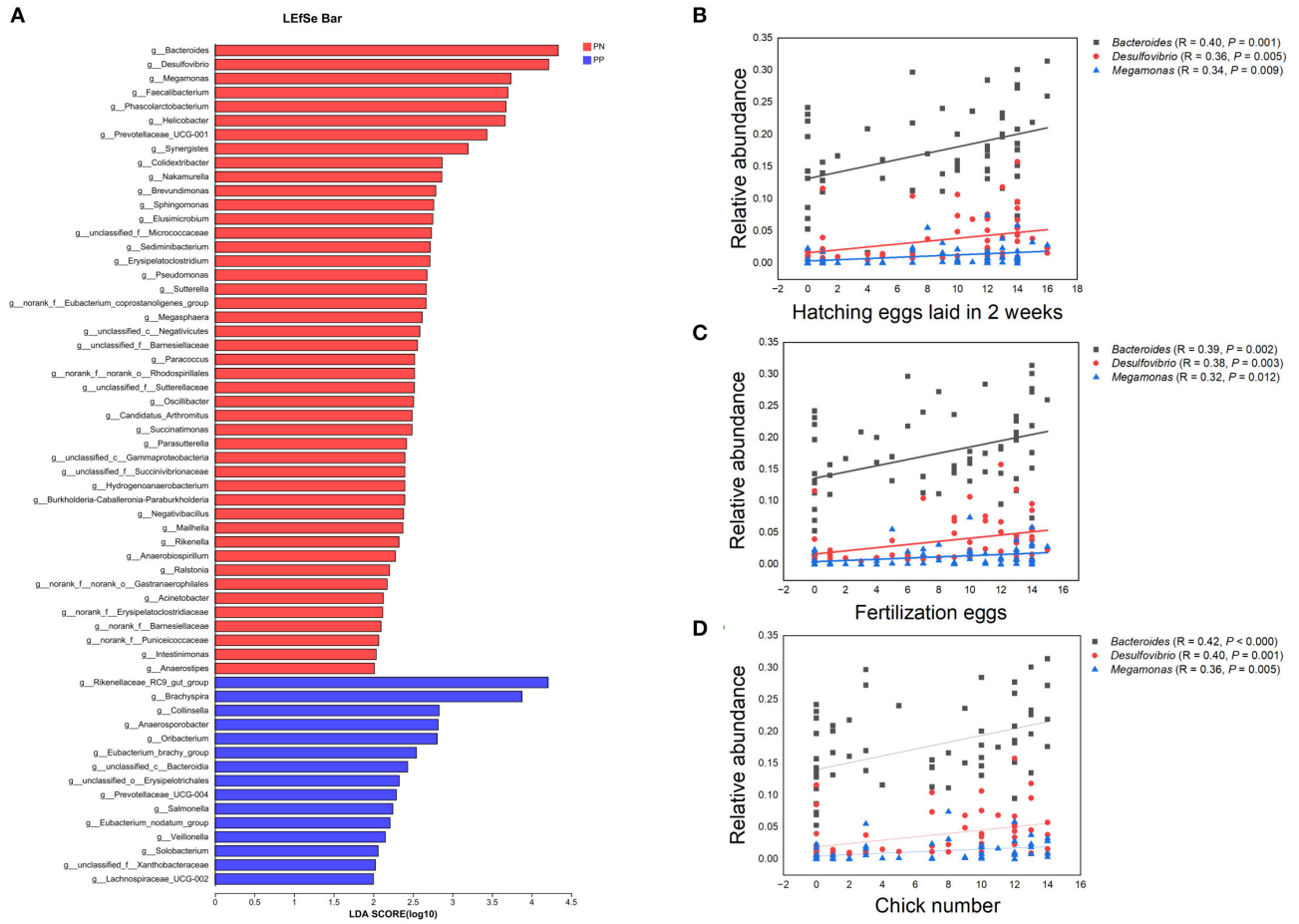

FIGURE 7
(A–D) Identification of differential microbes and the associations between microbial abundance and reproductive performance. **(A)** Linear discriminant analysis (LDA) effect size (LEfSe) analysis. **(B–D)** Correlation analysis microbial abundance and reproductive performance.PN, *S. pullorum*-negative group, PP, *S. pullorum*-positive group.

(Citi, 2018). A stable and healthier intestinal state can inhibit pathogenic bacteria and reduce the production of toxins to ensure better capacity for digestion and absorption in hens (Jackman et al., 2020; Papadopoulos et al., 2022; Cui et al., 2023). The gut microbiota is widely perceived as being closely related to gut health and the growth performance of the host (Wang et al., 2016). The results elaborated that the abundances of 29, 32, and 39 genera were separately positively correlated with HEL, FEN, and CN. Meanwhile, the abundances of 7, 6, and 2 genera were separately negatively correlated with HEL, FEN, and CN.

The 50 genera, which were significant enrichments for *S. pullorum*-negative hens, were used to make PICRUSt1 function prediction analyses in all samples. The most important functions and metabolic pathways of the above different potential biomarkers were amino acid transport and metabolism and amino acid metabolism, respectively. Among these 50 genera, previous studies showed that *Bacteroides* and *Megamonas* could produce short-chain fatty acids (SCFA) by fermenting carbohydrates to provide energy for the gut and promote the growth performance of animals (Hooper et al., 2002; Shimizu et al., 2019; Ni et al., 2021; Zhu et al., 2021). *Desulfovibrio* consumes free hydrogen for the reduction of sulfate, thus contributing to the removal of free hydrogen formed during anaerobic fermentation in the gut environment (Rychlik, 2020). *Faecalibacterium* as a probiotic candidate has shown promising results toward enhancing food safety and gut health (Khan et al., 2020). The major source energies of *Suterrella* and *Parasutterella* originate from protein, amino acid, and fatty acid metabolism (Line et al., 2010; Polansky et al., 2015). Rychlik showed that *Megasphaera* and *Phascolarctobacterium* were capable of butyrate production (Rychlik, 2020). Zhang indicated that high body weight chickens contained *Sphingomonas* more abundantly ($p < 0.05$) (Zhang et al., 2021). In-feed supplementations of probiotics strengthen the gut microbiota for improved host performance and colonization resistance to gut pathogens such as *Salmonella* and *Campylobacter*. The mechanisms of action of prebiotics and probiotics come through the production of organic acids, the activation of the host immune system, and the production of antimicrobial agents. Many probiotic preparations contain high numbers of lactobacilli that normally produce large quantities of volatile fatty acids such as formic

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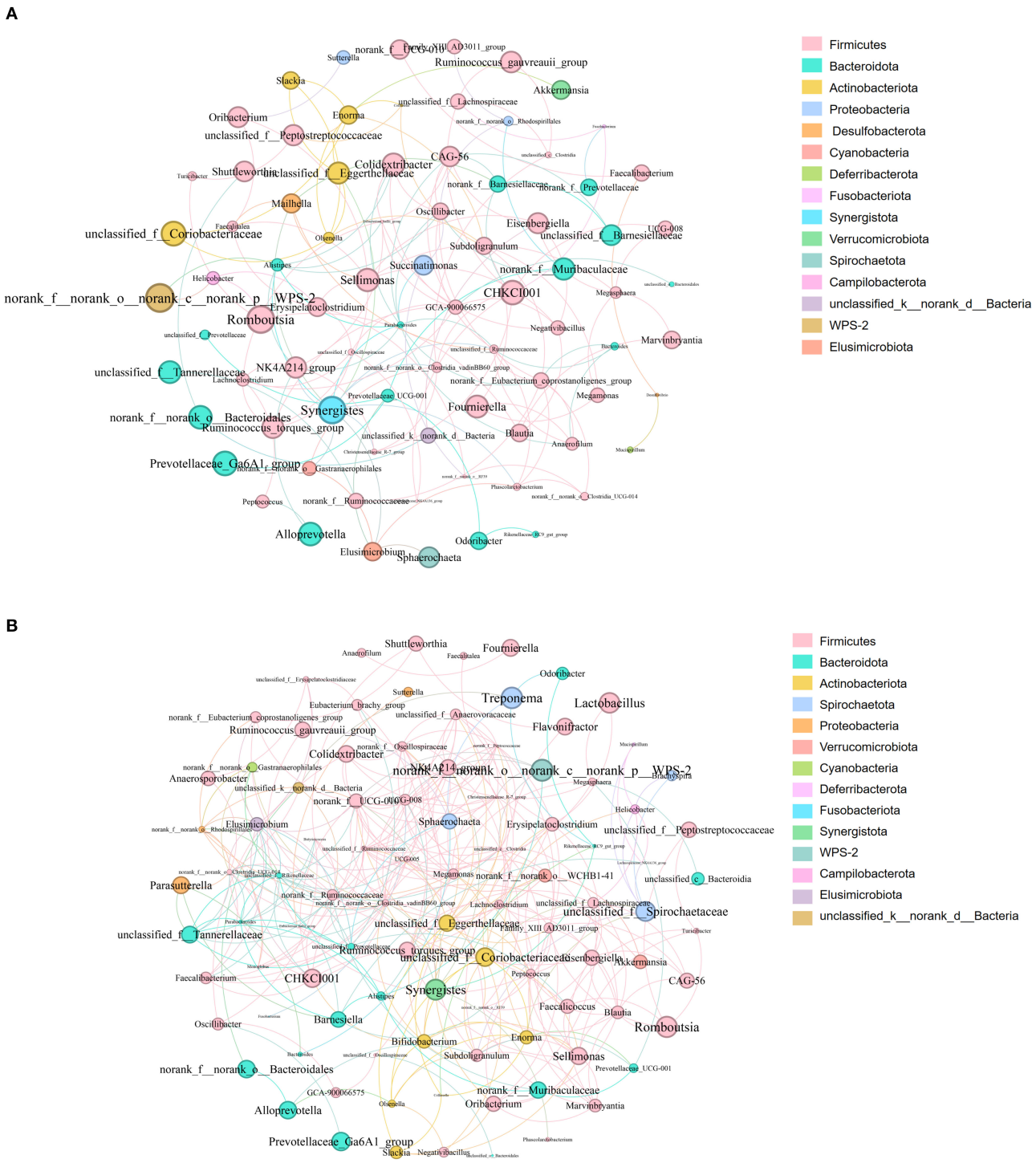

FIGURE 8
(A, B) Microbial co-occurrence network analysis at the genus level. **(A)** was PN, **(B)** was PP. Node size indicates relative abundance. Red edges indicate significant positive correlation ($P < 0.05$), and green edges indicate significant negative correlation ($p < 0.05$). PN, *S. pullorum*-negative group; PP, *S. pullorum*-positive group.

acid. The incorporation of these into feed has been shown to inhibit gut colonization by zoonotic serovars of *Salmonella* (Barrow and Freitas Neto, 2011). Mixed probiotics effectively reduced the mortality of pullorosis in chicks, promoted growth performance, regulated the balance of the intestinal flora, improved immune function, resisted pullorosis disease, completely prevented chicks from pullorosis after infection, and reduced economic loss in the poultry industry (Chen et al., 2020). The probiotics reported above

could be in culture, but most of these were poor or unclear, and most studies are empirical in nature.

Many studies have revealed the importance of probiotics in the context of infectious diseases, including pullorum. Zhou *et al.* studied the effect of a selected yeast fraction (*Safmannan*, SYF) on the prevention of pullorum disease in commercial breeder chickens and demonstrated that SYF supplementation could significantly decrease SP and SG infection rates and improve the body weight of birds challenged with *S. pullorum* (Zhou et al., 2020). Mon *et al.* examined the three-way interaction that occurred between host metabolites, resident gut microbiota, and *Salmonella* following inoculation of *Salmonella enteritidis* in 2-week-old layer chicks. It showed that there was differential regulation in many of the metabolites in association with *Salmonella enteritidis* colonization in chickens; perturbation in metabolic pathways related to arginine and proline metabolism as well as the TCA cycle was most prominently detected (Mon et al., 2020). Alrubaye *et al.* learned that the microbial metabolite deoxycholic acid shapes microbiota against *Campylobacter jejuni* chicken colonization and suggested that there was a bidirectional interaction between microbiota and microbial metabolites (Alrubaye et al., 2019). In this study, most of the genera we obtained by 16S rRNA sequencing were uncultured. The use of competitive exclusion gut flora preparations has the same protective effect as the normal flora in animal intestines. There is the possibility of a very intimate interaction between host bacteria and pathogens in the cecum (Barrow et al., 2012), and one area for future *Salmonella* control exploration is the development of probiotic organisms that have a rational basis for protection. Utilization of the information generated in this study should improve the efficacy of surveillance and biological interventions, both for intestinal carriage.

Conclusion

Pullorum disease reduced reproductive performance. Abnormal morphology of the ovaries and fallopian tubes and low integrity of epithelial tissue in the ileum and cecum were found in PP. Pullorum disease reduced the cecal microbial alpha diversity and relative abundance of *Bacteroides*, *Desulfovibrio*, and *Megamonas*, which were positively correlated with reproductive performance. Diminished phosphotransferase systems (PTS) and pentose phosphate pathways, butanoate metabolism, and oxidative phosphorylation were also found in PP. Taken together, this study clarified the morphological characteristics of the reproductive tract and intestines of hens infected with *S. pullorum* and preliminarily explored the potential association between cecal microbiota and reproductive performance in hens.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA799073.

Ethics statement

The animal study was reviewed and approved by the Ethics and Animal Welfare Committee of Shanghai Academy of Agricultural Sciences (No. SAASPZ0521009). Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

QN and QH conceived and designed the experiments. CG, ZZ, and KY raised the experimental animals. XW, CC, XQ, and QN participated in the sample collection. XQ and QN participated in the data analysis. QN wrote the article. QH, XW, CC, XQ, CG, KY, and ZZ assisted with experiments and provided advice on manuscript content. All authors read and approved the final manuscript.

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Conflict of interest

CC was employed by Shanghai Runzhuang Agricultural Technology Limited Liability Company.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1216542/full#supplementary-material

SUPPLEMENTARY FIGURE S1
Venn diagrams of OTUs **(A)** and genera **(B)** between PN and PP, respectively. PN, *S. pullorum*-negative group; PP, *S. pullorum*-positive group.

SUPPLEMENTARY TABLE S1
Composition of experimental diet.

SUPPLEMENTARY TABLE S2
The microbes with significant differences between PN and PP.

References

Alrubaye, B., Abraha, M., Almansour, A., Bansal, M., Wang, H., Kwon, Y. M., et al. (2019). Microbial metabolite deoxycholic acid shapes microbiota against Campylobacter jejuni chicken colonization. *PLoS ONE* 14, e0214705. doi: 10.1371/journal.pone.0214705

Barrow, P. A., and Freitas Neto, O. C. (2011). Pullorum disease and fowl typhoid–new thoughts on old diseases: a review. *Avian Pathol.* 40, 1–13. doi: 10.1080/03079457.2010.542575

Barrow, P. A., Jones, M. A., Smith, A. L., and Wigley, P. (2012). The long view: *Salmonella*–The last forty years. *Avian Pathol.* 41, 413–420. doi: 10.1080/03079457.2012.718071

Berchieri, A. Jr., Murphy, C.K., Marston, K., and Barrow, P. A. (2001). Observations on the persistence and vertical transmission of Salmonella enterica serovars Pullorum and Gallinarum in chickens: effect of bacterial and host genetic background. *Avian Pathol.* 30, 221–231. doi: 10.1080/03079450120054631

Chen, C., Li, J., Zhang, H., Xie, Y., Xiong, L., Liu, H., et al. (2020). Effects of a probiotic on the growth performance, intestinal flora, and immune function of chicks infected with Salmonella pullorum. *Poult. Sci.* 99, 5316–5323. doi: 10.1016/j.psj.2020.07.017

Chen, S., Zhou, Y., Chen, Y., and Gu, J. (2018). fastp: an ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics* 34, i884–i890. doi: 10.1093/bioinformatics/bty560

Chiu, Y. H., Lin, S. L., Tsai, J. J., and Lin, M. Y. (2014). Probiotic actions on diseases: implications for therapeutic treatments. *Food Funct.* 5, 625–634. doi: 10.1039/c3fo60600g

Citi, S. (2018). Intestinal barriers protect against disease. *Science.* 359, 1097–1098. doi: 10.1126/science.aat0835

Cui, Z., Zhang, R., Dai, B., Fu, C., Zhao, G., Xu, Y., et al. (2023). Effects of glyceryl monolaurate on production performance, egg quality, oviduct cytokines and intestinal microflora of 66 weeks old laying hens. *Animals* 13, 215. doi: 10.3390/ani13020215

De Lange, Pluske, C. F. M., and Gong, J. J., and Nyachoti, C. M. (2010). Strategic use of feed ingredients and feed additives to stimulate gut health and development in young pigs. *Livest. Sci.* 134, 124–134. doi: 10.1016/j.livsci.2010.06.117

Ding, J., Zhou, H., Luo, L., Xiao, L., Yang, K., Yang, L., et al. (2021). Heritable gut microbiome associated with *salmonella enterica* serovar pullorum infection in chickens. *mSystems* 6, 20. doi: 10.1128/mSystems.01192-20

Fuller, R. (1989). Probiotics in man and animals. *J. Appl. Bacteriol.* 66, 365–378. doi: 10.1111/j.1365-2672.1989.tb05105.x

Gao, P., Ma, C., Sun, Z., Wang, L., Huang, S., Su, X., et al. (2017). Feed-additive probiotics accelerate yet antibiotics delay intestinal microbiota maturation in broiler chicken. *Microbiome* 5, 91. doi: 10.1186/s40168-017-0315-1

Hazards, E. P. O. B., Koutsoumanis, K., Allende, A., Alvarez-Ordonez, A., Bolton, D., Bover-Cid, S., et al. (2019). Salmonella control in poultry flocks and its public health impact. *EFSA J.* 17, e05596. doi: 10.2903/j.efsa.2019.5596

Heo, J. M., Opapeju, F. O., Pluske, J. R., Kim, J. C., Hampson, D. J., Nyachoti, C. M., et al. (2013). Gastrointestinal health and function in weaned pigs: a review of feeding strategies to control post-weaning diarrhoea without using in-feed antimicrobial compounds. *J. Anim. Physiol. Anim. Nutr.* 97, 207–237. doi: 10.1111/j.1439-0396.2012.01284.x

Hooper, L. V., Midtvedt, T., and Gordon, J. I. (2002). How host-microbial interactions shape the nutrient environment of the mammalian intestine. *Annu. Rev. Nutr.* 22, 283–307. doi: 10.1146/annurev.nutr.22.011602.092259

Jackman, J. A., Hakobyan, A., Zakaryan, H., and Elrod, C. C. (2020). Inhibition of African swine fever virus in liquid and feed by medium-chain fatty acids and glycerol monolaurate. *J. Anim. Sci. Biotechnol.* 11, 114. doi: 10.1186/s40104-020-00517-3

Janssen, A. W., and Kersten, S. (2015). The role of the gut microbiota in metabolic health. *FASEB J.* 29, 3111–3123. doi: 10.1096/fj.14-269514

Jiang, Z., Kang, X., Song, Y., Zhou, X., and Yue, M. (2023). Identification and evaluation of novel antigen candidates against salmonella pullorum infection using reverse vaccinology. *Vaccines* 11, 865. doi: 10.3390/vaccines11040865

Khan, S., Moore, R. J., Stanley, D., and Chousalkar, K. K. (2020). The gut microbiota of laying hens and its manipulation with prebiotics and probiotics to enhance gut health and food safety. *Appl. Environ. Microbiol.* 86, 20. doi: 10.1128/AEM.00600-20

Langille, M. G., Zaneveld, J., Caporaso, J. G., Mcdonald, D., Knights, D., Reyes, J. A., et al. (2013). Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nat. Biotechnol.* 31, 814–821. doi: 10.1038/nbt.2676

Le Bon, M., Davies, H. E., Glynn, C., Thompson, C., Madden, M., Wiseman, J., et al. (2010). Influence of probiotics on gut health in the weaned pig. *Livest. Sci.* 133, 179–181. doi: 10.1016/j.livsci.2010.06.058

Li, P., Niu, Q., Wei, Q., Zhang, Y., Ma, X., Kim, S. W., et al. (2017). Microbial shifts in the porcine distal gut in response to diets supplemented with Enterococcus Faecalis as alternatives to antibiotics. *Sci. Rep.* 7, 41395. doi: 10.1038/srep41395

Li, X., Nie, C., Liu, Y., Chen, Y., Lv, X., Wang, L., et al. (2019). A genome-wide association study explores the genetic determinism of host resistance to Salmonella pullorum infection in chickens. *Genet. Sel. Evol.* 51, 51. doi: 10.1186/s12711-019-0492-4

Li, X., Nie, C., Zhang, Z., Wang, Q., Shao, P., Zhao, Q., et al. (2018). Evaluation of genetic resistance to Salmonella Pullorum in three chicken lines. *Poult. Sci.* 97, 764–769. doi: 10.3382/ps/pex354

Liao, S. F., and Nyachoti, M. (2017). Using probiotics to improve swine gut health and nutrient utilization. *Anim. Nutr.* 3, 331–343. doi: 10.1016/j.aninu.2017.06.007

Line, J. E., Hiett, K. L., Guard-Bouldin, J., and Seal, B. S. (2010). Differential carbon source utilization by Campylobacter jejuni 11,168 in response to growth temperature variation. *J. Microbiol. Methods* 80, 198–202. doi: 10.1016/j.mimet.2009.12.011

Lozupone, C., Hamady, M., and Knight, R. (2006). UniFrac–an online tool for comparing microbial community diversity in a phylogenetic context. *BMC Bioinform.* 7, 371. doi: 10.1186/1471-2105-7-371

Lv, Q., Ran, X., Qiu, H., Zhao, S., Hu, Z., Wang, J., et al. (2022). Seroprevalence of pullorum disease in chicken across mainland China from 1982 to 2020: a systematic review and meta-analysis. *Res. Vet. Sci.* 152, 156–166. doi: 10.1016/j.rvsc.2022.08.003

Magoc, T., and Salzberg, S. L. (2011). FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* 27, 2957–2963. doi: 10.1093/bioinformatics/btr507

Mon, K. K. Z., Zhu, Y., Chanthavixay, G., Kern, C., and Zhou, H. (2020). Integrative analysis of gut microbiome and metabolites revealed novel mechanisms of intestinal Salmonella carriage in chicken. *Sci. Rep.* 10, 4809. doi: 10.1038/s41598-020-60892-9

Ni, Z., Cheng, W., Ding, J., Yao, R., Zhang, D., Zhai, D., et al. (2021). Impact of Buzhong Yiqi prescription on the gut microbiota of patients with obesity manifesting polycystic ovarian syndrome. *Evid. Based Complement. Alternat. Med.* 2021, 6671367. doi: 10.1155/2021/6671367

Papadopoulos, G. A., Poutahidis, T., Chalvatzi, S., Kroustallas, F., Karavanis, E., Fortomaris, P., et al. (2022). Effects of a tributyrin and monolaurin blend compared to high ZnO levels on growth performance, faecal microbial counts, intestinal histomorphometry and immunohistochemistry in weaned piglets: a field study in two pig herds. *Res. Vet. Sci.* 144, 54–65. doi: 10.1016/j.rvsc.2022.01.011

Polansky, O., Sekelova, Z., Faldynova, M., Sebkova, A., Sisak, F., Rychlik, I., et al. (2015). Important metabolic pathways and biological processes expressed by chicken cecal microbiota. *Appl. Environ. Microbiol.* 82, 1569–1576. doi: 10.1128/AEM.03473-15

Rosenberg, E., and Zilber-Rosenberg, I. (2018). The hologenome concept of evolution after 10 years. *Microbiome* 6, 78. doi: 10.1186/s40168-018-0457-9

Rychlik, I. (2020). Composition and function of chicken gut microbiota. *Animals* 10, 103 doi: 10.3390/ani10010103

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., et al. (2009). Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol.* 75, 7537–7541. doi: 10.1128/AEM.01541-09

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., et al. (2011). Metagenomic biomarker discovery and explanation. *Genome Biol.* 12, R60. doi: 10.1186/gb-2011-12-6-r60

Setta, A. M., Barrow, P. A., Kaiser, P., and Jones, M. A. (2012). Early immune dynamics following infection with Salmonella enterica serovars Enteritidis, Infantis, Pullorum and Gallinarum: cytokine and chemokine gene expression profile and cellular changes of chicken cecal tonsils. *Comp. Immunol. Microbiol. Infect. Dis.* 35, 397–410. doi: 10.1016/j.cimid.2012.03.004

Shen, X., Zhang, A., Gu, J., Zhao, R., Pan, X., Dai, Y., et al. (2022). Evaluating Salmonella pullorum dissemination and shedding patterns and antibody production in infected chickens. *BMC Vet. Res.* 18, 240. doi: 10.1186/s12917-022-03335-z

Shen, Z., Wang, D., Ruan, Y., Xue, C., Zhang, J., Li, R., et al. (2014). Deep 16S rRNA pyrosequencing reveals a bacterial community associated with Banana Fusarium Wilt disease suppression induced by bio-organic fertilizer application. *PLoS ONE* 9, e98420. doi: 10.1371/journal.pone.0098420

Shimizu, J., Kubota, T., Takada, E., Takai, K., Fujiwara, N., Arimitsu, N., et al. (2019). Relative abundance of Megamonas hypermegale and Butyrivibrio species decreased in the intestine and its possible association with the T cell aberration by metabolite alteration in patients with Behcet's disease (210 characters). *Clin. Rheumatol.* 38, 1437–1445. doi: 10.1007/s10067-018-04419-8

Shin, M. S., Han, S. K., Ji, A. R., Kim, K. S., and Lee, W. K. (2008). Isolation and characterization of bacteriocin-producing bacteria from the gastrointestinal tract of broiler chickens for probiotic use. *J. Appl. Microbiol.* 105, 2203–2212. doi: 10.1111/j.1365-2672.2008.03935.x

Shivaprasad, H. L. (2000). Fowl typhoid and pullorum disease. *Rev. -. Off. Int. Epizoot.* 19, 405–424. doi: 10.20506/rst.19.2.1222

Soria, M. C., Soria, M. A., and Bueno, D. J. (2012). Comparison of 2 culture methods and PCR assays for Salmonella detection in poultry feces. *Poult. Sci.* 91, 616–626. doi: 10.3382/ps.2011-01831

Upadhaya, S. D., Kim, S. C., Valientes, R. A., and Kim, I. H. (2015). The effect of bacillus-based feed additive on growth performance, nutrient digestibility, fecal gas emission, and pen cleanup characteristics of growing-finishing pigs. *Asian-australas. J. Anim. Sci.* 28, 999–1005. doi: 10.5713/ajas.15.0066

Vazquez-Baeza, Y., Pirrung, M., Gonzalez, A., and Knight, R. (2013). EMPeror: a tool for visualizing high-throughput microbial community data. *Gigascience* 2, 16. doi: 10.1186/2047-217X-2-16

Wang, Q., Garrity, G. M., Tiedje, J. M., and Cole, J. R. (2007). Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* 73, 5261–5267. doi: 10.1128/AEM.00062-07

Wang, W., Li, Z., Han, Q., Guo, Y., Zhang, B., D'inca, R., et al. (2016). Dietary live yeast and mannan-oligosaccharide supplementation attenuate intestinal inflammation and barrier dysfunction induced by Escherichia coli in broilers. *Br. J. Nutr.* 116, 1878–1888. doi: 10.1017/S0007114516004116

Wang, X., Wang, H., Li, T., Liu, F., Cheng, Y., Guo, X., et al. (2020). Characterization of Salmonella spp. isolated from chickens in Central China. *BMC Vet. Res.* 16, 299. doi: 10.1186/s12917-020-02513-1

Wang, Y., Li, J., Xie, Y., Zhang, H., Jin, J., Xiong, L., et al. (2021). Effects of a probiotic-fermented herbal blend on the growth performance, intestinal flora and immune function of chicks infected with Salmonella pullorum. *Poult. Sci.* 100, 101196. doi: 10.1016/j.psj.2021.101196

Wei, S., Morrison, M., and Yu, Z. (2013). Bacterial census of poultry intestinal microbiome. *Poult. Sci.* 92, 671–683. doi: 10.3382/ps.2012-02822

Wigley, P., and Berchieri, A. Jr., Page, K.L., Smith, A.L., and Barrow, P.A. (2001). Salmonella enterica serovar Pullorum persists in splenic macrophages and in the reproductive tract during persistent, disease-free carriage in chickens. *Infect. Immun.* 69, 7873–7879. doi: 10.1128/IAI.69.12.7873-7879.2001

Wigley, P., Jones, M. A., and Barrow, P. A. (2002). Salmonella enterica serovar Pullorum requires the Salmonella pathogenicity island 2 type III secretion system for virulence and carriage in the chicken. *Avian Pathol.* 31, 501–506. doi: 10.1080/0307945021000005879

Zhang, Q. Q., Ying, G. G., Pan, C. G., Liu, Y. S., and Zhao, J. L. (2015). Comprehensive evaluation of antibiotics emission and fate in the river basins of China: source analysis, multimedia modeling, and linkage to bacterial resistance. *Environ. Sci. Technol.* 49, 6772–6782. doi: 10.1021/acs.est.5b00729

Zhang, X., Hu, Y., Ansari, A. R., Akhtar, M., Chen, Y., Cheng, R., et al. (2021). Caecal microbiota could effectively increase chicken growth performance by regulating fat metabolism. *Microb. Biotechnol.* 4, 841. doi: 10.1111/1751-7915.13841

Zhao, X., Guo, Y., Guo, S., and Tan, J. (2013). Effects of Clostridium butyricum and Enterococcus faecium on growth performance, lipid metabolism, and cecal microbiota of broiler chickens. *Appl. Microbiol. Biotechnol.* 97, 6477–6488. doi: 10.1007/s00253-013-4970-2

Zhou, C., Liang, J., Jiang, W., He, X., Liu, S., Wei, P., et al. (2020). The effect of a selected yeast fraction on the prevention of pullorum disease and fowl typhoid in commercial breeder chickens. *Poult. Sci.* 99, 101–110. doi: 10.3382/ps/pez567

Zhou, X., Kang, X., Zhou, K., and Yue, M. (2022). A global dataset for prevalence of *Salmonella Gallinarum* between 1945 and 2021. *Sci Data* 9, 495. doi: 10.1038/s41597-022-01605-x

Zhu, C., Huang, K., Bai, Y., Feng, X., Gong, L., Wei, C., et al. (2021). Dietary supplementation with berberine improves growth performance and modulates the composition and function of cecal microbiota in yellow-feathered broilers. *Poult. Sci.* 100, 1034–1048. doi: 10.1016/j.psj.2020.10.071

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Global trends and hotspots of gastrointestinal microbiome and toxicity based on bibliometrics

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Background: Toxicity concerns persist in the fields of public health, environmental science, and pharmacology. The intricate and vital role of the gastrointestinal microbiome in influencing toxicity and overall human health has gained increasing recognition in recent years. This study presents a comprehensive bibliometric analysis to evaluate the global scientific output, emerging trends, and research focal points in the area of gastrointestinal microbiome and toxicity.

Methods: The Web of Science Core Collection database was retrieved for publications on the gastrointestinal microbiome and toxicity from 1980 to 2022. Our analysis included scholarly research papers written in English and excluded duplicate publications. We used Biblioshiny and R to summarize the count and citation metrics of included articles, and visualized research trends and keywords. CiteSpace was used to identify reference literature, keywords, and citation bursts. VOSviewer was used to visualize the network of related countries, institutions, authors, co-cited authors, and keywords.

Results: A total of 2,140 articles were included, allowing us to identify significant countries, institutions, authors, and research focal points. Our results indicate a growing trend in the field, with China and the United States leading the research. The most productive journal in this area is Science of the Total Environment. Key findings revealed that research hotspots have shifted from drugs to environmental pollutants, emphasizing microplastics. Important mechanisms studied include oxidative stress, metabolism, inflammation, and apoptosis, with target organs being the gastrointestinal tract, liver, and brain. Furthermore, we highlight the rising significance of the gut-brain axis and the usage of zebrafish as a model organism.

Conclusion: Despite certain limitations, such as focusing solely on English-language publications and excluding unpublished literature, our findings provide valuable insights into the current state of research on toxicity and the

gastrointestinal microbiome. In the future, modifications to the gastrointestinal microbiome could offer new directions for treating and mitigating toxicity. These discoveries provide a comprehensive perspective on the broader scope of this research field.

KEYWORDS

gastrointestinal microbiome, toxicity, bibliometrics, CiteSpace, VOSviewer

Introduction

Toxicity issues have long been a primary concern in public health, environmental protection, and pharmacology. Toxicity research is essential for ensuring environmental, food, drug, and occupational safety and developing and evaluating new drugs (Arome and Chinedu, 2013). With the advancement of science and technology and changing societal needs, toxicity research has shifted from descriptive to mechanistic, from single substances to composite materials, from animal experiments to alternative methods, and from empiricism to system science. The gastrointestinal microbiome refers to the complex community of microorganisms in the human gastrointestinal tract, including bacteria, fungi, viruses, and more (Lindon et al., 2018). There is an increasing consensus suggesting that the gastrointestinal microbiome plays a crucial role in maintaining human health (Marchesi et al., 2016). These microorganisms participate in the body's metabolism and absorption of nutrients and are closely associated with various physiological functions, such as the immune and endocrine systems (Belkaid and Hand, 2014; Gomaa, 2020).

The past decade has witnessed a significant surge of interest in understanding the gastrointestinal microbiome and its crucial role in maintaining health and contributing to the development of diseases. This growing interest has prompted a rapid expansion of research efforts to explore the gastrointestinal microbiome's influence on various aspects of human wellbeing, including its potential relationship with toxicity. Concurrently, there has been an increased incidence of gastrointestinal disorders that could be influenced by the gastrointestinal microbiome and toxicity. For instance, recent estimates suggest that over 70 million people in the United States suffer from gastrointestinal (GI) disorders (O'Neill et al., 2021). The global rise in GI disorders has been linked to factors such as dietary changes, elevated stress levels, and exposure to environmental toxins, all of which can impact the gastrointestinal microbiome (Mohr et al., 2020). Given the burgeoning interest in this field and its potential implications for public health, there is a pressing need for a comprehensive and systematic analysis of the current state of knowledge, which is the primary focus of our study.

In recent years, research has unveiled complex interactions between the gastrointestinal microbiome and toxicity, which can be synergistic or antagonistic. On the one hand, the gastrointestinal microbiome can metabolize certain exogenous or endogenous toxic substances (Jeong et al., 2013), such as drugs, environmental pollutants, and endocrine disruptors, altering their structure, bioavailability, activity, or toxicity (Wilson and Nicholson, 2017). On the other hand, the gastrointestinal microbiome can also produce toxic metabolites such as D-lactic acid, ammonia, endotoxins, or carcinogens, thereby increasing the toxicity experienced by the host (Van de Wiele et al., 2005). Furthermore, an imbalance or disruption of the gastrointestinal microbiome, such as a decrease in microbial populations, overgrowth, or displacement, can lead to immune dysfunction, intestinal barrier damage, inflammatory responses, or systemic toxicity within the host (Chi et al., 2019).

Therefore, comprehending the mechanisms underlying the interactions between the gastrointestinal microbiome and toxicity, and finding ways to modulate the gastrointestinal microbiome to prevent or treat diseases associated with toxicity, are subjects of great importance and complexity. Various strategies centered around the gastrointestinal microbiome have been proposed or developed, including probiotics, fecal transplantation, microbial metabolites, and synthetic biology. These approaches promise to enhance the effectiveness and safety of drugs, reduce the risk of exposure to environmental pollutants, and augment the immune response against cancer.

Bibliometric analysis is a quantitative research method widely used in scientific research globally (Bornmann and Mutz, 2015). It has become one of the most extensively employed methods for evaluating the credibility, quality, and impact of academic work (van Raan, 1996). Bibliometric analysis can help us understand the current state, development trends, and research hotspots in a particular field. However, no bibliometric studies have hitherto been conducted on the association between gastrointestinal microbiome and toxicity. Therefore, we conducted a bibliometric analysis to reveal the major achievements, key technologies, and future development directions in this field. We hope this work can serve as a guide to identify critical knowledge and research priorities, assist researchers in formulating research strategies, optimize resource allocation, and achieve more breakthrough results in gastrointestinal microbiome and toxicity.

Materials and methods

Data acquisition

Bibliometric research is a quantitative method used to analyze and evaluate scientific literature. Web of Science (WOS) is one of the most commonly used academic database sources and is considered the most comprehensive and reliable database in

bibliometric analysis (Wang et al., 2015). To prevent bias caused by daily database updates, the literature relevant to this study was searched for and exported from the Web of Science Core Collection (WOSCC) as plain text files containing full records and cited references on February 20, 2023.

Our search strategy details are provided in **Supplementary Table 1**. For this study, the literature was selected based on the following inclusion and exclusion criteria: (i) The publication timeframe was limited to articles published until December 31, 2022; (ii) Only research articles and reviews were considered for inclusion; (iii) There were no limitations on the species or organisms studied; (iv) Publications written in English were included, while those in other languages were excluded; (v) Duplicate publications were removed from the analysis to ensure the uniqueness of the dataset. Given that this study uses publicly available secondary data and does not involve interaction with human subjects, it does not require an ethical review.

Data analysis

Our bibliometric analysis study was conducted using multiple tools, including Biblioshiny and R-version 4.2.2, VOSviewer (1.6.19), CiteSpace 6.2.R1 (64-bit), Tableau 2022.4, and Microsoft Excel 2021.

The Impact Factor (IF) is a widely used metric for assessing the significance and influence of scientific journals. It is calculated by dividing the total number of citations received by papers published in the journal during the previous 2 years by the total number of papers published in that journal during the same period (Garfield, 2006).

CiteSpace is a software tool developed by Professor Chaomei Chen of Drexel University to analyze and visualize scientific literature, particularly in the field of bibliometrics (Chen, 2017). It is widely used by researchers, librarians, and information professionals to gain valuable insights into the structure and evolution of scientific fields. In this study, we utilized CiteSpace to conduct visual analyses encompassing various aspects such as country distribution, institutional distribution, reference analysis, and keyword and citation bursts. The specific parameters used in CiteSpace were set as follows: time slicing (from 1980 to 2022, years per slice = 1), term source (title, abstract, author, keyword, and keywords plus), node type (one option chosen at a time from author, institution, country, keyword, cited reference, cited author, and cited journal), link strength (Cosine), link scope (Within Slices), selection criteria (g-index $K = 25$), and pruning (none).

VOSviewer is a powerful tool for visualizing and analyzing bibliometric networks based on distance-based bibliometric tools. It was developed by Nees Jan van Eck and Ludo Waltman of Leiden University, and can categorize related items into clusters of various colors, with the same color indicating a higher degree of association among these items (Van Eck and Waltman, 2011). This study used VOSviewer to examine and visualize the distribution of countries, institutions, authors, and co-cited authors and explore keyword co-occurrence and overlay networks.

Biblioshiny is a web-based software tool built using R programming language, designed to facilitate bibliometric analyses and generate interactive visualizations of the obtained results. It was jointly developed by Aria and Cuccurullo (2017) from the University of Naples and the University of Campania in Italy. In our study, we primarily utilized Biblioshiny to summarize the volume and citation counts of the bibliometric analyses, assess the performance of articles and journals, identify the occurrence of annual cumulative keywords/terms, calculate national influence and collaboration frequency, and visualize research trends and keyword timelines. The specific parameter information used in Biblioshiny is detailed in **Supplementary Table 2**. We also used generalized additive models using the MGCV package in R to estimate the trend and quantity of related literature publications (Wood, 2018). The source code file is provided in **Supplementary Table 3**.

Tableau is a widely used visualization tool for exploring and analyzing data. In this study, it was employed to analyze and visualize the distribution of publications over time and the number of publications by country.

Results

Publication output and trends

After the literature retrieval and screening process, 2,140 publications met the inclusion and exclusion criteria (**Figure 1**), including 1,606 research articles and 534 review articles. As shown in **Figure 2A**, the first article was published in 1980 (Carter et al., 1980). From 1980 to 2022, the number of publications increased from 1 in 1980 to 494 in 2022, with an annual compound growth rate of 15.52%. Although the number of annual publications fluctuated slightly, an overall increasing trend was observed. To facilitate a comprehensive understanding of the development of publications in this field, the timeline was divided into three distinct stages: (i) Early stage (1980–1990): during this stage, the number of publications was extremely low, with a maximum of only 1 record per year.; (ii) Moderate growth stage (1991–2012): during this stage, the number of publications began to increase with 4–24 publications/per year; (iii) Rapid growth stage (2013–2022): during this stage, the number of publications escalated, with the annual number of articles increasing from 45 to 494 in 2022.

A generalized additive model was also used to analyze the relationship between the number of publications and the corresponding year (**Figure 2B**). The formula for the model is "count $\sim$ s(year)," where "year" is the predictor variable, and the smooth term "s(year)" indicates that the relationship between "year" and "count" is not strictly linear, and is modeled using a smoothing function. The effective degrees of freedom (edf) for the smoothing term were 8.918, and the corresponding F-statistic and p-value indicated that the smoothing term was highly significant (p-value $<$ 2e-16). The model demonstrated a strong fit to the annual trend of publications (R^2 = 0.997, Deviance explained = 99.8%). Based on the model's prediction, the total number of publications related to the toxicity of gastrointestinal microbiome is projected to exceed 592 in 2023 and will continue to increase in the next 10 years to 2032, with an estimated 1,445 publications in 2032.

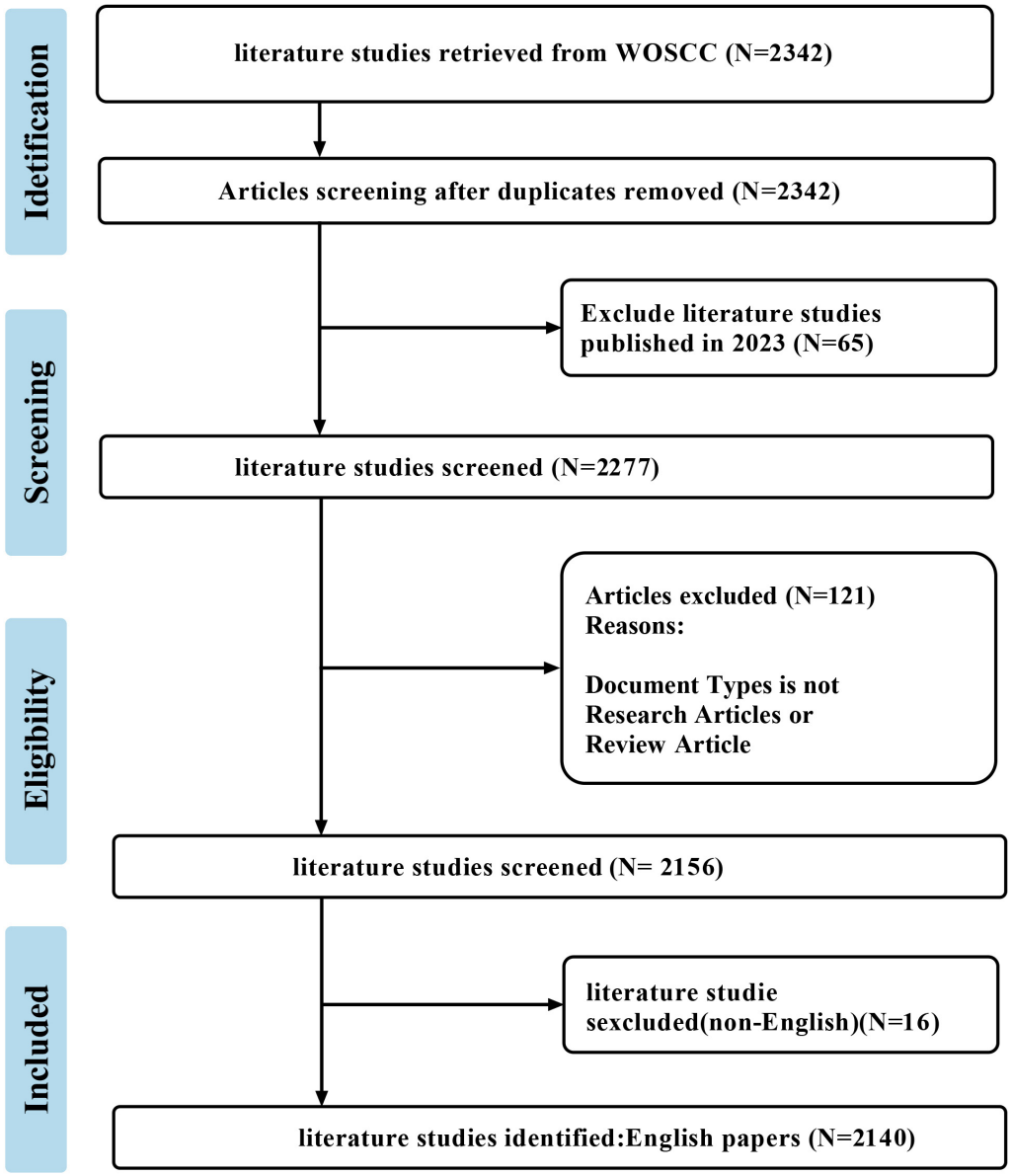


FIGURE 1
Flow diagram of the selection process.

Most prolific countries/regions analysis

Figure 3A provides an overview of the distribution of the 2,140 included articles among 84 countries/regions. The top 10 countries/regions, ranked by the number of publications, include a diverse representation from different parts of the world. Among these top contributors, there were five European countries, three Asian countries, two North American countries, and Australia. China emerged as the country with the highest number of publications (n = 908), followed by the United States (n = 519), while the remaining countries/regions published fewer than 100 articles each. Figure 3B illustrates the international collaborations among 47 countries/regions that have collaborated on at least five publications.

Most prolific institutions

The analysis revealed that a total of 2,585 institutions made contributions to research in the field of toxicology and gastrointestinal microbiome. The top 10 most productive institutions are presented in Table 1. Within the overlapping network of institutional collaboration analysis, 66 institutions published more than 10 related articles (Figure 4). Researchers affiliated with the University of London's Imperial College of Science, Technology and Medicine, and Harvard University, have been actively engaged in this field since its early stages. On the other hand, researchers from the Chinese Academy of Fishery Sciences and Chengdu University of Traditional Chinese Medicine have more recently begun exploring this study area.

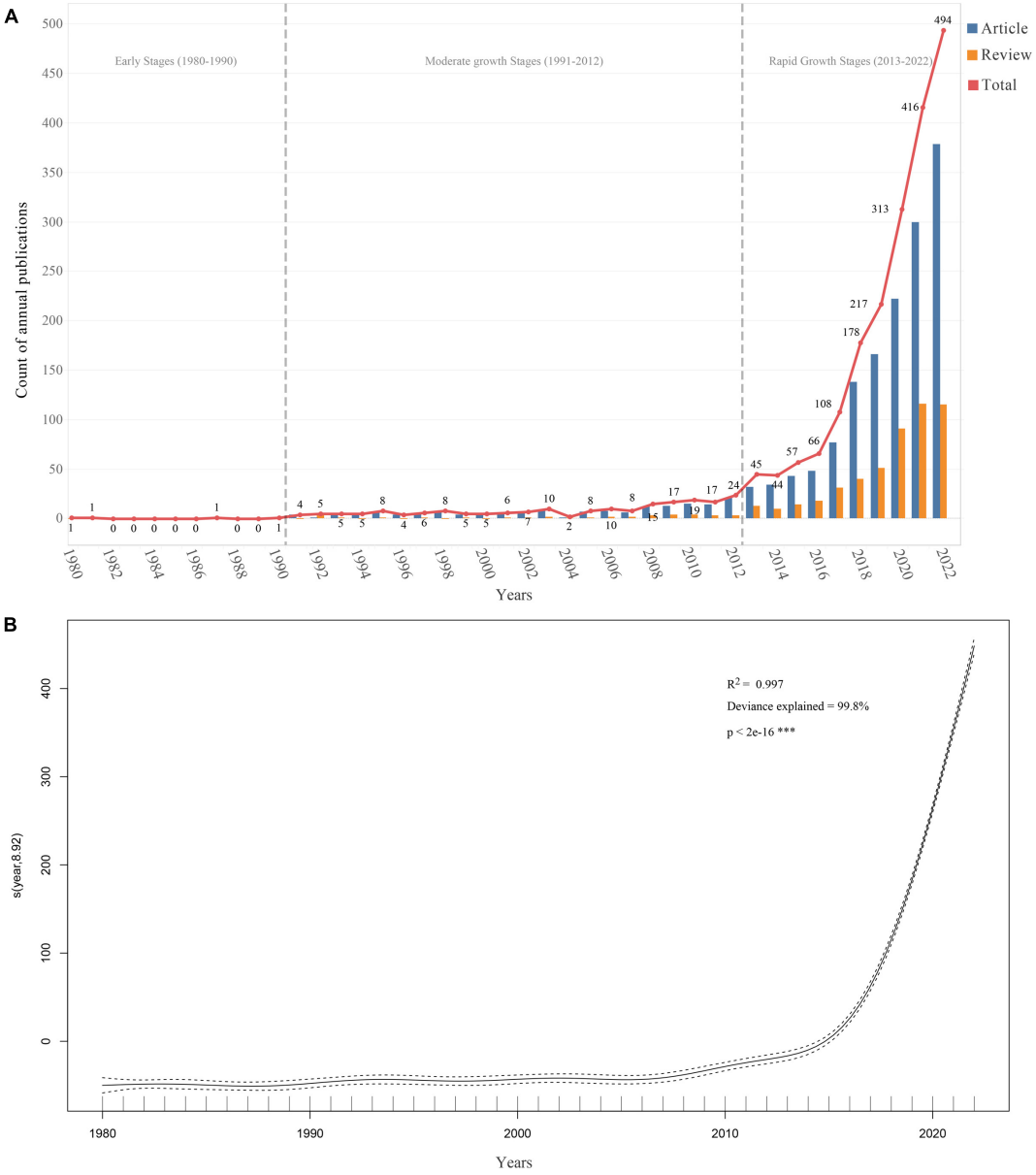


FIGURE 2
Trends in the number of publications on gastrointestinal microorganisms studied in the field of toxicity from 1980 to 2022. **(A)** The annual number of publications. **(B)** Generalized additive model fit curve plot.

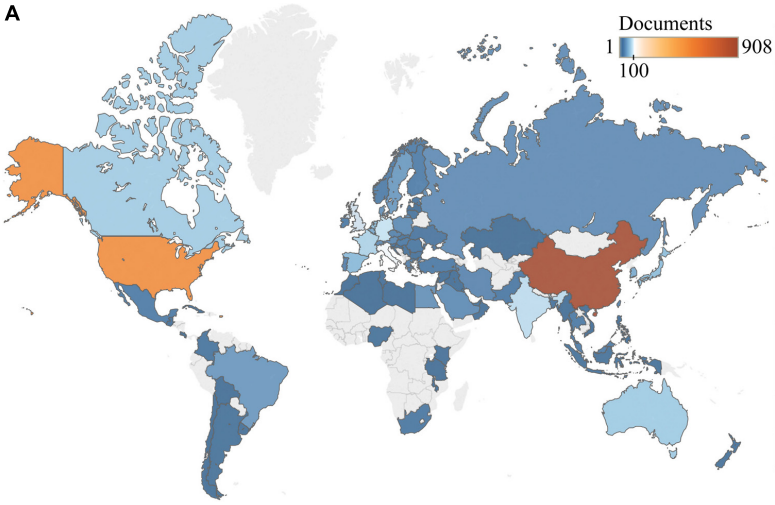
Journal analysis

The 2,140 selected publications were published across 745 journals. Among them, 415 articles were published in the top 10 journals, accounting for 19.4% (415/2140) of the total articles. Table 2 presents the top 10 journals and their respective IF for 2022. Among these journals, 90% (9/10) were classified as Q1 in the Journal Citation Reports (JCR), with five journals categorized as environmental sciences and nine with an IF greater than 5. In terms of publisher location, four of the top 10 journals were based in the United Kingdom, two in the United States, two in the Netherlands, and two in Switzerland. Furthermore, we employed Bradford's Law to identify the core journals in gastrointestinal microbiota and toxicology research (Bradford, 1934). As illustrated in Supplementary Figure 1, the application of Bradford's Law identified 28 core journals, with one-third of the articles published in core journals.

Most influential author analysis

Analysis of the authors in the included literature revealed that 11,894 authors contributed to publications related to gut microbiota and toxicity research. Table 3 shows the top 10 authors in terms of the number of published articles and co-citation frequency. Regarding the publication count, except for Kun Lu from the United States, the remaining nine authors were all from China. Yuanxiang Jin from Zhejiang University of Technology was

A



Countries/Regions	Documents
China	908
USA	519
Italy	98
England	98
Germany	85
India	80
France	73
Canada	68
Australia	67
Netherlands	58
Japan	58

B

Countries/Regions	Total link strength	Links	Documents
USA	303	40	519
China	221	34	908
England	138	35	98
Germany	103	31	85
Italy	97	26	98
Netherlands	85	24	58
France	80	23	73
Australia	67	18	67
Spain	58	22	55
Canada	58	21	68

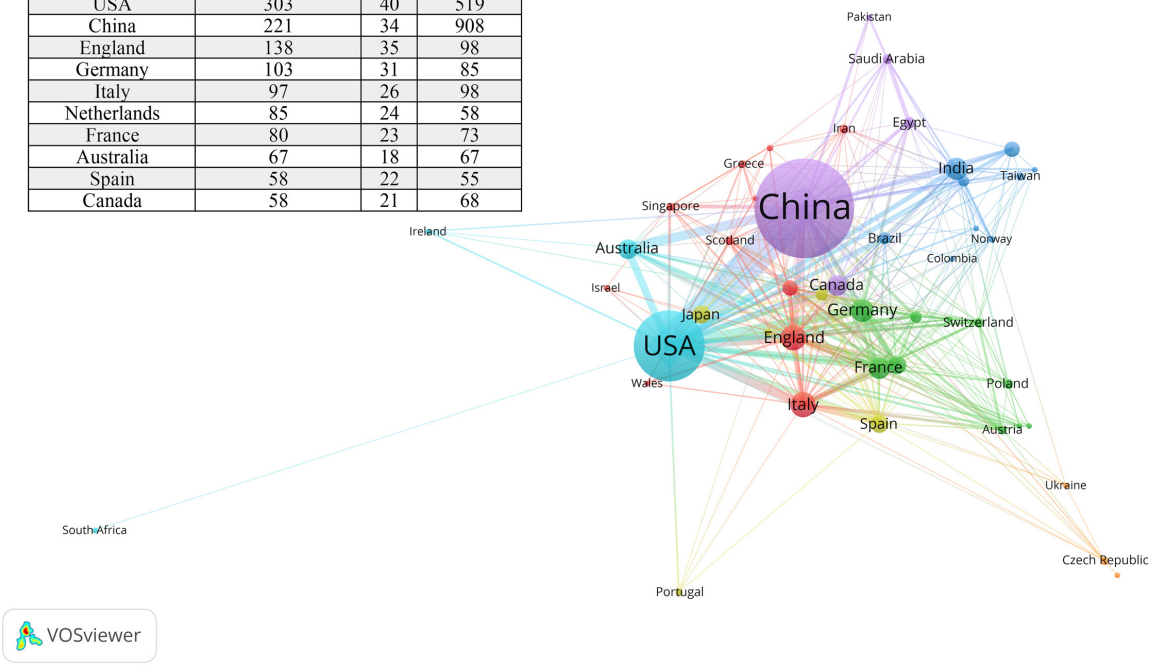


FIGURE 3
Countries/regions involved in the field of research. **(A)** World map of countries/regions based on the number of related publications. **(B)** The countries/regions collaboration network in this field. Circles represent countries, with the size of the circle indicating the number of publications. Different colors represent distinct clusters, while the lines connecting items represent international collaborations between countries. The thickness of the connecting lines indicates the strength of the collaboration.

the most prolific author (n = 28 articles), followed by Wei Chen and Hao Zhang from Jiangnan University (n = 25 articles), and Zhengwei Fu from Zhejiang University of Technology (n = 21 articles). Among the top 10 co-cited authors, four were from the United States, and two were from China. Notably, Yuanxiang Jin emerged as the most prolific author, having the highest count of publications and citation frequency, suggesting that he has been actively engaged in research and has achieved considerable recognition and influence within the gut microbiota and toxicity research field. Jeremy K. Nicholson from the Imperial College of Science, Technology and Medicine and Peter J. Turnbaugh from the University of California San Francisco ranked second and third regarding co-citation frequency.

The author collaboration analysis was performed using VOSviewer (Figure 5A), where authors with a publication count of 5 or more were included. The 164 included authors were classified into 30 clusters based on their collaborations, and the connections between different clusters were relatively scarce, indicating limited collaboration among research teams/laboratories involved in gut microbiota and toxicity studies. Figure 5B shows the co-cited author relationship network, which included 131 authors with a citation frequency of 50 or more. The network is visualized with

TABLE 1 Top 10 institutions in terms of number of articles issued.

Rank	Institution	Documents	Original country
1	Chinese Academy of Sciences	111	China
2	University of Chinese Academy of Sciences	67	China
3	Zhejiang University of Technology	46	China
4	Nanjing University	30	China
5	Jiangnan University	29	China
6	Shanghai Jiao Tong University	29	China
7	Univ N Carolina	27	USA
8	Zhejiang University	27	China
9	China Agricultural University	26	China
10	University of Adelaide	25	Australia

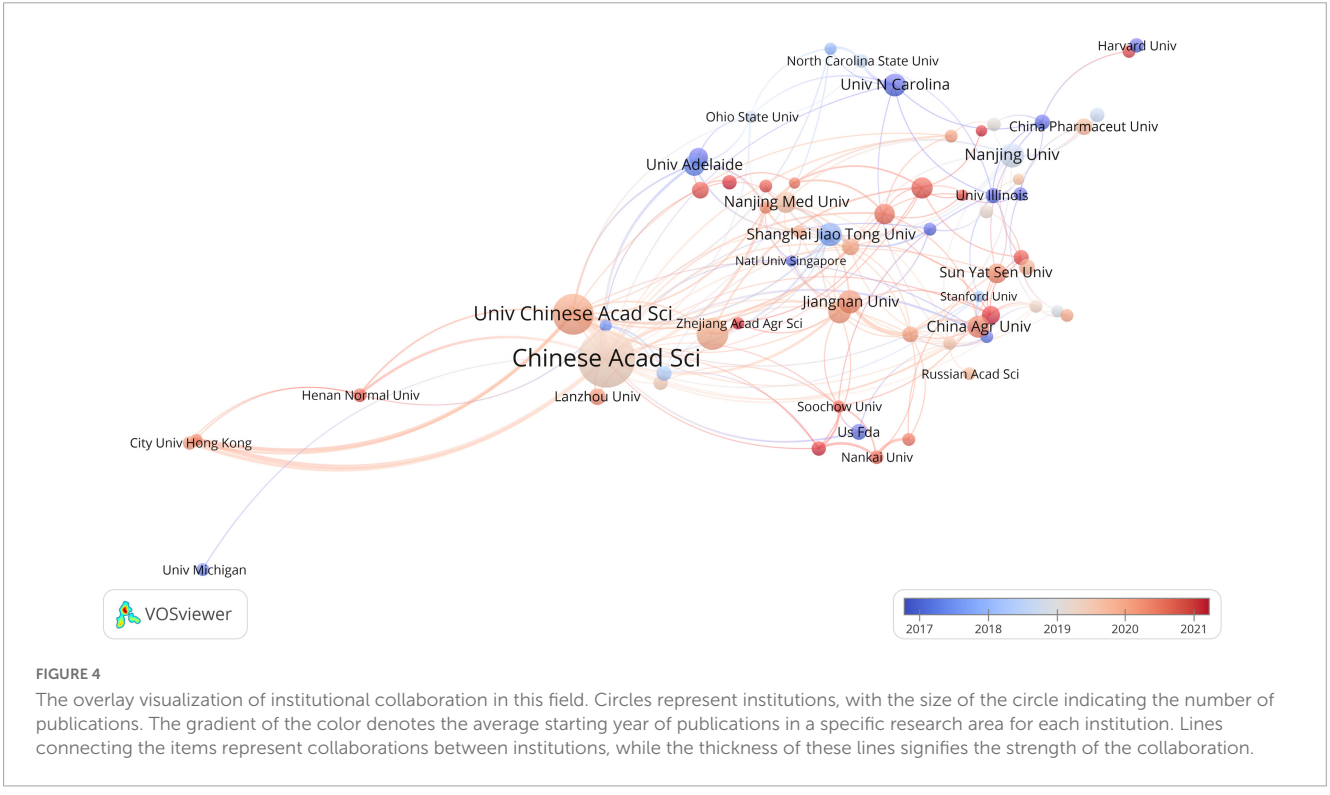


FIGURE 4
The overlay visualization of institutional collaboration in this field. Circles represent institutions, with the size of the circle indicating the number of publications. The gradient of the color denotes the average starting year of publications in a specific research area for each institution. Lines connecting the items represent collaborations between institutions, while the thickness of these lines signifies the strength of the collaboration.

colored sections representing the similarities in research interests among the co-cited authors. The analysis reveals a high level of homogeneity among the focus areas of the relevant researchers, as they can be divided into 5 distinct clusters. However, it is worth noting that the purple group appears to have fewer connections with other clusters, indicating a relatively sparse relationship with the research interests of other groups.

Analysis of research hotspots

Most cited local literature

The impact of an article in a particular research field can be gauged by the number of citations it receives (Sun et al., 2022). Table 4 lists the top 10 most globally cited documents. Half of these 10 articles have been cited more than 500 times, including 3 research articles and 7 review articles. Specifically, the most cited article in this field, "Plastic and Human Health: A Micro Issue?," published in 2017, has been cited 1,042 times (Wright and Kelly, 2017). This influential review utilized interdisciplinary literature to discuss and evaluate the potential impact of microplastics on human health, highlighting that microplastics can damage human health by triggering immune responses through gastrointestinal microbiome and inflammatory reactions. The second and third most cited articles were "Symbiotic gut microbes modulate human metabolic phenotypes" (Li et al., 2008) and "Gut microorganisms, mammalian metabolism, and personalized health care" (Nicholson et al., 2005), with 809 and 674 citations, respectively.

Cluster analysis of co-cited references

Co-citation is a research method to measure the degree of relationship between cited references, which requires two different

TABLE 2 The top 10 most published journals in the field of gastrointestinal microbiology and toxicity research.

Ranking	Sources	Articles	Country	IF	JCR-c
1	Science Of The Total Environment	76	Netherlands	10.754	ENVIRONMENTAL SCIENCES–SCIE (Q1)
2	Environmental Pollution	58	England	9.988	ENVIRONMENTAL SCIENCES–SCIE (Q1)
3	Ecotoxicology And Environmental Safety	56	England	7.129	ENVIRONMENTAL SCIENCES–SCIE (Q1); TOXICOLOGY–SCIE (Q1)
4	Chemosphere	47	England	8.943	ENVIRONMENTAL SCIENCES–SCIE (Q1)
5	Frontiers In Microbiology	39	Switzerland	6.064	MICROBIOLOGY–SCIE (Q1)
6	Journal Of Hazardous Materials	37	Netherlands	14.224	ENGINEERING, ENVIRONMENTAL–SCIE (Q1); ENVIRONMENTAL SCIENCES–SCIE (Q1)
7	Plos One	28	USA	3.752	MULTIDISCIPLINARY SCIENCES–SCIE (Q2)
8	Frontiers In Pharmacology	27	Switzerland	5.988	PHARMACOLOGY AND PHARMACY–SCIE (Q1)
9	Food And Chemical Toxicology	24	England	5.572	TOXICOLOGY–SCIE (Q1)
10	International Journal Of Molecular Sciences	23	USA	6.208	BIOCHEMISTRY AND MOLECULAR BIOLOGY–SCIE (Q1)

IF, impact factor (2021); JCR-c, Journal Citation Reports category (2021).

articles to be cited by the same document, forming a co-citation relationship (Small, 1973). **Table 5** shows the top 10 highly cited references in the field of gastrointestinal microbiome and toxicity research. These articles are often regarded as fundamental to the development of this research area. Among the top four ranked articles, three were published in *Science* and focused on the relationship between the gastrointestinal microbiome and anti-PD-1 immunotherapy.

Furthermore, we analyzed the relationships between co-cited documents using CiteSpace 6.2.R1, and the co-citation network of cited references consisted of 1,422 nodes and 5,979 links (**Figure 6A**). Cluster analysis generated 192 co-citation clusters, with the top ten clusters shown in **Figure 6B** (Chen, 2020). The largest cluster was #0, characterized by the keyword "drug metabolism," with the most representative article being "Effects of single and combined toxic exposures on the gut microbiome: current knowledge and future directions" by Tsiaoussis et al. (2019). **Figure 6C** presents a timeline view of the co-citation clusters of cited references, reflecting the temporal characteristics of research hotspots in this field.

Citation bursts analysis of references

Citation bursts refer to sudden increases in the number of times an article is cited, indicating heightened attention to the topic by scholars and reflecting new trends and hotspots in the field. **Figure 6D** presents the top 25 cited references with the strongest citation bursts. The shortest burst duration was 1 year, while the longest lasted 18 years. Among these articles, the one with the strongest citation burst was "Gut microorganisms, mammalian metabolism and personalized health care," published in *Nature Reviews Microbiology*, with a burst spanning from 2007 to 2016 (Nicholson et al., 2005). In addition, several articles have experienced citation bursts in the past 5 years and are still ongoing, such as "The microbiome, cancer, and cancer therapy" (Helmink et al., 2019) and "Accumulation of different shapes of microplastics initiates intestinal injury and gut microbiota dysbiosis in the gut of zebrafish" (Qiao et al., 2019). These results suggest that these research directions have gained considerable popularity in recent years and are expected to continue to be a prominent area of study in the coming years.

Keyword analysis

A total of 9,628 keywords were initially considered during the analysis of keywords. To ensure the inclusion of relevant keywords while avoiding overly general or infrequent ones, a minimum occurrence threshold of 30 was applied. This resulted in 101 keywords meeting the criteria and being included in the analysis. The chosen threshold has been validated in previous bibliometric studies and has proven effective (Sweileh, 2019; Sun et al., 2022). The National Library of Medicine's Medical Subject Headings (MeSH) vocabulary was used to consolidate similar keywords. This vocabulary is widely recognized and comprehensive in biomedical research (**Supplementary Table 4**). **Table 6** lists the top 10 most frequently occurring keywords. Excluding those related to microbiology, the highest-frequency keywords were "toxicity," "oxidative stress," "metabolism," "exposure," "inflammation," and "health." **Figure 7A** displays the network visualization of these keywords. We observed that in the largest red cluster, the keywords were related to microbiology and cancer, such as "gastrointestinal microbiome," "microbiota," "neoplasms," and "chemotherapy." The second green cluster was associated with inflammation and metabolomics, featuring keywords like "inflammation," "expression" and "metabolomics." The third blue cluster was related to environmental toxicity, with primary keywords including "toxicity," "exposure," "microplastics," "pesticides" and "metals, heavy." Additionally, **Figure 7B** displays the overlay visualization of the keywords. The results revealed that "pharmacokinetics," "diarrhea," "Escherichia coli," "cytotoxicity" and "inflammatory bowel disease" were the major themes in the early stages. In contrast, recent years have seen research hotspots focusing on keywords such as "oxidative stress," "exposure," "immunotherapy," "dysbiosis," and "microplastics." **Figure 7C** illustrates the development trends of keywords over the years. As shown in **Figures 7B, C** we observed a gradual transition in

TABLE 3 Top 10 authors in terms of number of publications and frequency of co-citations.

Rank	Author	Publications	Countries/ regions	Institutions	Author	Co-citations	Countries/ regions	Institutions
1	Jin, Yuanxiang	28	China	Zhejiang University of Technology	Jin, Yuanxiang	299	China	Zhejiang University of Technology
2	Chen, Wei	25	China	Jiangnan University	Nicholson, Jeremy K	241	England	Univ London Imperial Coll Sci
3	Zhang, Hao	25	China	Jiangnan University	Turnbaugh, Peter J	218	USA	University of California San Francisco
4	Fu, Zhengwei	21	China	Zhejiang University of Technology	Zhai, Qixiao	214	China	Jiangnan University
5	Zhao, Jianxin	20	China	Jiangnan University	Caporaso, J. Gregory	212	USA	Univ Colorado
6	Zhai, Qixiao	16	China	Jiangnan University	Ley, Ruth E	203	Germany	Max Planck Inst Dev Biol
7	Lu, Kun	16	USA	University of Georgia	Cani, Patrice D	174	Belgium	Catholic Univ Louvain
8	Liu, Yang	16	China	Henan Normal University	Robert C Edgar	166	USA	University of California, Berkeley
9	Tian, Fengwei	14	China	Jiangnan University	Stringer, Andrea M	153	Australia	University of South Australia
10	Li, Yuan	14	China	Chinese Academy of Medical	Wallace, Bret D	152	USA	University of North Carolina

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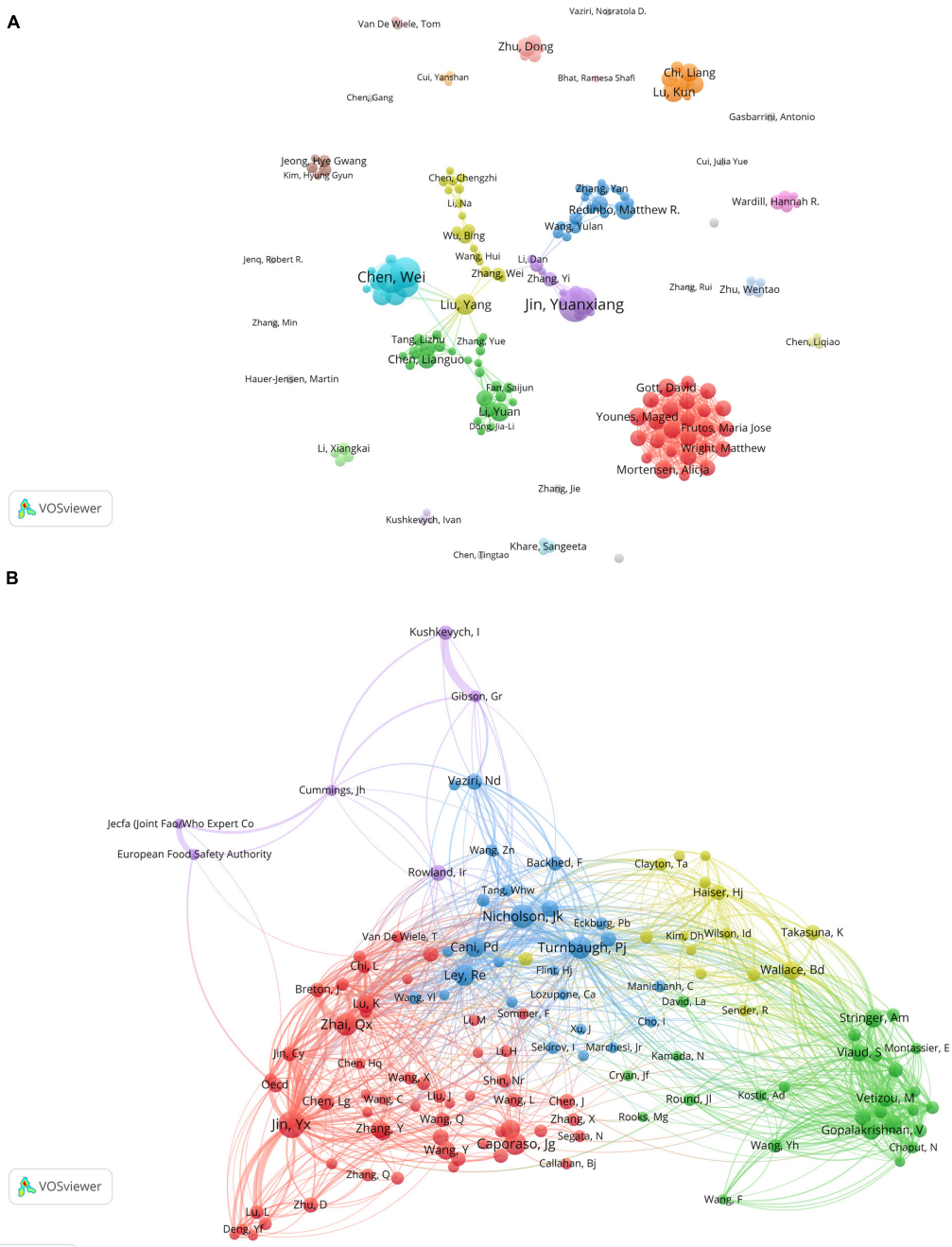

FIGURE 5
Visualization of co-authorship and co-citation relationships among authors in gastrointestinal microbiota and toxicology research. **(A)** Network visualization of collaborations between authors. The size of the items represents the number of publications by the author, and different colors mark different clusters. The thickness of the connections between items indicates the strength of the relationships. **(B)** Co-citation author network. The size of the items represents the frequency of co-citations for each author.

the research focus of the gastrointestinal microbiome and toxicity fields, shifting from a focus on drugs to environmental pollutants.

Moreover, we conducted a burst analysis of keywords using the CiteSpace software. Figure 7D displays the top 26 keywords with the highest burst strength. Among them, the keywords "Diarrhea" (1996–2017), "active metabolite" (1996–2013), "beta-glucuronidase" (2000–2016), and "bacteria" (2002–2016) attracted attention for an extended duration. The keyword "Microplastics" (2021–2022) was most recently used, suggesting that this keyword has recently garnered significant attention and may become a future research hotspot.

Discussion

This bibliometric analysis investigated research development in the gastrointestinal microbiome and toxicity from 1980 to 2022. The advancement of scientific systems is closely intertwined with

TABLE 4 Top 10 most global cited documents.

Rank	Title	Author	Year	Cited	Article type	Journal	IF
1	Plastic and Human Health: A Micro Issue?	Wright, Stephanie L	2017	1,042	Review	Environmental Science and Technology	11.357
2	Symbiotic gut microbes modulate human metabolic phenotypes	Li, Min	2008	809	Article	Proceedings of The National Academy of Sciences of The United States of America	12.779
3	Gut microorganisms, mammalian metabolism and personalized health care	Nicholson, J K	2005	674	Review	Nature Reviews Microbiology	78.297
4	Baseline gut microbiota predicts clinical response and colitis in metastatic melanoma patients treated with ipilimumab	Chaput, N	2017	604	Article	Annals of Oncology	51.769
5	Environmental exposure to microplastics: An overview on possible human health effects	Prata, Joana Correia	2020	524	Review	Science of The Total Environment	10.753
6	Intestinal alkaline phosphatase detoxifies lipopolysaccharide and prevents inflammation in zebrafish in response to the gut microbiota	Bates, Jennifer M	2007	499	Article	Cell Host and Microbe	31.316
7	Postinfectious Irritable Bowel Syndrome	Spiller, Robin	2009	485	Review	Gastroenterology	33.883
8	Microbiota: a key orchestrator of cancer therapy	Roy, Soumen	2017	478	Review	Nature Reviews Cancer	69.8
9	Commensal Clostridia: leading players in the maintenance of gut homeostasis	Lopetuso, Loris R	2013	453	Review	Gut Pathogens	5.324
10	The microbiome, cancer, and cancer therapy	Helmink, Beth A	2019	448	Review	Nature Medicine	87.241

IF, impact factor (2021).

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TABLE 5 Top 10 highly cited references.

Rank	Title	Author	Year	Cited	Journal	IF
1	QIIME allows analysis of high-throughput community sequencing data	J Gregory Caporaso	2010	144	Nature Methods	47.990
2	Gut microbiome influences efficacy of PD-1-based immunotherapy against epithelial tumors	Bertrand Routy	2018	118	Science	63.714
3	Gut microbiome modulates response to anti-PD-1 immunotherapy in melanoma patients	V Gopalakrishnan	2018	117	Science	63.714
4	Commensal Bifidobacterium promotes antitumor immunity and facilitates anti-PD-L1 efficacy	Ayelet Sivan	2015	115	Science	63.714
5	Anticancer immunotherapy by CTLA-4 blockade relies on the gut microbiota	Marie Vétizou	2015	111	Science	63.714
6	The intestinal microbiota modulates the anticancer immune effects of cyclophosphamide	Sophie Viaud	2013	107	Science	63.714
7	Alleviating cancer drug toxicity by inhibiting a bacterial enzyme	Bret D Wallace	2010	107	Science	63.714
8	An obesity-associated gut microbiome with increased capacity for energy harvest	Peter J Turnbaugh	2006	102	Nature	69.504
9	Commensal bacteria control cancer response to therapy by modulating the tumor microenvironment	Noriho Iida	2013	92	Science	63.714
10	Effects of environmental pollutants on gut microbiota	Yuanxiang Jin	2017	91	Environmental Pollution	9.988

IF, impact factor (2021).

the progress of human civilization, and the field of toxicology is no exception. The late 20th century witnessed a golden era of development, attributable to factors such as rapid global economic growth, ecological degradation, increased exposure to human health issues, and accelerated advancements in computer science and medicine (Watson and Wexler, 2009). During this period, molecular research techniques played a crucial role in the study of gastrointestinal microbiome, resulting in significant breakthroughs in the analysis of gastrointestinal microbiome polymorphism and conducting qualitative and quantitative studies. On the other hand, the development of model organisms and genetically engineered animals has been paralleled by increased research on the physiological functions of gastrointestinal microbiome based on animal models (Stappenbeck et al., 2002; Bäckhed et al., 2004; Rakoff-Nahoum et al., 2004). In the late 20th and early 21st centuries, researchers increasingly focused on understanding the contribution of the gastrointestinal microbiome to overall health and disease states (Cho and Blaser, 2012). These early studies provided the groundwork for more complex explorations into the relationships between the gastrointestinal microbiome, disease progression, and responses to toxins.

With the official launch of the Human Microbiome Project in 2007 [Integrative HMP (iHMP) Research Network Consortium, 2019] and the thriving development of high-throughput sequencing technologies (Caporaso et al., 2010), the scientific community's enthusiasm for gastrointestinal microbiome research reached unprecedented heights. A review by Sekirov et al. (2010) examined the gastrointestinal microbiome's influence on host physiology and its role in health and disease. They explored how changes in the microbiome can lead to disorders like inflammatory bowel disease and obesity, setting a foundation for further research into the microbiome's role in disease development (Sekirov et al., 2010). In the first half of 2012, *Science* published a special issue on "The Gut Microbiota" and *Nature* followed suit in the second half of the same year with a special issue on "Gut microbes and health" (Lupp et al., 2012; Mueller et al., 2012). Since 2010, there has been a growing focus on the gastrointestinal microbiome's role in influencing the efficacy of various treatments, including cancer therapies (Moayyedi et al., 2010; Simrén et al., 2013; Perez-Chanona and Jobin, 2014; Rajagopala et al., 2017). The results of these studies have expanded our understanding of how the gastrointestinal microbiome can modulate treatment responses, including potential toxicities. Since 2013, research on the relationship between toxicology and the gastrointestinal microbiome has gained momentum, driven by contemporary demands. Amidst this research boom, the field has amassed considerable findings and data. A comprehensive summary and analysis of its developmental trends, disciplinary frontiers, and research hotspots are important for facilitating further in-depth investigations.

China and the United States emerged as the leading countries in terms of the number of publications in the field of gastrointestinal microbiome and toxicity research, along with the highest frequency of collaboration. The substantial volume of articles from China can be attributed to increased research investment, particularly in the biomedical field, by the Chinese government in recent years (Xie and Freeman, 2019). China's rapid economic development has also led to a heightened focus on ecological and public health issues. In terms of total link strength, the United States ranked first, primarily

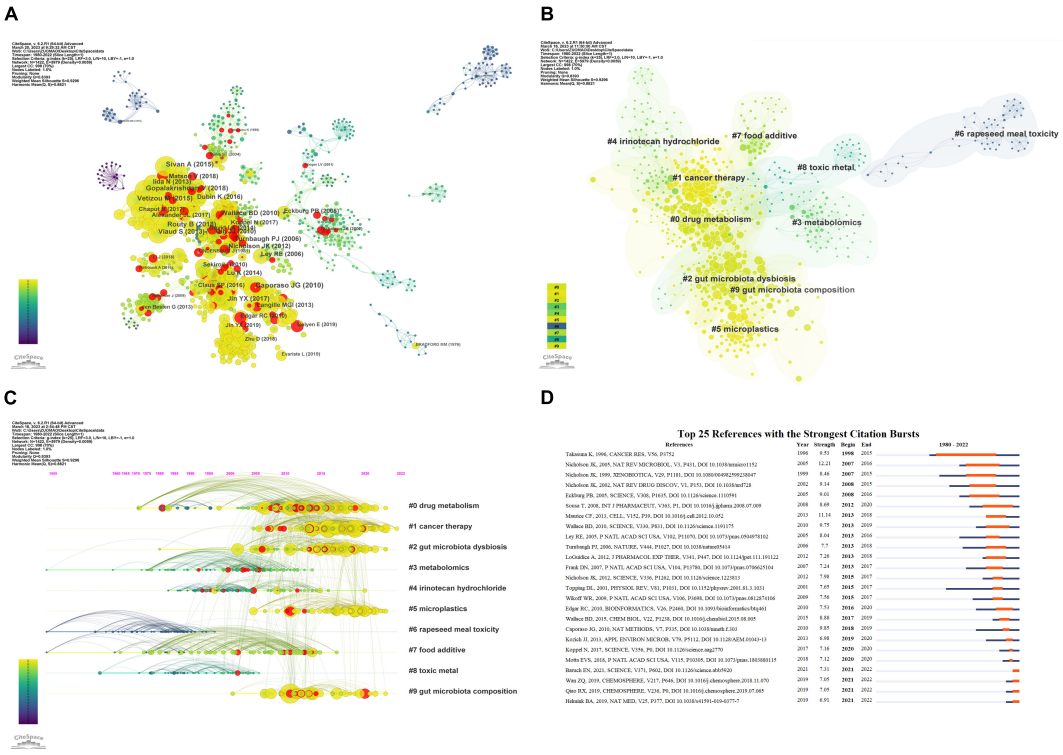

FIGURE 6
Co-citation analysis and citation burst ranking of references. **(A)** Co-citation network of references. Nodes with different colors represent different years; node size indicates citation frequency, and red nodes denote documents with citation bursts. **(B)** Top 10 clusters of co-cited references in the network. **(C)** Timeline view of co-cited references. Clusters are placed vertically in descending order of size. The position of nodes on the horizontal axis indicates the time when they were first cited, and connecting lines represent co-citation relationships. The number of citations determines the size of the nodes. **(D)** Top 25 references with the highest citation burst strength. Blue lines represent the timeline, while the orange segments denote the citation burst periods, displaying the starting year, ending year, and duration of the burst.

TABLE 6 Top 10 keywords in terms of frequency of occurrence.

Rank	Keyword	Occurrences	Total link strength
1	Gastrointestinal microbiome	1,319	4,891
2	Toxicity	663	2,723
3	Microbiota	412	1,780
4	Oxidative stress	267	1,187
5	Metabolism	263	1,103
6	Exposure	234	1,099
7	Inflammation	200	938
8	Bacteria	170	757
9	Health	157	725
10	Probiotics	143	669

due to its high reputation in scientific research and innovation and numerous world-renowned research institutions and universities that attract researchers and scholars from around the world (Adams, 2012). This contributes to collaboration and development in this field (Ioannidis et al., 2018). As barriers to international exchange are eliminated, it is highly conceivable that influential countries and regions will experience complementary advantages, which will significantly impact the long-term development of this field.

Although China, India, and Japan are major countries in the gastrointestinal microbiome and toxicity research, the publishers of the ten most active journals in this field are all from Western Europe and the United States, without representation from Asia. This observation highlights the importance of developing internationally influential journals in Asia. Furthermore, we found that the majority of the top 10 journals in terms of article quantity are environmental science journals, including *Science of the Total Environment*, *Environmental Pollution*, *Ecotoxicology*

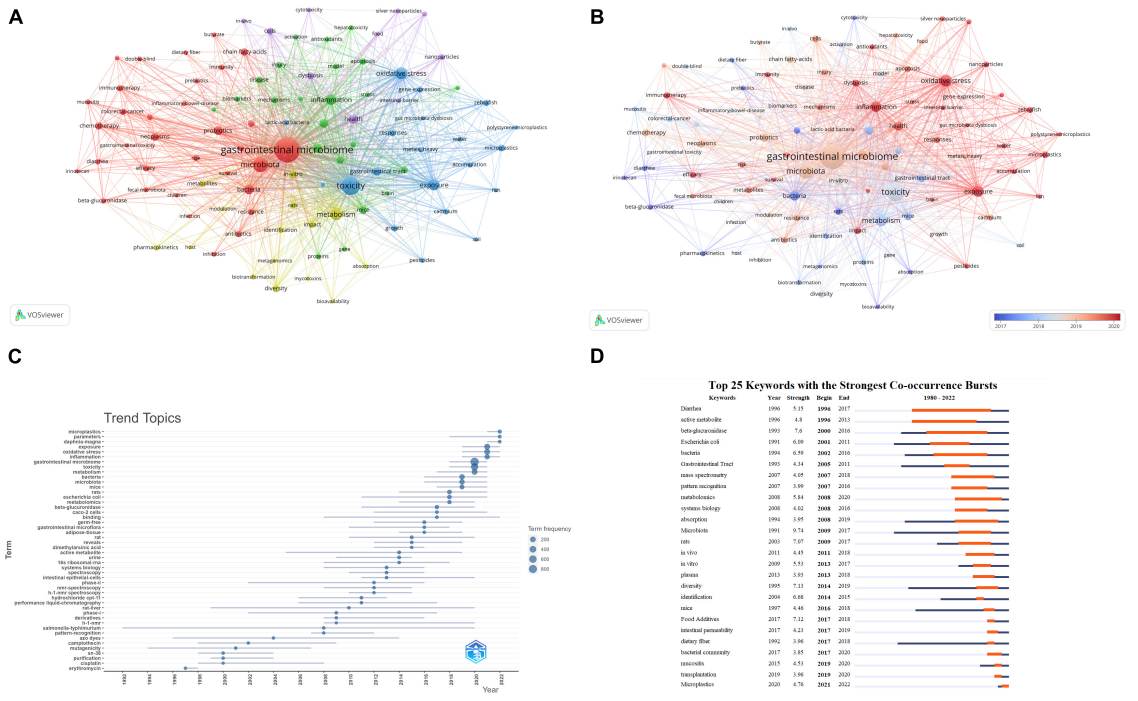

FIGURE 7
Keyword analysis. **(A)** Keyword co-occurrence network relationship diagram. Items represent keywords, and the size of items represents the frequency of keyword co-occurrence, clusters are marked using different colors, and links represent co-occurrence between keywords. **(B)** Overlay visualization of Keyword co-occurrence network. Items represent keywords; the size of items indicates the frequency of keyword co-occurrence. Early keywords are displayed in blue, while orange indicates recent keywords. **(C)** Trends in Keywords Plus development over the years. The blue line indicates the timeline of keywords, and the bubble size indicates the frequency of keywords. **(D)** Top 26 keywords with the strongest Co-occurrence frequency burst. The blue bars represent the time period when the keywords appear; the orange bars represent the time interval when the keywords are found to erupt, indicating the beginning year, end year, and duration of the outbreak.

and Environmental Safety, and others. This finding indicates that gastrointestinal microbiome and toxicity research has become a significant topic in environmental science.

In bibliometric analysis, hotspots typically refer to the most active and widely followed research directions or topics within a specific field and the characteristics and changes of hotspots can be reflected through citation analysis, keywords, and other indicators (Wu et al., 2021). Citation analysis can demonstrate the academic influence of research (Breugelmans et al., 2015). Interestingly, among the top 10 most globally cited documents, "microplastics" and "cancer therapy" were recurring topics. The top-ranked article, a review published in *Environmental Science and Technology* in 2017, was cited 1,042 times (Wright and Kelly, 2017). This article suggests that exposure to microplastics may affect the intestinal microbiome by promoting the growth of certain types of bacteria and altering the balance of microbial communities, potentially leading to adverse health outcomes such as inflammation and weakened immunity. In addition, three of the top 10 explored the role of the microbiome, particularly the gastrointestinal microbiome, in cancer and cancer therapy (Chaput et al., 2017; Roy and Trinchieri, 2017; Helmink et al., 2019). The gastrointestinal microbiome can modulate the response to various forms of cancer treatment, such as chemotherapy and radiotherapy, by influencing drug metabolism, pharmacokinetics, anticancer activity, and toxicity.

Interestingly, among the top 10 most co-cited articles, the majority (70%) focused on "cancer therapy" and were published in *Science* from 2010 to 2018. Three of these articles, cited over 110 times, were all dedicated to studying how the gastrointestinal microbiome influences the efficacy of PD-1-based immunotherapy (Sivan et al., 2015; Gopalakrishnan et al., 2018b; Routy et al., 2018). Anti-PD-1 immunotherapy is a type of cancer treatment that blocks the interaction between programmed cell death protein 1 (PD-1) and its ligands (PD-L1 and PD-L2), thereby enhancing T-cell anti-tumor activity. However, this therapy can also cause various adverse events, such as immune-related colitis. The severity and frequency of these toxicities depend on the type and combination of PD-1 or PD-L1 inhibitors used (Naidoo et al., 2015). The gastrointestinal microbiome has been shown to modulate the efficacy of anti-PD-1 immunotherapy, with higher diversity and abundance of certain bacterial taxa (such as *Bifidobacterium* and *Faecalibacterium*) being associated with better prognosis and lower incidence of immune-related adverse events (Sivan et al., 2015). Possible mechanisms by which the gastrointestinal microbiome regulates anti-PD-1 immunotherapy include affecting the composition and function of immune cells, altering the expression of PD-L1 on tumor cells, and producing metabolites that regulate inflammation and immunity (Gopalakrishnan et al., 2018a; Miller and Carson, 2020). These findings demonstrate the significant role of cancer therapy in the field of gastrointestinal microbiome and toxicity research. Therefore, interventions targeting the gastrointestinal microbiome, such as probiotics, prebiotics, antibiotics, or fecal microbiota transplantation, represent a promising strategy to improve cancer treatment outcomes and reduce toxicity.

In addition, the analysis of citation bursts, keyword clustering, and keyword bursts provides valuable insights into the key focus areas in the gastrointestinal microbiome and toxicity research. Several notable characteristics that attract significant attention in this field were identified: (1) toxic substances: drugs, nanoparticles, microplastics, heavy metals, pesticides, and biotoxins; (2) target organs: gastrointestinal tract, liver, and brain; (3) mechanisms: oxidative stress, metabolism, inflammation, and cell apoptosis. It is essential to interpret the identified burst keywords with caution. While they can be instrumental in signaling new and fast-emerging research areas, they do not always correlate with the quality or significance of the research. Thus, while these findings provide an overview of the research trajectory, a further qualitative assessment is required to discern the impact and importance of these emerging trends.

Interestingly, the research hotspots in the field of gastrointestinal microbiome and toxicity have gradually shifted from drugs to environmental pollutants. Indeed, since 2018, studies on the correlation between microplastic toxicity and gastrointestinal microbiome have begun to intensify. Microplastics are a common environmental pollutant that can lead to particle toxicity, oxidative stress, and inflammatory responses in organisms (Prata et al., 2020). Jin et al. (2018) found that exposure to two sizes of polystyrene microplastics for 14 days resulted in a significantly decreased abundance of *Bacteroidetes* and *Proteobacteria* in the intestine, while *Firmicutes* significantly increased. Besides, the levels of inflammatory factors IL-1α, IL-1β, and IFN were increased in the gut. Moreover, several studies have confirmed that microplastics can lead to gastrointestinal microbiome dysbiosis and metabolic disorders (Jin et al., 2019; Lu et al., 2019; Qiao et al., 2019). Notably, microplastics may also induce neurobehavioral toxicity through the gut-brain axis, potentially activating neuroactive ligand-receptor interaction and serotonergic synapse-related pathways (Huang et al., 2022). Importantly, Lianguo Chen et al. (2018) found that the toxicity of titanium dioxide nanoparticles and bisphenol A exposure in zebrafish is associated with the gastrointestinal microbiome, with the combined exposure leading to oxidative stress closely related to the ratio of pathogenic Lawsonia and normal metabolic Hyphomicrobium (Chen et al., 2018). Recent research findings indicate that the ingestion of differentially charged nanoplastics leads to the development of inflammatory lesions in the gut, disruption of electron transfer processes, inhibition of energy metabolism during mitochondrial oxidative phosphorylation, oxidative stress, increased expression of pro-inflammatory factors, and disturbances in pathways related to glycolipid metabolism (Xu et al., 2023). Our findings highlight that zebrafish is the predominant model organism in environmental toxicity studies. Their appeal as a crucial animal model in environmental toxicology research stems from several advantages: low maintenance cost, high breeding rate, swift growth, and considerable genetic homology with humans.

The primary target organs in the gastrointestinal microbiome and toxicity research are the gastrointestinal tract, liver, and brain. Studies on the microbiota-target organ axis have flourished in recent years, spurring many researchers to explore toxicology in this field. Zhong et al. (2021) investigated the toxic effects of arsenic trioxide (ATO) on the intestines and liver of ducks. The results demonstrated that ATO could mediate hepatic and jejunal inflammation and pyroptosis through the gut-liver axis and LPS/TLR4/NF-κB signaling pathway (Zhong et al., 2021). Another study found that exposure to perfluorooctanoic acid (PFOA) in mice led to a decrease in intestinal probiotics (including *Lactobacillus* and *Bifidobacterium*), while *Dehalobacterium* and *Pseudomonas* genera were significantly disturbed, which are associated with liver inflammation and oxidative stress. This confirmed that the hepatotoxicity of PFOA might be related to gastrointestinal microbiome dysbiosis (Wang et al., 2021). As previously mentioned, the importance of the gut-brain axis in toxicity research is increasing. Diazinon is an organophosphorus insecticide known to cause neurotoxicity. Studies have found that diazinon disrupts the gut microbiota composition and its metabolic functions in a sex-specific manner (Gao et al., 2017). Sub-chronic and chronic exposure to glyphosate-based herbicides (GBH) induces anxiety and depression-like behaviors in mice and leads to a decrease in the abundance of *Bacteroides*, *Firmicutes*, *Pseudomonas*, and *Lactobacillus* in the mouse gastrointestinal microbiome. Researchers hypothesized that gut dysbiosis might be highly associated with the observed changes in neurobehavior (Aitbali et al., 2018). In a recent study, a correlation between the gut microbiota and two specific organs, the lungs and the brain, has been established. The study found that inhalation of zinc oxide nanoparticles by the lungs can potentially lead to cerebral cortical impairment by disrupting the intricate lung-gut-brain axis (Zhang L. et al., 2023).

Oxidative stress, metabolism, and inflammation are the most reported mechanisms involving gastrointestinal microbiome and toxicity. The gastrointestinal microbiome can induce or exacerbate oxidative stress by metabolizing drugs or environmental pollutants to produce reactive oxygen species or free radicals or influence the host's antioxidant system. For instance, the oral antiviral brivudine can be metabolized by the host and gastrointestinal microbiome into bromovinyluracil, exhibiting hepatotoxicity. The hepatotoxicity of brivudine may be associated with oxidative stress induced by its metabolic products (Jameson and Hsiao, 2019). Moreover, the gastrointestinal microbiome can induce or modulate inflammatory responses by regulating the host's immune system or releasing endotoxins or pro-inflammatory cytokines. For example, the gastrointestinal microbiome can indirectly influence an individual's response to immunotherapy in cancer treatment. Some commensal bacteria in the gut can enhance the antitumor effects of immune checkpoint inhibitors (ICIs), while some pathogenic bacteria may suppress the efficacy of ICIs or increase their toxic side effects (Lu et al., 2022). Nevertheless, certain probiotics can potentially exert a beneficial effect on the toxicity of specific substances. In this respect, the efficacy of *Lactobacillus fermentum HNU312* has been demonstrated in mitigating oxidative damage and behavioral abnormalities induced by chronic lead exposure during early brain development (Zhang Z. et al., 2023). The gastrointestinal microbiome can influence the metabolism of drugs and environmental pollutants in various ways, thereby altering their toxicity (Li et al., 2008; Inamura, 2021).

An important direction for future research in the gastrointestinal microbiome field is exploring the therapeutic potential of manipulating the microbiome to mitigate the harmful effects of toxins. Building upon the existing knowledge of how the gastrointestinal microbiome influences responses to cancer therapies and the effects of environmental pollutants,

investigations can focus on interventions such as probiotics, prebiotics, antibiotics, or fecal microbiota transplantation to enhance treatment efficacy and reduce toxicity. In addition, given the complexity of the interactions between the gastrointestinal microbiome and host health, interdisciplinary research approaches will be crucial in advancing our understanding of this field. Collaborations among toxicologists, microbiologists, environmental scientists, and health professionals could significantly advance our understanding of the gastrointestinal microbiome and its role in toxicity.

Despite the insights provided by our bibliometric analysis, this study is not without limitations. This study employed bibliometric analysis to assess research progress on the gastrointestinal microbiome and toxicity. However, it is important to note that bibliometric analysis mainly provides a quantitative assessment of the literature and does not necessarily reflect the quality or impact of the research. It allows for an objective and comprehensive exploration of the literature but does not substitute for a thorough qualitative assessment. Evaluating the quality of bibliometrics should not solely depend on quantitative measures. Instead, it requires an amalgamation of different methods and standards, such as peer review, expert opinion, and societal benefits, among others, to attain a more comprehensive and equitable scientific assessment. We acknowledge that our study was limited to English publications, potentially omitting significant contributions from non-English sources. A more extensive and inclusive approach would entail translating and analyzing scientific literature published in other languages. Furthermore, our study did not include unpublished literature, such as conference presentations, thesis work, and data from ongoing studies, which could also contribute valuable insights. While this literature is an essential part of the scientific discourse, its inclusion often presents practical challenges due to accessibility and verification issues. Nonetheless, future studies should also consider these sources to capture a more comprehensive view of the research landscape. In addition, this study only analyzed publications indexed in the Web of Science Core Collection database, which may not represent the entire body of literature on this topic. Therefore, these results should be interpreted with consideration of this limitation.

Conclusion

In conclusion, the relationship between the gastrointestinal microbiome and toxicity has become a research hotspot, with a substantial growth in annual publications indicating the global importance of this research field. China and the United States are the core competitive countries in this field. This research has identified the major researchers and institutions involved in this field globally. *Science of the Total Environment* is the most productive and core journal in this research area. Gastrointestinal microbiome and their relationships with metabolism, immune systems, and cancer treatments are considered hot topics, while environmental pollution and health may be the focus of future research. While we have discussed the potential for modifications to the gastrointestinal microbiome as new directions for treating and mitigating toxicity, we acknowledge the complexity of the underlying mechanisms. These complex interactions require further rigorous investigation. In summary, bibliometric analysis can provide valuable insights into the current state of toxicity and gastrointestinal microbiome research, highlight knowledge gaps, and identify potential future developments. These insights can guide future research directions, facilitate collaboration, and ultimately lead to the development of effective gastrointestinal microbiome intervention strategies for preventing and treating toxicity.

Data availability statement

Publicly available datasets were analyzed in this study. This data can be found here: Web of Science™ (WOS, http://www.webofknowledge.com).

Author contributions

JD, CL, TJ, and XB analyzed the data, carried out the literature research, and wrote the manuscript. JD, CL, and XZ contributed to interpreting and discussing the results. TJ reviewed and edited before submission. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1231372/full#supplementary-material

References

Adams, J. (2012). Collaborations: The rise of research networks. *Nature* 490, 335–336. doi: 10.1038/490335a

Aitbali, Y., Ba-M'Hamed, S., Elhidar, N., Nafis, A., Soraa, N., and Bennis, M. (2018). Glyphosate based-herbicide exposure affects gut microbiota, anxiety and depression-like behaviors in mice. *Neurotoxicol. Teratol.* 67, 44–49. doi: 10.1016/j.ntt.2018.04.002

Aria, M., and Cuccurullo, C. (2017). Bibliometrix: An r-tool for comprehensive science mapping analysis. *J. Informetr.* 11, 959–975. doi: 10.1016/j.joi.2017.08.007

Arome, D., and Chinedu, E. (2013). The importance of toxicity testing. *J. Pharm. Biosci.* 4, 146–148.

Bäckhed, F., Ding, H., Wang, T., Hooper, L. V., Koh, G. Y., Nagy, A., et al. (2004). The gut microbiota as an environmental factor that regulates fat storage. *Proc. Natl. Acad. Sci. U.S.A.* 101, 15718–15723. doi: 10.1073/pnas.0407076101

Belkaid, Y., and Hand, T. W. (2014). Role of the microbiota in immunity and inflammation. *Cell* 157, 121–141. doi: 10.1016/j.cell.2014.03.011

Bornmann, L., and Mutz, R. (2015). Growth rates of modern science: A bibliometric analysis based on the number of publications and cited references. *J. Assoc. Inf. Sci. Technol.* 66, 2215–2222. doi: 10.1002/asi.23329

Bradford, S. C. (1934). Sources of information on specific subjects. *Engineering* 137, 85–86.

Breugelmans, J. G., Makanga, M. M., Cardoso, A. L., Mathewson, S. B., Sheridan-Jones, B. R., Gurney, K. A., et al. (2015). Bibliometric assessment of European and Sub-Saharan African research output on poverty-related and neglected infectious diseases from 2003 to 2011. *PLoS Negl. Trop. Dis.* 9:e3997. doi: 10.1371/journal.pntd.0003997

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010). Qiime allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336. doi: 10.1038/nmeth.f.303

Carter, J. H., Mclafferty, M. A., and Goldman, P. (1980). Role of the gastrointestinal microflora in amygdalin (laetrile)-induced cyanide toxicity. *Biochem. Pharmacol.* 29, 301–304. doi: 10.1016/0006-2952(80)90504-3

Chaput, N., Lepage, P., Coutzac, C., Soularue, E., Le Roux, K., Monot, C., et al. (2017). Baseline gut microbiota predicts clinical response and colitis in metastatic melanoma patients treated with ipilimumab. *Ann. Oncol.* 28, 1368–1379. doi: 10.1093/annonc/mdx108

Chen, C. (2017). Science mapping:a systematic review of the literature. *J. Data Inf. Sci. (Warsaw, Poland)* 2, 1–40. doi: 10.1515/jdis-2017-0006

Chen, C. (2020). *How to use citespace (c) 2015-2020*. Available online at: https://citespace.podia.com/ebook-how-to-use-citespace (accessed August 8, 2022).

Chen, L., Guo, Y., Hu, C., Lam, P. K. S., Lam, J. C. W., and Zhou, B. (2018). Dysbiosis of gut microbiota by chronic coexposure to titanium dioxide nanoparticles and bisphenol A: Implications for host health in zebrafish. *Environ. Pollut.* 234, 307–317. doi: 10.1016/j.envpol.2017.11.074

Chi, L., Xue, J., Tu, P., Lai, Y., Ru, H., and Lu, K. (2019). Gut microbiome disruption altered the biotransformation and liver toxicity of arsenic in mice. *Arch. Toxicol.* 93, 25–35. doi: 10.1007/s00204-018-2332-7

Cho, I., and Blaser, M. J. (2012). The human microbiome: At the interface of health and disease. *Nat. Rev. Genet.* 13, 260–270. doi: 10.1038/nrg3182

Gao, B., Bian, X., Mahbub, R., and Lu, K. (2017). Sex-specific effects of organophosphate diazinon on the gut microbiome and its metabolic functions. *Environ. Health Perspect.* 125, 198–206. doi: 10.1289/EHP202

Garfield, E. (2006). The history and meaning of the journal impact factor. *JAMA* 295, 90–93. doi: 10.1001/jama.295.1.90

Gomaa, E. Z. (2020). Human gut microbiota/microbiome in health and diseases: A review. *Antonie Van Leeuwenhoek* 113, 2019–2040. doi: 10.1007/s10482-020-01474-7

Gopalakrishnan, V., Spencer, C. N., Nezi, L., Reuben, A., Andrews, M. C., Karpinets, T. V., et al. (2018b). Gut microbiome modulates response to anti-pd-1 immunotherapy in melanoma patients. *Science* 359, 97–103. doi: 10.1126/science.aan4236

Gopalakrishnan, V., Helmink, B. A., Spencer, C. N., Reuben, A., and Wargo, J. A. (2018a). The influence of the gut microbiome on cancer, immunity, and cancer immunotherapy. *Cancer Cell* 33, 570–580. doi: 10.1016/j.ccell.2018.03.015

Helmink, B. A., Khan, M. A. W., Hermann, A., Gopalakrishnan, V., and Wargo, J. A. (2019). The microbiome, cancer, and cancer therapy. *Nat. Med.* 25, 377–388. doi: 10.1038/s41591-019-0377-7

Huang, J., Wen, B., Xu, L., Ma, H., Li, X., Gao, J., et al. (2022). Micro/nano-plastics cause neurobehavioral toxicity in discus fish (*Symphysodon aequifasciatus*): Insight from brain-gut-microbiota axis. *J. Hazard. Mater.* 421:126830. doi: 10.1016/j.jhazmat.2021.126830

Inamura, K. (2021). Gut microbiota contributes towards immunomodulation against cancer: New frontiers in precision cancer therapeutics. *Semin. Cancer Biol.* 70, 11–23. doi: 10.1016/j.semcancer.2020.06.006

Integrative HMP (iHMP) Research Network Consortium (2019). The integrative human microbiome project. *Nature* 569, 641–648. doi: 10.1038/s41586-019-1238-8

Ioannidis, J., Klavans, R., and Boyack, K. W. (2018). Thousands of scientists publish a paper every five days. *Nature* 561, 167–169. doi: 10.1038/d41586-018-06185-8

Jameson, K. G., and Hsiao, E. Y. (2019). A novel pathway for microbial metabolism of levodopa. *Nat. Med.* 25, 1195–1197. doi: 10.1038/s41591-019-0544-x

Jeong, H. G., Kang, M. J., Kim, H. G., Oh, D. G., Kim, J. S., Lee, S. K., et al. (2013). Role of intestinal microflora in xenobiotic-induced toxicity. *Mol. Nutr. Food Res.* 57, 84–99. doi: 10.1002/mnfr.201200461

Jin, Y., Lu, L., Tu, W., Luo, T., and Fu, Z. (2019). Impacts of polystyrene microplastic on the gut barrier, microbiota and metabolism of mice. *Sci. Total Environ.* 649, 308–317. doi: 10.1016/j.scitotenv.2018.08.353

Jin, Y., Xia, J., Pan, Z., Yang, J., Wang, W., and Fu, Z. (2018). Polystyrene microplastics induce microbiota dysbiosis and inflammation in the gut of adult zebrafish. *Environ. Pollut.* 235, 322–329. doi: 10.1016/j.envpol.2017.12.088

Li, M., Wang, B., Zhang, M., Rantalainen, M., Wang, S., Zhou, H., et al. (2008). Symbiotic gut microbes modulate human metabolic phenotypes. *Proc. Natl. Acad. Sci. U.S.A.* 105, 2117–2122. doi: 10.1073/pnas.0712038105

Lindon, J. C., Nicholson, J. K., and Holmes, E. (2018). *The handbook of metabolic phenotyping*. Amsterdam: Elsevier.

Lu, L., Luo, T., Zhao, Y., Cai, C., Fu, Z., and Jin, Y. (2019). Interaction between microplastics and microorganism as well as gut microbiota: A consideration on environmental animal and human health. *Sci. Total Environ.* 667, 94–100. doi: 10.1016/j.scitotenv.2019.02.380

Lu, Y., Yuan, X., Wang, M., He, Z., Li, H., Wang, J., et al. (2022). Gut microbiota influence immunotherapy responses: Mechanisms and therapeutic strategies. *J. Hematol. Oncol.* 15:47. doi: 10.1186/s13045-022-01273-9

Lupp, C., Skipper, M., and Weiss, U. (2012). Gut microbes and health. *Nature* 489:219. doi: 10.1038/489219a

Marchesi, J. R., Adams, D. H., Fava, F., Hermes, G. D., Hirschfield, G. M., Hold, G., et al. (2016). The gut microbiota and host health: A new clinical frontier. *Gut* 65, 330–339. doi: 10.1136/gutjnl-2015-309990

Miller, P. L., and Carson, T. L. (2020). Mechanisms and microbial influences on ctla-4 and pd-1-based immunotherapy in the treatment of cancer: A narrative review. *Gut Pathog.* 12:43. doi: 10.1186/s13099-020-00381-6

Moayyedi, P., Ford, A. C., Talley, N. J., Cremonini, F., Foxx-Orenstein, A. E., Brandt, L. J., et al. (2010). The efficacy of probiotics in the treatment of irritable bowel syndrome: A systematic review. *Gut* 59, 325–332. doi: 10.1136/gut.2008.167270

Mohr, A. E., Jäger, R., Carpenter, K. C., Kerksick, C. M., Purpura, M., Townsend, J. R., et al. (2020). The athletic gut microbiota. *J. Int. Soc. Sport Nutr.* 17:24. doi: 10.1186/s12970-020-00353-w

Mueller, K., Ash, C., Pennisi, E., and Smith, O. (2012). The gut microbiota. *Science* 336:1245. doi: 10.1126/science.336.6086.1245

Naidoo, J., Page, D. B., Li, B. T., Connell, L. C., Schindler, K., Lacouture, M. E., et al. (2015). Toxicities of the anti-pd-1 and anti-pd-l1 immune checkpoint antibodies. *Ann. Oncol.* 26, 2375–2391. doi: 10.1093/annonc/mdv383

Nicholson, J. K., Holmes, E., and Wilson, I. D. (2005). Gut microorganisms, mammalian metabolism and personalized health care. *Nat. Rev. Microbiol.* 3, 431–438. doi: 10.1038/nrmicro1152

O'Neill, J. D., Pinezich, M. R., Guenthart, B. A., and Vunjak-Novakovic, G. (2021). Gut bioengineering strategies for regenerative medicine. *Am. J. Physiol. Gastroint. Liver Physiol.* 320, G1–G11. doi: 10.1152/ajpgi.00206.2020

Perez-Chanona, E., and Jobin, C. (2014). From promotion to management: The wide impact of bacteria on cancer and its treatment. *Bioessays* 36, 658–664. doi: 10.1002/bies.201400015

Prata, J. C., Da Costa, J. P., Lopes, I., Duarte, A. C., and Rocha-Santos, T. (2020). Environmental exposure to microplastics: An overview on possible human health effects. *Sci. Total Environ.* 702:134455. doi: 10.1016/j.scitotenv.2019.134455

Qiao, R., Deng, Y., Zhang, S., Wolosker, M. B., Zhu, Q., Ren, H., et al. (2019). Accumulation of different shapes of microplastics initiates intestinal injury and gut microbiota dysbiosis in the gut of zebrafish. *Chemosphere* 236:124334. doi: 10.1016/j.chemosphere.2019.07.065

Rajagopala, S. V., Vashee, S., Oldfield, L. M., Suzuki, Y., Venter, J. C., Telenti, A., et al. (2017). The human microbiome and cancer. *Cancer Prev. Res.* 10, 226–234. doi: 10.1158/1940-6207.CAPR-16-0249

Rakoff-Nahoum, S., Paglino, J., Eslami-Varzaneh, F., Edberg, S., and Medzhitov, R. (2004). Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. *Cell* 118, 229–241. doi: 10.1016/j.cell.2004.07.002

Routy, B., Le Chatelier, E., Derosa, L., Duong, C., Alou, M. T., Daillère, R., et al. (2018). Gut microbiome influences efficacy of pd-1-based immunotherapy against epithelial tumors. *Science* 359, 91–97. doi: 10.1126/science.aan3706

Roy, S., and Trinchieri, G. (2017). Microbiota: A key orchestrator of cancer therapy. *Nat. Rev. Cancer* 17:271. doi: 10.1038/nrc.2017.13

Sekirov, I., Russell, S. L., Antunes, L. C., and Finlay, B. B. (2010). Gut microbiota in health and disease. *Physiol. Rev.* 90, 859–904. doi: 10.1152/physrev.00045.2009

Simrén, M., Barbara, G., Flint, H. J., Spiegel, B. M., Spiller, R. C., Vanner, S., et al. (2013). Intestinal microbiota in functional bowel disorders: A Rome foundation report. *Gut* 62, 159–176. doi: 10.1136/gutjnl-2012-302167

Sivan, A., Corrales, L., Hubert, N., Williams, J. B., Aquino-Michaels, K., Earley, Z. M., et al. (2015). Commensal bifidobacterium promotes antitumor immunity and facilitates anti-pd-l1 efficacy. *Science* 350, 1084–1089. doi: 10.1126/science.aac4255

Small, H. (1973). Co-citation in the scientific literature: A new measure of the relationship between two documents. *J. Am. Soc. Inf. Sci.* 24, 265–269.

Stappenbeck, T. S., Hooper, L. V., and Gordon, J. I. (2002). Developmental regulation of intestinal angiogenesis by indigenous microbes via paneth cells. *Proc. Natl. Acad. Sci. U.S.A.* 99, 15451–15455. doi: 10.1073/pnas.202604299

Sun, H. L., Bai, W., Li, X. H., Huang, H., Cui, X. L., Cheung, T., et al. (2022). Schizophrenia and inflammation research: A bibliometric analysis. *Front. Immunol.* 13:907851. doi: 10.3389/fimmu.2022.907851

Sweileh, W. M. (2019). A bibliometric analysis of health-related literature on natural disasters from 1900 to 2017. *Health Res. Policy Syst.* 17:18. doi: 10.1186/s12961-019-0418-1

Tsiaoussis, J., Antoniou, M. N., Koliarakis, I., Mesnage, R., Vardavas, C. I., Izotov, B. N., et al. (2019). Effects of single and combined toxic exposures on the gut microbiome: Current knowledge and future directions. *Toxicol. Lett.* 312, 72–97. doi: 10.1016/j.toxlet.2019.04.014

Van de Wiele, T., Vanhaecke, L., Boeckaert, C., Peru, K., Headley, J., Verstraete, W., et al. (2005). Human colon microbiota transform polycyclic aromatic hydrocarbons to estrogenic metabolites. *Environ. Health Perspect.* 113, 6–10. doi: 10.1289/ehp.7259

Van Eck, N. J., and Waltman, L. (2011). Text mining and visualization using vosviewer. *arXiv* [preprint]. arxiv:1109.2058

van Raan, A. (1996). Advanced bibliometric methods as quantitative core of peer review based evaluation and foresight exercises. *Scientometrics* 36, 397–420.

Wang, G., Pan, R., Liang, X., Wu, X., Wu, Y., Zhang, H., et al. (2021). Perfluorooctanoic acid-induced liver injury is potentially associated with gut microbiota dysbiosis. *Chemosphere* 266:129004. doi: 10.1016/j.chemosphere.2020.129004

Wang, Y. X., Arora, R., Choi, Y., Chung, H. W., Egorov, V. I., Frahm, J., et al. (2015). Implications of web of science journal impact factor for scientific output evaluation in 16 institutions and investigators' opinion. *Quant. Imaging Med. Surg.* 4, 453–461. doi: 10.3978/j.issn.2223-4292.2014.11.16

Watson, K. D., and Wexler, P. (2009). "Chapter 2–History of toxicology," in *Information resources in toxicology*, Fourth Edn, eds P. Wexler, S. G. Gilbert, P. J. Hakkinen, and A. Mohapatra (San Diego, CA: Academic Press), 11–29.

Wilson, I. D., and Nicholson, J. K. (2017). Gut microbiome interactions with drug metabolism, efficacy, and toxicity. *Transl. Res.* 179, 204–222. doi: 10.1016/j.trsl.2016.08.002

Wood, S. (2018). *Mgcv: Mixed gam computation vehicle with automatic smoothness estimation*.

Wright, S. L., and Kelly, F. J. (2017). Plastic and human health: A micro issue? *Environ. Sci. Technol.* 51, 6634–6647. doi: 10.1021/acs.est.7b00423

Wu, H., Zhou, Y., Wang, Y., Tong, L., Wang, F., Song, S., et al. (2021). Current state and future directions of intranasal delivery route for central nervous system disorders: A scientometric and visualization analysis. *Front. Pharmacol.* 12:717192. doi: 10.3389/fphar.2021.717192

Xie, Q., and Freeman, R. B. (2019). Bigger than you thought: China's contribution to scientific publications and its impact on the global economy. *China World Econ.* 27, 1–27. doi: 10.1111/cwe.12265

Xu, Z., Zhang, Y., Xu, Z., Zhong, Y., Wang, L., Liu, C., et al. (2023). Dysregulation of gut health in zebrafish by differentially charged nanoplastic exposure: An integrated analysis of histopathology, immunology, and microbial informatics. *Environ. Sci. Nano* 10, 933–947. doi: 10.1039/d2en00542e

Zhang, L., Zhang, Y., Jiang, X., Mao, L., Xia, Y., Fan, Y., et al. (2023). Disruption of the lung-gut-brain axis is responsible for cortex damage induced by pulmonary exposure to zinc oxide nanoparticles. *Toxicology* 485:153390. doi: 10.1016/j.tox.2022.153390

Zhang, Z., Li, J., Jiang, S., Xu, M., Ma, T., Sun, Z., et al. (2023). *Lactobacillus fermentum* hnu312 alleviated oxidative damage and behavioural abnormalities during brain development in early life induced by chronic lead exposure. *Ecotoxicol. Environ. Saf.* 251:114543. doi: 10.1016/j.ecoenv.2023.114543

Zhong, G., Wan, F., Lan, J., Jiang, X., Wu, S., Pan, J., et al. (2021). Arsenic exposure induces intestinal barrier damage and consequent activation of gut-liver axis leading to inflammation and pyroptosis of liver in ducks. *Sci. Total Environ.* 788:147780. doi: 10.1016/j.scitotenv.2021.147780

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Plateau pika fecal microbiota transplantation ameliorates inflammatory bowel disease manifestations in a mouse model of colitis

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Inflammatory bowel disease (IBD) is a serious global public health concern. Although the pathogenesis of the disease is currently unknown, it has been reported to be associated with both intestinal microbiota and inflammatory mediators. There is evidence suggesting that the feces of the Plateau pika is useful for treating gastrointestinal injuries and pain. Although fecal microbiota transplantation is highly efficacious intervention for IBD prevention, however, potential the transfer of pathogenic microbes or toxic substances is potentially hazardous. Fortunately, micropore filtering of the donor feces can minimize the risk of bacterial infection allowing retention of the therapeutic effects of the residual bacteriophages. Here, we demonstrated that Plateau pika feces not only alleviated the IBD symptoms but also promoted optimal structure and composition of the intestinal microbiota. Additionally, Plateau pika feces transfer also enhanced phenotypic features, such as, body-weight, disease activity index, and histological scores. In conclusion, Plateau pika feces was found to protect mice against colitis induced by dextran sodium sulfate by reducing inflammation and regulating microbial dysbiosis. These findings suggest the potential of Plateau pika feces as an alternative therapy for IBD.

KEYWORDS

inflammatory bowel disease, Plateau pika feces, fecal microbiota transplantation, gut microbiota, colitis murine model

Introduction

Inflammatory bowel disease (IBD) is a progressive immune-associated disease, characterized by persistent remission and relapse, that increases the risk of colorectal cancer (Cleynen et al., 2016). The incidence of the disease is currently increasing, and it is particularly common in recently industrialized countries (Gecse and Vermeire, 2018; Xiao et al., 2019). The risk factors of IBD include dysregulation of the mucosal immune response against bacteria-derived antigens, leading to the increased production of pro-inflammatory cytokines (Olén et al., 2020). Considering the crucial relevance of intestinal microbiota in keeping pathogens at bay and in maintaining

overall health, there is growing research interest in supporting and enhancing the healthy microbial ecosystem to achieve better physical health (Jess et al., 2012). Moreover, these interventions may also assist in relieving the clinical manifestations of ulcerative colitis (UC) and Crohn's disease (CD). The small lagomorph the Plateau pika (*Ochotona curzoniae*) from the alpine meadows of the Tibetan Plateau is a keystone species as it is food for predators, and provides shelter for small nesting tiny birds in its burrows (Olén et al., 2020).

Despite its overall protective actions against harmful circumstances such as pathogen infection, inflammation often involves pathways that aggravate pathogenic processes, thereby increasing the vulnerability of the host to further attacks (Ng et al., 2017). Many cytokines, such as, TNF-α, IL-6, and IL-1 are targets of the NF-κB pathway (Nadeem et al., 2020). While inhibitor proteins (IBs) typically restrict the activity of NF-κB in the cytoplasm, after translocation of the protein to the nucleus NF-κB promotes a series of complex phosphorylation and degradation events leading to the expression of downstream target genes and subsequent pro-inflammatory signaling (Kuipers et al., 2015). The NF-κB axis is reported to be closely associated with the pathogenesis of both UC and CD in humans and animals (Odenwald and Turner, 2017; Schirmer et al., 2019).

The interaction between intestinal microbiota and pro-inflammatory factors in UC is complex (Xavier and Podolsky, 2007). Prior investigations have revealed that the alleviation of UC symptoms occurs via an intricate balance between beneficial bacteria, host genetic factors, and common environmental stimuli. The recently introduced technique of fecal microbiota transplantation (FMT) has been shown to effectively enhance the structural regulation of the intestinal microbiota, thus relieving IBD and persistent gastrointestinal dysbiosis (Roda et al., 2016; Na and Moon, 2019). Nevertheless, the precise mechanism where FMT treatment relieves UC symptoms remains undetermined, and the majority of published information to date has been from case reports and allied research.

Previous studies have shown a reduced risk of UC when Plateau pika feces, host genetic factors, and common environmental stresses interact in a balanced manner (Paramsothy et al., 2019). Here, we explored the role of Plateau pika FMT in relieving IBD in a mouse model of colitis.

Materials and methods

Animals

All protocols involving the C57BL/6 J mice followed the Care and Use of Laboratory Animals (Gansu Province Animal Care Committee, Lanzhou, China) guidelines, and the study received ethical approval from the Lanzhou Institute of Husbandry and Pharmaceutical Sciences, CAAS. The mice were housed in an environment with constant temperature (20–24°C) and humidity and a 12-h / 12-h light/dark cycle. Mice received food and water *ad libitum*.

The animal protocol is presented in Figure 1. Briefly, mice($n = 10$/group) were randomly assigned to one of three groups: (i) control; (ii) dextran sodium sulfate (DSS); (iii) FMT. For the first 7 days, all mice, apart from the controls received drinking water containing 3% DSS solution thus establishing the UC model. Subsequently, we collected, accumulated, gently homogenized, and froze the cecal contents of 10 Plateau pikas in 10% sterile glycerol. To generate a working FMT solution, the frozen fecal material was thawed and diluted to 0.05 g/ml with sterile saline, followed

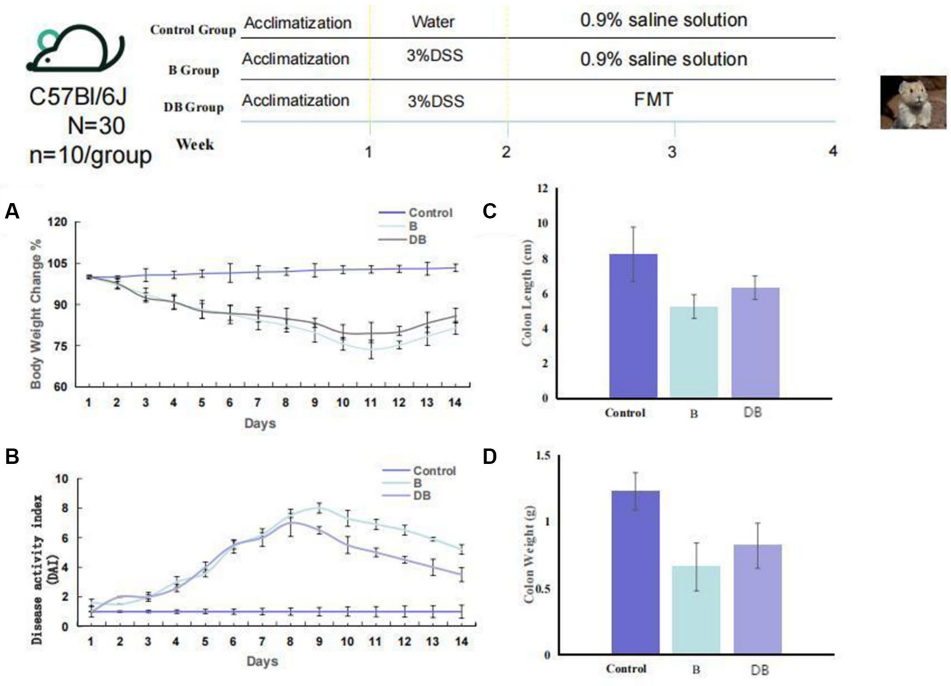

FIGURE 1
FMT reduced colonic damage of the colitis mouse model. Experimental design for FMT treatment on DSS-induced colitis in mice. **(A)** Weight loss ($n = 10$). **(B)** DAI ($n = 6$). **(C)** Colon length, and **(D)** Colon weight. Statistics were calculated with a two-tailed Student's t test. *, $p < 0.01$; **, p, 0.01 **(C,D)**. Data are presented as the mean 6 SEM.

by homogenization, centrifugation (5,000 g 30 min, 4°C), and subsequent filtration of the supernatant using a 0.45 μm PES filter (Minisart® High Flow Syringe Filter, Sartorius™, Germany; Slack et al., 2022). The mice received FMT via a gavage of 200 μl of the supernatant from the Plateau pika fecal samples once a day over 2 weeks. Meanwhile, the control and DSS mice received a gavage of 200 μl of 0.9% saline solution. To establish the colitis mouse model, we supplemented sterile water with 3% DSS for 7 days, with 7 additional days of water without DSS. the pika feces supernatant was administered via gavage to the FMT mice, while control mice were given 0.9% saline solution. Daily parameters, including body-weight, changes in the consistency of the feces, and the presence of blood in the feces were monitored. To analyze the intestinal microbiota using 16S rRNA gene sequencing, feces were collected from the mice on day 14, and colonic tissue was harvested from the mice following euthanasia (Figure 1). DAI was scored as earlier described (Supplementary Table S1; Murthy et al., 1993; Camuesco et al., 2004).

After completion of the treatment, all animals were first anesthetized with sodium pentobarbital (5%), and then sacrificed by cervical dislocation immediately before dissection. Their colons opened, and the feces were gently moved using forceps. The distal colon (about 30 mm) together with additional intestinal regions were used for histological analysis using hematoxylin–eosin (HE) staining, respectively. We also collected colon samples for proinflammatory cytokine evaluation.

Histopathological analysis and HE staining

Mouse colons were fixed in 10% buffered formalin, before paraffin embedding, sectioning into 5 μm sections, HE staining, and examination under light microscopy.

Assessment of colonic oxidative stress (OS) parameter

Colonic parameters, namely, malondialdehyde (MDA) levels, total antioxidant capacity (T-AOC), catalase (CAT), and superoxide dismutase (SOD) activities, were measured using the appropriate kits (Nanjing Jiancheng Bioengineering Institute, China).

16S rRNA gene amplicon sequencing

We next determined the composition of the intestinal microbial communities in the distal ileal mucosa and intestinal lumen using 16S rRNA gene (V3-region) amplicon sequencing using NextSeq with v2 MID, 300-cycle, paired-end chemistry on a NextSeq (Illumina United States) platform. Total DNA was isolated using a Bead-Beat® Micro AX Gravity Kit (A&A Biotechnology, Poland), according to the provided directions. A library was generated, as reported previously (Slack et al., 2022). The mean amplicon sequencing depth per sample was 35,840 reads (minimum 9,286; maximum 63,584 reads).

Statistical analysis

Data were analyzed using GraphPad Prism and SPSS. $p < 0.05$ was set as the significance threshold. Data are presented as mean ± standard deviation (SD) and compared using one-way ANOVA and Duncan's test.

Results

FMT-mediated regulation of mouse colitis development and clinicopathological manifestations

Among other consequences, DSS-triggered colitis causes the formation of ulcers in the intestinal epithelium, resulting in disruption of the inner mucosal layer, and promoting the invasion of acute and inflammatory immune cells (Das et al., 2020; Bell et al., 2022). Typical biomarkers of IBD are body weight, disease activity index (DAI) scoring, and the length/colon weight ratio (Ni et al., 2017; Chen et al., 2021). These variables were, therefore, used in the present study to evaluate the FMT-mediated regulation of colitis development and progression *in vivo*. It was found that all three variables were significantly reduced in DSS-treated mice ($p < 0.01$) compared with the controls (Figure 1). Significant weight gain was observed after FMT administration, which was equivalent to the weight of the control mice ($p < 0.05$). FMT-treated mice also showed substantially reduced DAI scores. Together, these results are consistent with previous findings (Weingarden and Vaughn, 2017), and indicated that treatment with FMT could successfully prevent DSS-driven IDB.

Plateau pika feces mitigated the pathological manifestations of DSS-induced experimental UC

We developed a DSS-induced mouse model of UC using 14-week-old C57BL/6 J mice to elucidate the influences of Plateau pika microbiota transplantation on UC. Relative to the control mice, the mice receiving Plateau pika feces microbiota transplantation showed significant improvements in their colons (Figure 1). The changes in body weight of the FMT-treated mice were also moderate. It was also found that the FMT treatment strongly prevented weight loss, relative to the control mice ($p = 0.038$; Figure 1A). Additionally, the DAI scores of the FMT-treated mice were substantially reduced, in comparison with the controls ($p = 0.027$; Figure 1B).

FMT-mediated regulation of histopathological changes in the colon

Representative images of the colon histology from each group of mice are shown in Figure 2. As expected, control mice showed normal colon histomorphology (Figure 2). In contrast, the DSS-treated mice showed thickening of the intestinal walls, submucosal edema, mucosal invasion, cytoplasmic mucin depletion, and ulceration of the colon (Figure 2). These characteristic symptoms of UC were significantly reduced after FMT administration (Figure 3). These results are consistent with those of Newman et al. (2017).

FMT-mediated regulation of oxidative status in colonic tissue

The present as well as prior UC studies demonstrated a link between intestinal OS and disease (Figure 4). Reactive oxygen species (ROS) are typically produced when proinflammatory factors activate phagocytes

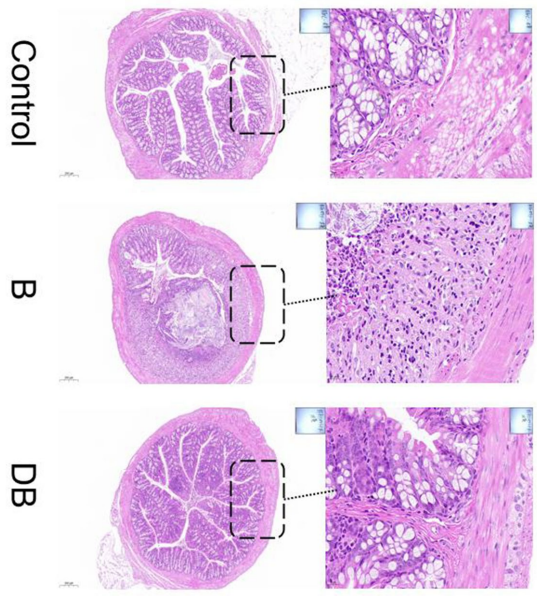

FIGURE 2
Effects of FMT intervention on the mouse colon histological changes (Representative hematoxylin and eosin staining) (Control) Control group, (B) B group, (DB) DB group.

(Sun et al., 2017). Hence, any intervention that enhances the cellular antioxidant capacity can potentially prevent the development and severity of UC (Paramsothy et al., 2017; Mishra et al., 2022). Markedly reduced levels of CAT, SOD, and T-AOC together with significantly raised MDA levels were observed in DSS mice relative to the controls. CAT requires the splitting of the superoxide anion to catalyze O_2 reduction to H_2O_2, as well as H_2O_2 conversion to water (Ortiz-Rivera et al., 2017), while T-AOC modulates body function and the susceptibility to certain diseases (Özçam et al., 2019). The MDA content is a well-established indicator of OS (Özçam et al., 2022). This evidence suggests that the colon undergoes OS during UC. Here, it was found that after FMT treatment, the CAT, SOD, and T-AOC levels rose significantly ($p < 0.05$) while those of MDA declined sharply ($p < 0.01$), thus indicating that FMT treatment could successfully reduce OS.

Plateau pika feces modulated the composition of the intestinal microbiota in a DSS-induced mouse model of UC

Colitis is closely linked to modifications in the composition and organization of the intestinal microbiota (Sartor and Wu, 2017). We used 16S rRNA sequencing of fecal samples collected after 2 weeks of DSS treatment to explore the significance of Plateau pika feces for regulating intestinal microbiota in a DSS-driven mouse model of colitis. The rarefaction analysis showed that the bacterial composition was quite diverse (Zhu et al., 2018). The intestinal microbiota of all

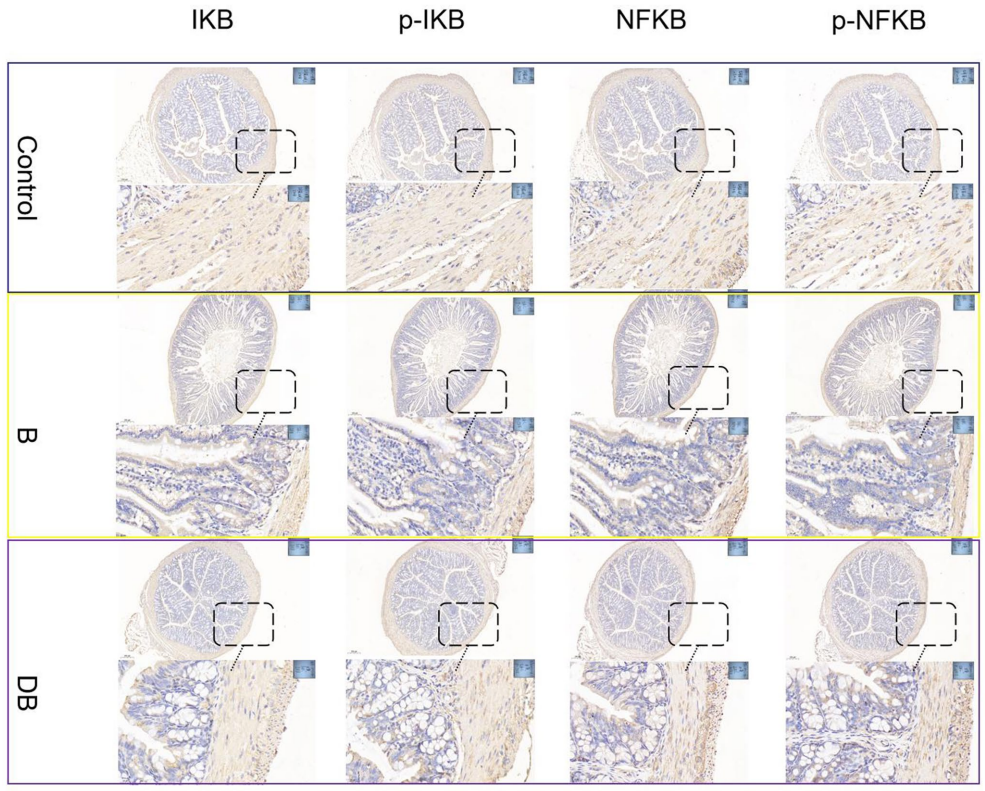

FIGURE 3
FMT modulated DSS-induced NF-κB activation in the colonic tissue. NF-κB protein levels in the colonic tissue were analyzed by immunohistochemical.

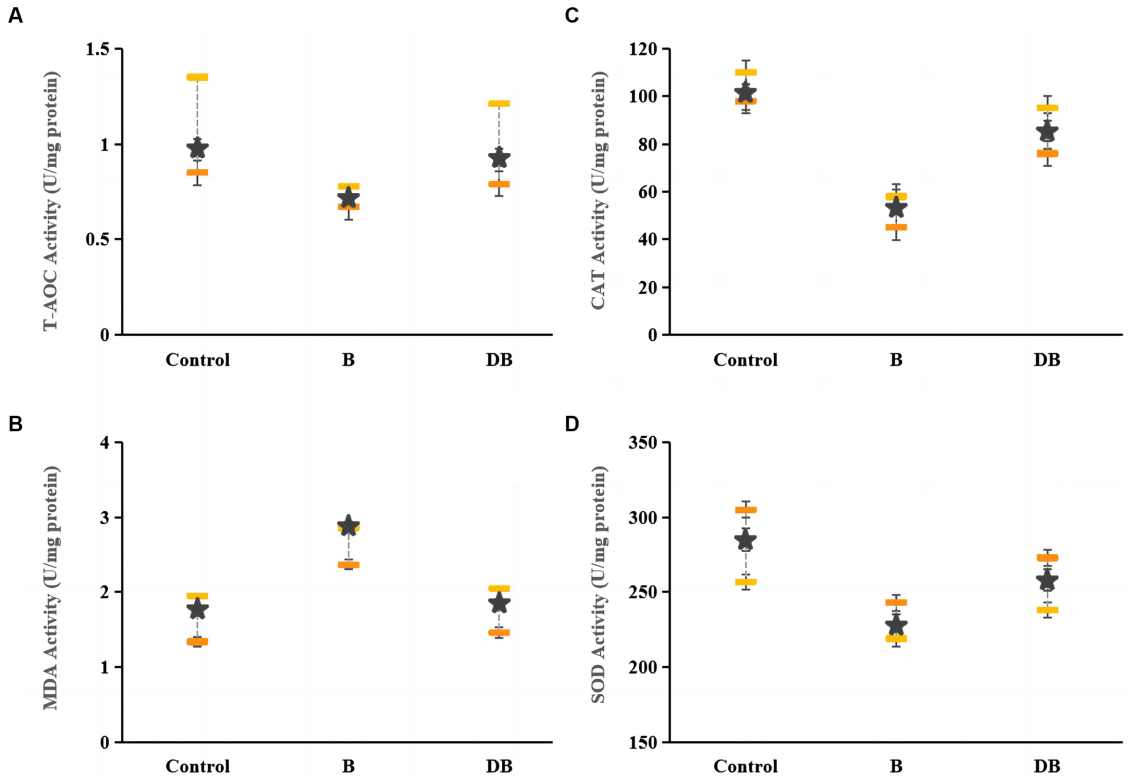

FIGURE 4
Effects of FMT intervention on colonic oxidative stress parameters in DSS-induced colitis mouse. **(A)** T-AOC, **(B)** MDA, **(C)** CAT, and **(D)** SOD. Data are presented as mean ± SD. $^{\#\#}p < 0.01$ and $^{\#}p < 0.05$ vs. the control group. $^{**}p < 0.01$ and $^{*}p < 0.05$ vs. the B model group.

three groups differed in their species compositions (Figure 5). The Simpson index was used to evaluate the alpha diversity at the species level in samples from all three groups collected on day 14. This showed that the Simpson index of FMT-treated mice differed markedly from that of the control mice (Figure 5). Treatment with Plateau pika feces increased the composition and variety of the microbiota throughout the chronic inflammatory phase, which, in turn, accelerated healing. Measurement of beta diversity using principal coordinate analysis (P-CoA) with the Bray Curtis distance metric revealed two distinct clusters for the FMT and control mice (Figure 5), further indicative of significantly altered microbial compositions, even in species diversity, in the FMT-treated mice by day 14. Our overall findings revealed that FMT treatment strongly regulated the intestinal microbiota throughout the chronic and recuperation stages of intestinal inflammation. Moreover, FMT-treated mice exhibited unusual microbial compositions throughout recovery from intestinal inflammation, namely, the presence of *Dubosiella* and *Lactobacillus*, while *Ileibacterium*, unclassified_*f_Lachnospiraceae*, *Bifidobacterium*, *Turicibacter*, *Lachnospiraceae_NK4A136_group*, and *Candidatus_Saccharimonas* were more prevalent on day 14.

Discussion

The aim of this investigation was to determine the overall effects of Plateau pika feces on recovery from intestinal inflammation in a DSS-induced mouse model of colitis. The relief of UC symptoms may have resulted from the restoration of the intestinal structure, function, and micro-environment. The findings of this study provide important information on alternative treatment methods for colonic inflammation employing functional probiotics and the use of Plateau pika feces in IBD.

We demonstrated that Plateau pika feces alleviated the symptoms of DSS-triggered colitis, shown in reduced colon length, weight-loss, DAI scores, mucosal loss, and the invasion of inflammatory cells, as documented in previous publications (Park et al., 2018; Wang et al., 2020, 2021; Jeong et al., 2022).

Importantly, the Plateau pika feces cohort outperformed single-strain mice in terms of minimizing body-weight fluctuations, DAI, and colonic histological scores (Kühl et al., 2015; Gasaly et al., 2021). This may be because, after entry into the gut, bacterial strains may directly diminish inflammation by modulating the total microbial network, apart from their interaction with mucosal epithelial cells (Liang et al., 2021). We also confirmed that on day 8 of the development of colonic inflammation and on day 14 of healing in colitis mice, Plateau pika feces effectively rescued micro-ecological dysregulation. Based upon reports, altered microbiota may prevent colitis progression. The alpha and beta diversities revealed that the structural composition of the FMT mice intestinal microbiota differed significantly from that of the controls (Sun et al., 2020; Wang et al., 2021). The single strain-based intestinal microbiota recovery mostly occurred on day 14, which made it less efficacious than the Plateau pika feces. Metabolites that enter hosts are quickly absorbed into the intestine. They also reach the small

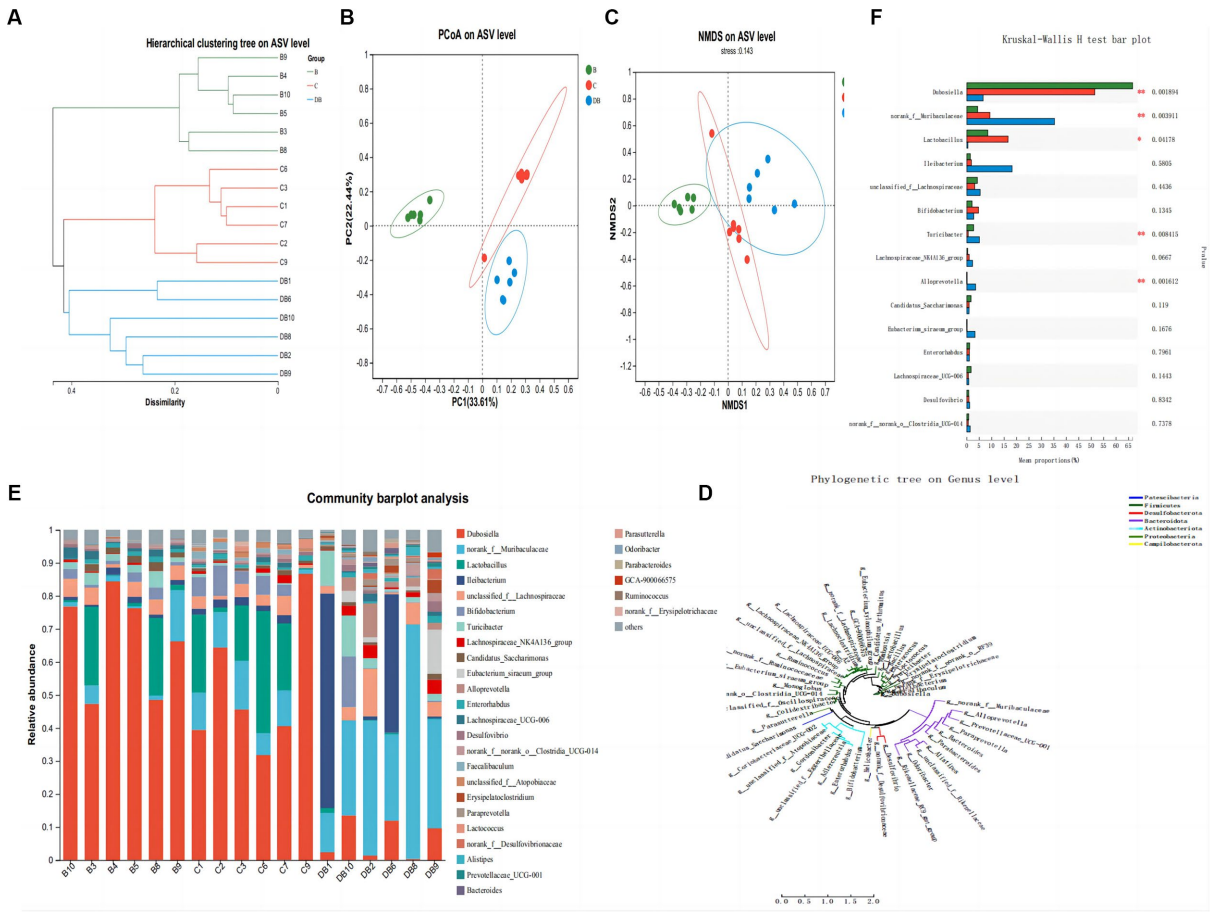

FIGURE 5
Influences of FMT administration on gut microbiota β-diversity. **(A)** Hierarchical clustering tree of weighted UniFrac distance, and **(B)** Principal coordinates analysis (PCoA) of ASV level. **(C)** NMDS of ASV level. **(D)** Phylogenetic tree on genus level. **(E)** Community barplot analysis. **(F)** Kruskal-Wallis H test bar plot. Data are presented as mean ± SD. $^{\#\#}p < 0.01$ and $^{\#}p < 0.05$ vs. the control group. $^{**}p < 0.01$ and $^{*}p < 0.05$ vs. the DSS model group.

intestinal epithelial cells, whereby their utilization is further augmented.

The intestinal microbiota is modulated directly by metabolites as they include bacteriocins with significant antibacterial activity (Salminen et al., 2021). The logical conclusion is that following absorption into the gastrointestinal tract, metabolites may potentially exhibit indirect anti-inflammatory activities through additional regulation of microbial communities.

Considering such significant alterations of the microbiota in FMT-treated colitis, we consider that treatments with Plateau pika feces have great potential in treating UC. Consequently, RFCV discovered 11 unique bacterial taxa. Given that only three taxa, namely, *Lactobacillaceae Lactobacillus*, *Dubosiella Firmicutes*, and *Rikenellaceae Rikenellaceae* were strongly influenced by the single strains suggests that single strains are less beneficial for enhancing the intestinal microbiota. The Plateau pika feces are particularly controlled by the other eight taxa. it was found that six taxa, namely, *Muribaculaceae Muribaculaceae*, *Bacteroidaceae Bacteroides*, *Oscillospirales* UCG.005, *Lachnospiraceae Ruminococcus*, *Tissierellales Anaerovoracaceae*, and *Clostridia Clostridia*, were abundant in the FMT-treated group, relative to controls. *Muribaculaceae* is known for its effect in lowering inflammation, inhibiting dangerous microorganisms, and fostering anticancer immunity (Setoyama et al., 2003). Bacteroides are potent candidates for next-generation probiotics due to their unique ability to generate short-chain fatty acids and sphingolipids that support intestinal barrier integrity, as well as modulation of the immune response, which, in turn, repairs intestinal damage (Brown et al., 2019; Wang et al., 2022). The other four species produce butyrate, with *Oscillospirales* UCG.005 and *L. ruminococcus* showing a relatively low abundance in patients with UC and CRC patients (Mancabelli et al., 2017). The remaining two Clostridia taxa enhance the proliferation and differentiation of regulatory T cells to alleviate IBD and allergic diarrhea (Atarashi et al., 2013). Conversely, here, DSS treatment strongly increased the relative abundances of *Parasutterella Burkholderiales*, and *Bacteroidales Tannerellaceae* in mice, consistent with the conclusions of prior publications (Silvestri et al., 2020).

IBD patients have increased numbers of *P. burkholderiales*, which is correlated with persistent colonic inflammation (Chen et al., 2018; Zhao et al., 2021). Importantly, there are no significant variations in the relative abundances of the eight taxa in the metabolite intervention experiment between the mix and control groups, indicating that mixed metabolites cannot fully control the colonic microbiota. As a result, we hypothesized that metabolite delivery would influence microbial ecology in the small intestine. The relative abundance of

Acidiferrobacterales Acidiferrobacteraceae, *Sphingomonadaceae Sphingosinicella*, and *Reyranellaceae Reyranella* was markedly reduced in the controls, confirming the supposition that metabolites act by balancing the small intestine microenvironment.

Despite widespread acknowledgement that IBD is correlated with alterations in the composition and metabolism of the intestinal microbiota, there have been no reports of a causal association between dysbiosis and IBD in humans (Ni et al., 2017). There is very limited research on the association between intestinal microbiota and the IBD phenotype. Here, our RDA provided strong evidence for an association between microbial and colitis phenotypic data in the probiotic FMT group, whereby core species-level microbial data provided essential phenotypic data, such as disease severity and colonic pathology. Co-occurrence networks to established to investigate the relationship between the phenotypic data and the microbiological markers. In FMT-treated mice, we clearly identified the presence of *B. bacteroides*, *M. muribaculaceae*, *B. tannerellaceae*, and *P. burkholderiales*, as well as a stronger and broader interaction network on day 8. Since many microbial markers in the metabolite intervention experiments were stand-alone taxonomic units and not symbiotic communities, they obviously did not affect phenotypic parameters. In all the interventions, *M. muribaculaceae* was found to be pivotal in the co-occurrence networks, indicating its importance in the interplay between the general microbiota and disease phenotypes. Collectively, our findings and those of other published work demonstrated that Plateau pika feces might effectively reduce intestinal inflammation and restore function in a colitis animal model.

Conclusion

Here, we demonstrated that Plateau pika FMT relieved the intestinal inflammatory symptoms associated with colitis by altering the structure and composition of the intestinal microbiota. Our findings demonstrated a strong link between the intestinal microbiota and the colitis phenotype. These findings in an animal model highlight the potential use of Plateau pika FMT in the maintenance of intestinal health and may contribute toward the development of clinically applicable Plateau pika FMT-based interventions for IBD and other intestinal inflammatory diseases.

Limitations of our research

The current investigation has several limitations. Firstly, we did not employ metagenomics for the evaluation of bacterial community composition. This will be done in future investigations. Secondly, to identify suitable potential therapeutic targets, the associated intestinal microbiota- and inflammation-related pathways require further examination. Lastly, additional metabolomics research is warranted to clarify the methods of the purification and identification of vital components that operate via the mixed-metabolite intervention.

Importance

IBD is a chronic, nonspecific inflammatory disease. It is characterized by ulceration and erosion of the colonic mucosa and can also cause a wide variety of other lesions that frequently affect the entire colon. In this study, we investigated the efficacy of Plateau pika feces administration in reducing gut inflammation and the restoration of gut microecology restoration. The results showed that Plateau pika feces significantly reduced inflammation and accelerated recovery when compared with the control group. These findings support the use of Plateau pika feces as an alternative therapeutic approach for IBD.

Data availability statement

The data presented in the study are deposited in the NIH repository, accession number SUB13763520.

Ethics statement

The animal study was approved by Lanzhou Institute of Husbandry and Pharmaceutical Sciences, CAAS. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

XD, PD, and YW designed the experiment and acquired grants. YY, BC, WS, and MF performed the experiments. YY, YL, and XL collected and analyzed the data. YY drafted and edited the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1228778/full#supplementary-material

References

Atarashi, K., Tanoue, T., Oshima, K., Suda, W., Nagano, Y., Nishikawa, H., et al. (2013). Treg induction by a rationally selected mixture of Clostridia strains from the human microbiota. *Nature* 500, 232–236. doi: 10.1038/nature12331

Bell, H. N., Rebernick, R. J., Goyert, J., Singhal, R., Kuljanin, M., Kerk, S. A., et al. (2022). Reuterin in the healthy gut microbiome suppresses colorectal cancer growth through altering redox balance. *Cancer Cell* 40, 185–200.e6. doi: 10.1016/j.ccell.2021.12.001

Brown, E. M., Ke, X., Hitchcock, D., Jeanfavre, S., Avila-Pacheco, J., Nakata, T., et al. (2019). Bacteroides-derived sphingolipids are critical for maintaining intestinal homeostasis and symbiosis. *Cell Host Microbe* 25, 668–680.e7. doi: 10.1016/j.chom.2019.04.002

Camuesco, D., Comalada, M., Rodríguez-Cabezas, M. E., Nieto, A., Lorente, M. D., Concha, A., et al. (2004). The intestinal anti-inflammatory effect of quercitrin is associated with an inhibition in iNOS expression. *Br. J. Pharmacol.* 143, 908–918. doi: 10.1038/sj.bjp.0705941

Chen, Y., Cui, W., Li, X., and Yang, H. (2021). Interaction between commensal bacteria, immune response and the intestinal barrier in inflflammatory bowel disease. *Front. Immunol.* 12:761981. doi: 10.3389/fifimmu.2021.761981

Chen, Y.-J., Wu, H., Wu, S.-D., Lu, N., Wang, Y.-T., Liu, H.-N., et al. (2018). Parasutterella, in association with irritable bowel syndrome and intestinal chronic inflflammation. *J. Gastroenterol. Hepatol.* 33, 1844–1852. doi: 10.1111/jgh.14281

Cleynen, I., Boucher, G., Jostins, L., Schumm, L. P., Zeissig, S., Ahmad, T., et al. (2016). Inherited determinants of Crohn's disease and ulcerative colitis phenotypes: a genetic association study. *Lancet* 387, 156–167. doi: 10.1016/S0140-6736(15)00465-1

Das, N. K., Schwartz, A. J., Barthel, G., Inohara, N., Liu, Q., Sankar, A., et al. (2020). Microbial metabolite signaling is required for systemic iron homeostasis. *Cell Metab.* 31, 115–130.e6. doi: 10.1016/j.cmet.2019.10.005

Gasaly, N., de Vos, P., and Hermoso, M. A. (2021). Impact of bacterial metabolites on gut barrier function and host immunity: a focus on bacterial metabolism and its relevance for intestinal inflflammation. *Front. Immunol.* 12:658354. doi: 10.3389/fifimmu.2021.658354

Gecse, K. B., and Vermeire, S. (2018). Differential diagnosis of inflflammatory bowel disease: imitations and complications. *Lancet Gastroenterol. Hepatol.* 3, 644–653. doi: 10.1016/S2468-1253(18)30159-6

Jeong, Y., Kim, D. H., and Lee, K. (2022). Homeostasis effects of fermented Maillard reaction products by *Lactobacillus gasseri* 4M13 in dextran sulfate sodium-induced colitis mice. *J. Sci. Food Agric.* 102, 434–444. doi: 10.1002/jsfa.11374

Jess, T., Rungoe, C., and Peyrin–Biroulet, L. (2012). Risk of colorectal cancer in patients with ulcerative colitis: a meta-analysis of population-based cohort studies. *Clin. Gastroenterol. Hepatol.* 10, 639–645. doi: 10.1016/j.cgh.2012.01.010

Kühl, A. A., Erben, U., Kredel, L. I., and Siegmund, B. (2015). Diversity of intestinal macrophages in inflflammatory bowel diseases. *Front. Immunol.* 6:613. doi: 10.3389/fifimmu.2015.00613

Kuipers, E. J., Grady, W. M., Lieberman, D., Seufferlein, T., Sung, J. J., Boelens, P. G., et al. (2015). Colorectal cancer. *Nat. Rev. Dis. Primers.* 1:15065. doi: 10.1038/nrdp.2015.65

Liang, Y., Liu, M., Pu, J., Zhu, Z., Gao, Z., Zhou, Q., et al. (2021). Probiotics and their metabolites ameliorate inflflammatory bowel disease: a critical review. *Infect. Microbes. Dis.* 3, 4–13. doi: 10.1097/IM9.0000000000000046

Mancabelli, L., Milani, C., Lugli, G. A., Turroni, F., Cocconi, D., van Sinderen, D., et al. (2017). Identifification of universal gut microbial biomarkers of common human intestinal diseases by meta-analysis. *FEMS Microbiol. Ecol.* 93:fifix153. doi: 10.1093/femsec/fifix153

Mishra, J., Stubbs, M., Kuang, L., Vara, N., Kumar, P., and Kumar, N. (2022). Inflflammatory bowel disease therapeutics: a focus on probiotic engineering. *Med. Inf.* 2022:9621668. doi: 10.1155/2022/9621668

Murthy, S. N., Cooper, H. S., Shim, H., Shah, R. S., Ibrahim, S. A., and Sedergran, D. J. (1993). Treatment of dextran sulfate sodium-induced murine colitis by intracolonic cyclosporin. *Dig. Dis. Sci.* 38, 1722–1734. doi: 10.1007/BF01303184

Na, S.-Y., and Moon, W. (2019). Perspectives on current and novel treatments for inflflammatory bowel disease. *Gut Liver* 13, 604–616. doi: 10.5009/gnl19019

Nadeem, M. S., Kumar, V., Al-Abbasi, F. A., Kamal, M. A., and Anwar, F. (2020). Risk of colorectal cancer in inflflammatory bowel diseases. *Semin. Cancer Biol.* 64, 51–60. doi: 10.1016/j.semcancer.2019.05.001

Newman, K. M., Rank, K. M., Vaughn, B. P., and Khoruts, A. (2017). Treatment of recurrent *Clostridium difficile* infection using fecal microbiota transplantation in patients with inflammatory bowel disease. *Gut Microbes* 8, 303–309. doi: 10.1080/19490976.2017.1279377

Ng, S. C., Shi, H. Y., Hamidi, N., Underwood, F. E., Tang, W., Benchimol, E. I., et al. (2017). Worldwide incidence and prevalence of inflflammatory bowel disease in the 21st century: a systematic review of population-based studies. *Lancet* 390, 2769–2778. doi: 10.1016/S0140-6736(17)32448-0

Ni, J., Wu, G. D., Albenberg, L., and Tomov, V. T. (2017). Gut microbiota and IBD: causation or correlation? *Nat. Rev. Gastroenterol. Hepatol.* 14, 573–584. doi: 10.1038/nrgastro.2017.88

Odenwald, M. A., and Turner, J. R. (2017). The intestinal epithelial barrier: a therapeutic target? *Nat. Rev. Gastroenterol. Hepatol.* 14, 9–21. doi: 10.1038/nrgastro.2016.169

Olén, O., Erichsen, R., Sachs, M. C., Pedersen, L., Halfvarson, J., Askling, J., et al. (2020). Colorectal cancer in ulcerative colitis: a Scandinavian population-based cohort study. *Lancet* 395, 123–131. doi: 10.1016/S0140-6736(19)32545-0

Ortiz-Rivera, Y., Sánchez-Vega, R., Gutiérrez-Méndez, N., León-Félix, J., AcostaMuñiz, C., and Sepulveda, D. R. (2017). Production of reuterin in a fermented milk product by *Lactobacillus reuteri*: inhibition of pathogens, spoilage microorganisms, and lactic acid bacteria. *J. Dairy Sci.* 100, 4258–4268. doi: 10.3168/jds.2016-11534

Özçam, M., Oh, J.-H., Tocmo, R., Acharya, D., Zhang, S., Astmann, T. J., et al. (2022). A secondary metabolite drives intraspecies antagonism in a gut symbiont that is inhibited by cell-wall acetylation. *Cell Host Microbe* 30, 824–835.e6. doi: 10.1016/j.chom.2022.03.033

Özçam, M., Tocmo, R., Oh, J.-H., Afrazi, A., Mezrich, J. D., Roos, S., et al. (2019). Gut symbionts *Lactobacillus reuteri* R2lc and 2010 encode a polyketide synthase cluster that activates the mammalian arylhydrocarbon receptor. *Appl. Environ. Microbiol.* 85, e01661–e01618. doi: 10.1128/AEM.01661-18

Paramsothy, S., Kamm, M. A., Kaakoush, N. O., Walsh, A. J., van den Bogaerde, J., Samuel, D., et al. (2017). Multidonor intensive faecal microbiota transplantation for active ulcerative colitis: a randomised placebo-controlled trial. *Lancet* 389, 1218–1228. doi: 10.1016/S0140-6736(17)30182-4

Paramsothy, S., Nielsen, S., Kamm, M. A., Deshpande, N. P., Faith, J. J., Clemente, J. C., et al. (2019). Specific Bacteria and metabolites associated with response to fecal microbiota transplantation in patients with ulcerative colitis. *Gastroenterology* 156, 1440–1454.e2. doi: 10.1053/j.gastro.2018.12.001

Park, J.-S., Choi, J. W., Jhun, J., Kwon, J. Y., Lee, B.-I., Yang, C. W., et al. (2018). *Lactobacillus acidophilus* improves intestinal inflflammation in an acute colitis mouse model by regulation of Th17 and Treg cell balance and fifibrosis development. *J. Med. Food* 21, 215–224. doi: 10.1089/jmf.2017.3990

Roda, G., Jharap, B., Neeraj, N., and Colombel, J.-F. (2016). Loss of response to antiTNFs: defifinition, epidemiology, and management. *Clin. Transl. Gastroenterol.* 7:e135. doi: 10.1038/ctg.2015.63

Salminen, S., Collado, M. C., Endo, A., Hill, C., Lebeer, S., Quigley, E. M. M., et al. (2021). The international Scientifific Association of Probiotics and Prebiotics (ISAPP) consensus statement on the defifinition and scope of postbiotics. *Nat. Rev. Gastroenterol. Hepatol.* 18, 649–667. doi: 10.1038/s41575-021-00440-6

Sartor, R. B., and Wu, G. D. (2017). Roles for intestinal bacteria, viruses, and fungi in pathogenesis of inflflammatory bowel diseases and therapeutic approaches. *Gastroenterology* 152, 327–339.e4. doi: 10.1053/j.gastro.2016.10.012

Schirmer, M., Garner, A., Vlamakis, H., and Xavier, R. J. (2019). Microbial genes and pathways in inflflammatory bowel disease. *Nat. Rev. Microbiol.* 17, 497–511. doi: 10.1038/s41579-019-0213-6

Setoyama, H., Imaoka, A., Ishikawa, H., and Umesaki, Y. (2003). Prevention of gut inflflammation by Bififidobacterium in dextran sulfate-treated gnotobiotic mice associated with Bacteroides strains isolated from ulcerative colitis patients. *Microbes Infect.* 5, 115–122. doi: 10.1016/S1286-4579(02)00080-1

Silvestri, C., Pagano, E., Lacroix, S., Venneri, T., Cristiano, C., Calignano, A., et al. (2020). Fish oil, cannabidiol and the gut microbiota: an investigation in a murine model of colitis. *Front. Pharmacol.* 11:585096. doi: 10.3389/fphar.2020.585096

Slack, R. J., Macdonald, S. J. F., Roper, J. A., Jenkins, R. G., and Hatley, R. J. D. (2022). Emerging therapeutic opportunities for integrin inhibitors. *Nat. Rev. Drug Discov.* 21, 60–78. doi: 10.1038/s41573-021-00284-4

Sun, M., Liu, Y., Song, Y., Gao, Y., Zhao, F., Luo, Y., et al. (2020). The ameliorative effect of *Lactobacillus plantarum*-12 on DSS-induced murine colitis. *Food Funct.* 11, 5205–5222. doi: 10.1039/d0fo00007h

Sun, M., Wu, W., Liu, Z., and Cong, Y. (2017). Microbiota metabolite short chain fatty acids, GPCR, and inflflammatory bowel diseases. *J. Gastroenterol.* 52, 1–8. doi: 10.1007/s00535-016-1242-9

Wang, G., Huang, S., Cai, S., Yu, H., Wang, Y., Zeng, X., et al. (2020). Lactobacillusreuteri ameliorates intestinal inflflammation and modulates gut microbiota and metabolic disorders in dextran sulfate sodium-induced colitis in mice. *Nutrients* 12:2298. doi: 10.3390/nu12082298

Wang, N., Wang, S., Xu, B., Liu, F., Huo, G., and Li, B. (2021). Alleviation effects of Bififi-dobacterium animalis subsp. lactis XLTG11 on dextran sulfate sodiuminduced colitis in mice. *Microorganisms* 9:2093. doi: 10.3390/microorganisms9102093

Wang, C., Xiao, Y., Yu, L., Tian, F., Zhao, J., Zhang, H., et al. (2022). Protective effects of different *Bacteroides vulgatus* strains against lipopolysaccharide-induced acute intestinal injury, and their underlying functional genes. *J. Adv. Res.* 36, 27–37. doi: 10.1016/j.jare.2021.06.012

Weingarden, A. R., and Vaughn, B. P. (2017). Intestinal microbiota, fecal microbiota transplantation, and inflammatory bowel disease. *Gut Microbes* 8, 238–252. doi: 10.1080/19490976.2017.1290757

Xavier, R. J., and Podolsky, D. K. (2007). Unravelling the pathogenesis of inflammatory bowel disease. *Nature* 448, 427–434. doi: 10.1038/nature06005

Xiao, P., Zhang, H., Zhang, Y., Zheng, M., Liu, R., Zhao, Y., et al. (2019). Phosphatase Shp2 exacerbates intestinal inflflammation by disrupting macrophage responsiveness to interleukin-10. *J. Exp. Med.* 216, 337–349. doi: 10.1084/jem.20181198

Zhao, L., Tian, W., Qu, W., Li, T., Zhu, J., and Ma, H. (2021). Protease hydrolysates ameliorates inflflammation and intestinal flflora imbalance in DSS-induced colitis mice. *J. Food Qual.* 2021, 1–11. doi: 10.1155/2021/5536148

Zhu, J., Liao, M., Yao, Z., Liang, W., Li, Q., Liu, J., et al. (2018). Breast cancer in postmenopausal women is associated with an altered gut metagenome. *Microbiome* 6:136. doi: 10.1186/s40168-018-0515-3

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The signaling pathway of hypoxia inducible factor in regulating gut homeostasis

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Hypoxia represent a condition in which an adequate amount of oxygen supply is missing in the body, and it could be caused by a variety of diseases, including gastrointestinal disorders. This review is focused on the role of hypoxia in the maintenance of the gut homeostasis and related treatment of gastrointestinal disorders. The effects of hypoxia on the gut microbiome and its role on the intestinal barrier functionality are also covered, together with the potential role of hypoxia in the development of gastrointestinal disorders, including inflammatory bowel disease and irritable bowel syndrome. Finally, we discussed the potential of hypoxia-targeted interventions as a novel therapeutic approach for gastrointestinal disorders. In this review, we highlighted the importance of hypoxia in the maintenance of the gut homeostasis and the potential implications for the treatment of gastrointestinal disorders.

KEYWORDS

hypoxia, HIFs, signaling pathways, inflammation, gut homeostasis

1. Introduction

Oxygen is essential for mammals to survive, including humans. It is also a key factor in several biochemical reactions involved in the normal physiology of the body. At the same time, it may be also converted into reactive oxygen species (ROS), which was reported to have negative impact on cells. Hypoxia-inducible factor (HIF) could activate gene transcription and modulate the cellular oxygen levels and metabolic activity (Choudhry and Harris, 2018), playing a role in the oxygen sensing within mammalian cells (Agani and Jiang, 2013), demonstrated by investigations about the erythropoietin regulation. Since its discovery, the number of known HIF and hypoxia-regulated target genes increased, including the ones encoding proteins of several and important biological processes, such as erythropoiesis, angiogenesis, glycolytic pathway, glucose transport, metastasis, and cell survival (Cummins et al., 2008; Colgan and Eltzschig, 2012). As a result, HIF was identified as the most involved factor in the regulation of the cellular responses to oxygen deprivation (Agani and Jiang, 2013). Indeed, the HIF and its related signaling pathways are essential to facilitate the metabolic adaptation to hypoxia-induced stress (Jin et al., 2018; Zhong et al., 2020). Additionally, HIF is involved in many important physiological processes such as cardiovascular generation (Semenza, 2014), tumor progression (Palazon et al., 2017), and pulmonary hypertension (Luo et al., 2019). Over the past decade, the importance of intestinal microbiota in human physiological and metabolic functions has been widely recognized. At the same time, research shows that HIF also plays an important role in

regulating intestinal function (Kumar et al., 2020). Specifically, HIF-1α facilitates the host-microbe crosstalk maintaining the gut homeostasis (Fan et al., 2015) as it up-regulates the tight junction proteins, thereby improving the integrity of the epithelial barrier, leading to a resolution of gut inflammation while facilitating the microbial colonization (Kumar et al., 2020). The mucin induced by HIF-1α promotes the colonization of commensal bacteria within the mucus layer, an essential component of the innate immune system, forming a defensive physical barrier for pathogens at the intestinal epithelium level (Kang et al., 2022). Substantial evidence indicated that HIF-1α contributed to the development of disease, and that it can be considered as a promising therapeutic target due to its involvement in the intestinal homeostasis maintenance. This review comprehensively discussed the HIF-1α structure and function, the associated signaling pathways, its role in the disease development, as well as the impact on the intestinal homeostasis.

2. Structure and function of HIF

Semenza et al. (1991), Ratcliffe (2007), and Pugh et al. (1991) discovered a transcriptional enhancer able to control the expression levels of erythropoietin, which is in turn regulated by the oxygen concentration as it is induced under hypoxic conditions. The hypoxia-inducible gene expression is regulated by a group of proteins recognized as hypoxia-inducible factors (HIFs). In 1992, Semenza et al. (1991) found that HIF-1 is composed of two proteins (HIF-1α and HIF-1β) that mediates the body adaptation to hypoxic conditions through red blood cell and angiogenesis. There are five major isoforms of HIFs, including HIF1α, HIF2α, and HIF3α which are oxygen-sensitive, and HIF1β and HIF1β2 that showed oxygen-insensitive (Hankinson, 2008; Prabhakar and Semenza, 2012; Choudhry and Harris, 2018). HIF-1α and HIF-2α are stable in hypoxic conditions, they form heterodimers with HIF-1β and thus to activate the gene transcription (Smythies et al., 2019). Interestingly, HIF-1α showed high activation during brief stage of severe hypoxia or anoxia, while HIF-2α was found to be more active in mild or physiological hypoxia, and retain continuous active in 48–72 h of hypoxia (Hu et al., 2022). Thus, it may be possible to state that HIF-1 is responsible for initiating the hypoxic response, while HIF-2α plays a more predominant role in driving the prolonged hypoxic response (Koh et al., 2011; Koh and Powis, 2012; Hu et al., 2022).

Being an oxygen-sensitive transcription factor, HIF-1α is correlated with the maintenance of oxygen homeostasis in mammalian cells, and it mediates the adaptive responses to hypoxia (Choudhry and Harris, 2018; Sousa et al., 2019). The protein stability of HIF-1α is mainly modulated by the oxygen-dependent degradation domain (Sun et al., 2017; Zheng et al., 2021). In the presence of oxygen, HIF-1/2α encounters hydroxylation via a specific prolyl hydroxylases (PHDs) at two conserved proline residues (P402/P564 and P405/P531 for human HIF-1α and HIF-2α, respectively) (Hu et al., 2022). After hydroxylation, HIFα is thus identified by von Hippel–Lindau (VHL) to be subsequently degraded through an oxygen-dependent ubiquitin-proteasome pathway (Figure 1) (Sousa et al., 2019). Therefore, under regular conditions, HIF-1α has a very short half-life. The hydroxylation of proline residues in HIFα is critical for VHL binding and relies on PHD, α-ketoglutarate-dependent dioxygenase, and asparagine hydroxylase, that have inhibitory effects on HIF (Choudhry and Harris, 2018). In case of hypoxia, the proteasomal of HIFα is stably expressed and not degraded by the ubiquitin ligase system. HIF-1α is therefore accumulated and it can be translocated to the nucleus, where it binds to aryl carbon receptor nuclear translocator (ARNT) to form the HIF-1α/β heterodimer (Aj et al., 2010; Lee et al., 2019), that binds to p300 to form a transcriptional activation complex targeting the hypoxia response element (HRE) within the DNA that activated the transcription of HIF-1 target genes, such as vascular endothelial growth factor (VEGF), erythropoietin, inducible nitric oxide synthase (iNOS), and glucose transporter (GLUT) (Choudhry and Harris, 2018; Singh et al., 2018). HIF-2α is an heterodimeric transcription factor formed following the dimerization between HIF-2α subunit and its obligate partner subunit nuclear translocator (ARNT) (Wu et al., 2019). Similarly to HIF-1α, HIF-2α is activated in hypoxic conditions through a common signaling pathway (Hu et al., 2022), causing a more relevant response to hypoxia due to its higher affinity for the promoters of genes involved in the hypoxia response (Hu et al., 2022), which results also in an enhanced inducing activity of HIF-2α in some cancers (Wu et al., 2019). The inhibitory PAS domain protein (IPAS), a short splice variant of hypoxia-inducible factor 3 alpha (HIF-3α), has been shown to drive transcriptional activity through its interactions with HIF-1α and HIF-2α in mice, as demonstrated by Tanaka et al. (2009) and Kobayashi et al. (2015). HIF-3α has been traditionally considered as a negative regulator of the hypoxia response pathway (Ravenna et al., 2016). However, recently it was found that long variants of HIF-3α have the capacity to create αβ dimers with an inverse activation ability (Tolonen et al., 2020). HIF-β subunit, also named as the aromatic hydrocarbon ARNT, is not regulated by e oxygen levels. Recent research has revealed that a prolonged hypoxic environment can increase HIF-1β expression in high-risk multiple myeloma cells, with this effect mediated via the expression of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Wu et al., 2018).

HIFs is classified within the bHLH/PAS protein family, and it is composed of one N-terminal basic-helix–loop–helix (bHLH) domain and two Per-ARNT-Sim (PAS) domains that are crucial for DNA binding and dimerization (Bersten et al., 2013; Yang et al., 2021). Activation of the target gene is facilitated by the N-terminal transactivation domain (NTAD) present in HIFα isoforms, as well as by the C-terminal transactivation domain (CTAD) that is included in both isoforms (Prabhakar and Semenza, 2012). HIF-3α features a unique C-terminal leucine zipper (LZIP) domain that facilitates protein–protein interactions instead of the CTAD. On the other hand, both HIF-1β and HIF-2β lack the ODDD/NTAD and LZIP domains, and while they possess a CTAD, they lack an asparagine residue (Figure 2) (Ravenna et al., 2016). Within the DNA binding and dimerization domains, HIF-1α and HIF-2α shared a high level of homology in both DNA sequence and structure (Smythies et al., 2019). However, substantial evidence suggests that HIF-1α and HIF-2α heterodimers show distinct physiological functions and different roles in the same disease (Rosenberger et al., 2002; Takeda et al., 2010). For example, following the activation of both subtypes in VHL-deficient renal cancer, it was suggested that HIF-2α is oncogenic, whereas HIF-1α has tumor suppressor properties (Shen et al., 2011; Salama et al., 2015). Structurally, HIF-2α and HIF-1α are highly similar, with an overall amino acid (aa) identity of 48%. In particular, their bHLH domains share up to 83% of aa identity, while their PAS regions approximately 70% of aa identity (Hu et al., 2022). Despite a

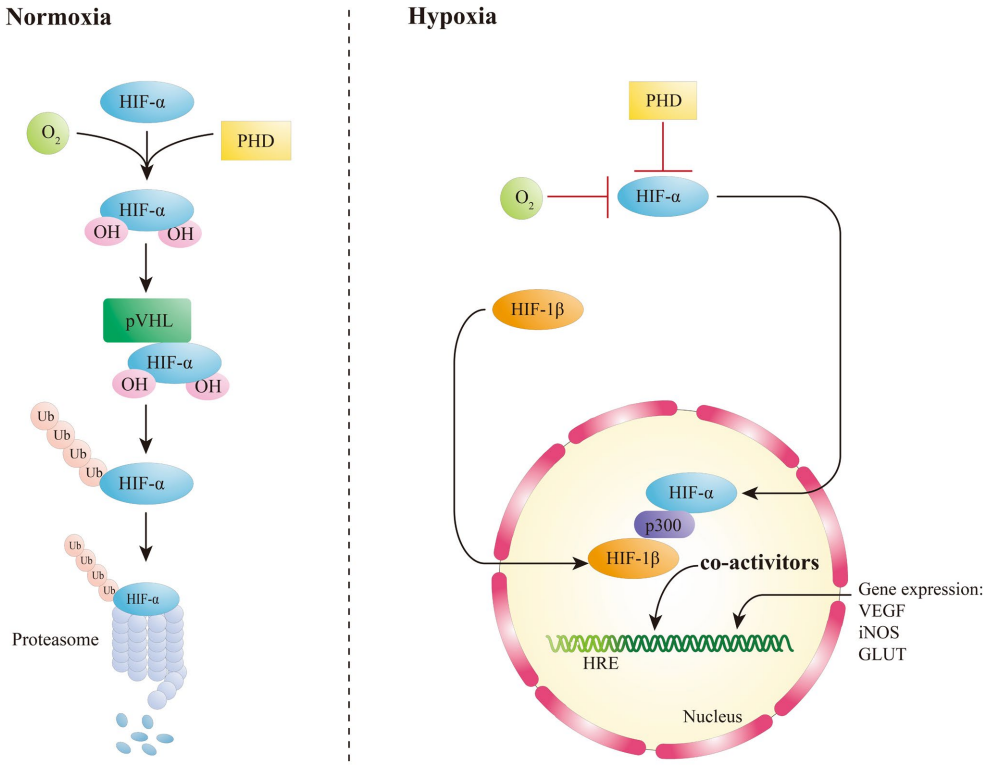

FIGURE 1
Oxygen-dependent regulation of HIFs.

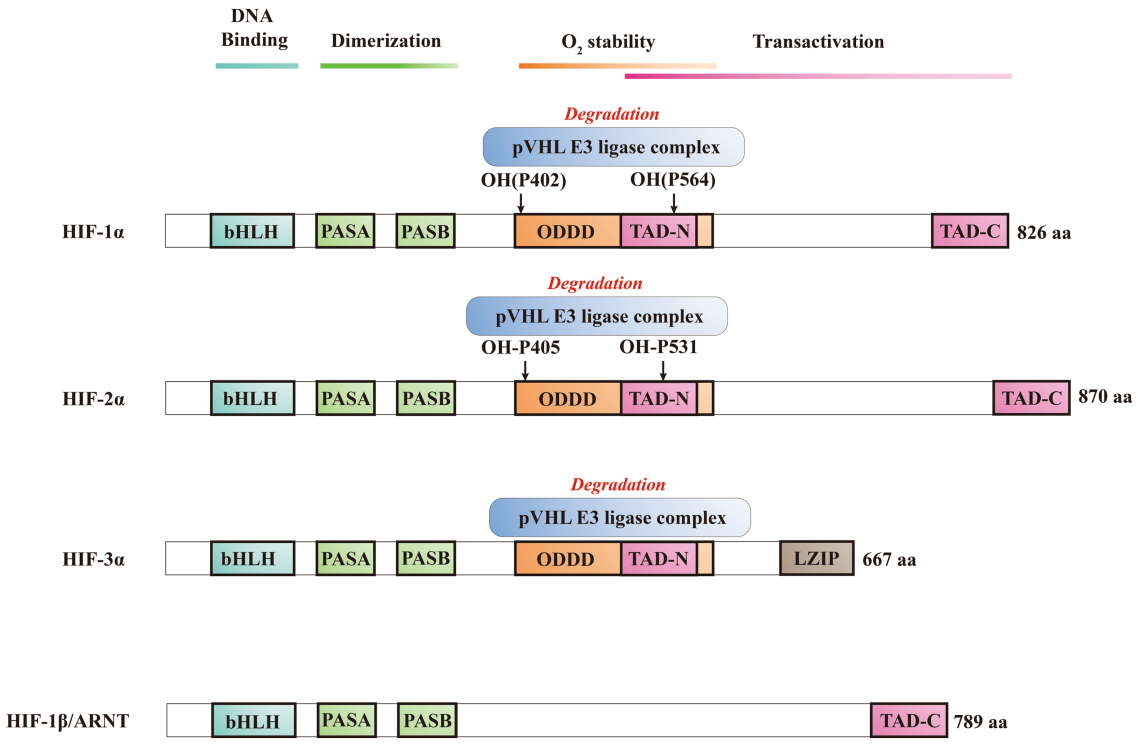

FIGURE 2
Schematic illustration of the domain structure of HIFs.

common consensus DNA-binding motif, HIF-1α and HIF-2α bind two distinct but overlapping sites in chromatin with common and unique patterns of downstream gene induction (Koh et al., 2011). Through the examination of the gene induction pattern in renal cell

carcinoma 786-O WT-8 cellsstrongly indicates that the upregulation of ADRP, NDRG-1, and VEGF can be attributed to the activity of HIF-2α (Keith et al., 2011). A previous investigation that decreased the expression of specific HIF-α isoforms in Hep3B cells by siRNA revealed a predominant function of HIF-2α in the stimulation of erythropoietin (EPO) production (Shu et al., 2019). Several investigations demonstrated that HIF-1α or HIF-2α could regulate the expression of several genes induced by hypoxia, however, each HIFα isoform showed also distinct targets (Figure 3) (Rr et al., 2005). Several studies suggested that different transcriptional responses mediated by HIF-1α and HIF-2α allow the adaptation to hypoxia (Colgan and Eltzschig, 2012). For example, the ones that coordinate the glycolytic pathway include multiple target genes, and it appears that the HIF-1α subtype is more selective than HIF-2α (Keith et al., 2011). Hif-1α$^{-/-}$ ES cells lost the hypoxic response of the glycolytic genes GLUT-1 and VEGF, suggesting a regulation only by HIF-1α (Keith et al., 2011). In addition, compared with HIF-1α, HIF-2α shows a more prominent effect on the induction of erythropoietin (Shu et al., 2019).

3. HIF-1α and signaling pathways

HIF-1α is located in a wide range of human cells and it interacts with several up-stream and downstream proteins to establish different signaling pathways. HIF-1α mediates the hypoxia signals, leading to a range of compensatory responses to hypoxia, and it plays a significant role in physiological and pathological processes within the body (Xu et al., 2022). The HIF stabilization during the hypoxia is important to upregulate several hundred of downstream target genes, in light of the complexity and importance of HIF signaling (Lee et al., 2019). Most recent research studies indicate that HIF-1α is involved in several signaling pathways, including phosphatidylinositol-3 kinase/protein kinase/mechanistic target of rapamycin (PI3K/Akt/mTOR), extracellular signal-regulated kinase (ERK), Wnt/β-catenin, Notch, and NF-κB (Figure 4) (Malekan et al., 2021; Zhang et al., 2021). These pathways affect several functions including cellular metabolism, regulation of cell proliferation, and control of inflammatory responses (Luo et al., 2022).

Previous research indicated that the phosphatidylinositol-3 kinase/protein kinase B signaling pathway (PI3K/Akt), governs a variety of cellular processes, is able to modulate the HIF-1α expression (Zhang et al., 2018). The downstream localization of HIF-1α is modulated by the mechanistic target of rapamycin complex 1 (mTORC1) pathway, while the PI3K/Akt signaling pathway regulates mTORC1 in an independent way (Malekan et al., 2021). In human mesenchymal stem cells, the exposure to hypoxia stimulated an increase in the levels of both p-Akt and HIF-1α, with p-Akt reaching its maximum earlier than HIF-1α (Zhang et al., 2018). The Akt inhibitor, wortmannin, is also able to inhibit the expression of HIF-1α while mTOR is Akt specific and represent a target of Akt during phosphorylation (Zhang et al., 2018). mTOR functions as an up-stream mediator of HIF-1α activation, and it was recently demonstrated that PI3K/Akt signaling pathway may regulate HIF-1α through mTOR. This regulation may be on a post-transcriptional protein level, altering HIF-1α (Liu et al., 2014). The activation of platelet-derived growth factor (PDGF), transforming growth factor (TGF), tumor necrosis factor-α (TNF- α), and inter-leukin-1β (IL-1β) triggers the HIF-1α regulation through the PI3K/Akt pathway (Oktay et al., 2007; Niu et al., 2008), that lead to an enhanced expression of HIF-1α when activated by receptor tyrosine protein kinase (RTK) (Xie R. et al., 2019). All together these findings suggest that PI3K/Akt/mTOR signaling pathway regulates the HIF-α mRNA levels.

NF-κB is a transcription factor involved in different biological processes including apoptosis, viral replication, tumorigenesis, inflammation, and autoimmune diseases (Barnabei et al., 2021). Endothelial cells (EC) were found able to autocrine TNF-α in case of hypoxia, and to activate the HIF pathway through a NF-κB-dependent process, while producing VEGF that led to the neovascularization (Jin et al., 2019). The silencing of HIF1α and specific glycolytic enzymes can reduce the NF-κB activation and ex-pression of pro-inflammatory genes in endothelial cells when exposed to a disordered flow (Wu et al., 2017). Consequently, endothelial cells when exposed to low and turbulent flow increase the HIF-1α expression and inflammatory signaling by enhancing the NF-κB activation while upregulating the number of glycolytic enzymes (Li et al., 2017). The principal mechanism underlying the canonical NF-κB activation involves a site-specific phosphorylation of IκBα by the multi-subunit IκB kinase (IKK) complex, that leads to its inducible degradation (LaGory and Giaccia, 2016). Furthermore, the activation of the non-canonical NF-κB pathway (i.e., TNFSF14/LIGHT) induces an in-creased

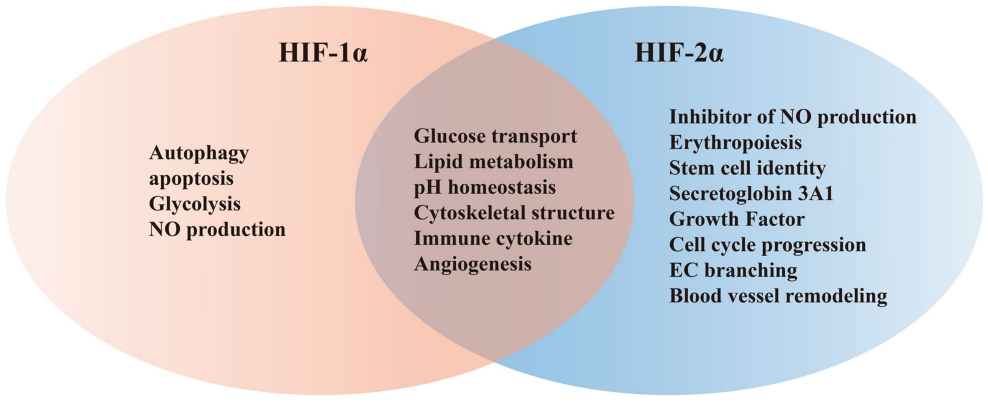

FIGURE 3
Representative shared and unique target genes regulated by HIF-1α and HIF-2α.

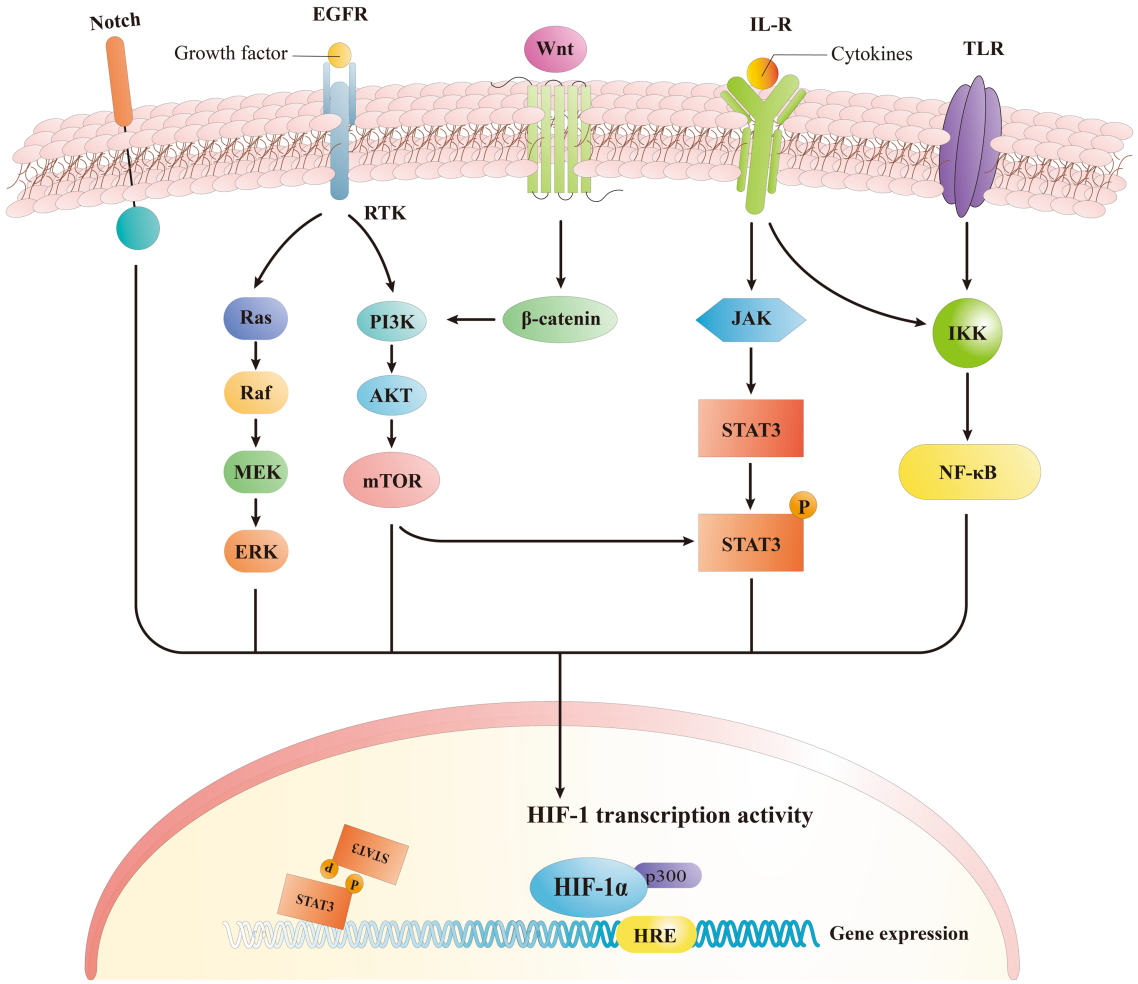

FIGURE 4
Signaling pathway of HIF-1α. HIF-1α induced expression of downstream genes by activating different signaling pathways.

expression of HIF, specifically HIF-2α. This is also facilitated by direct interaction of NF-κB subunit p52 and HIF-2α, that initiates multiple site binding on the subunit (Wu et al., 2017). In previous studies, we observed that TNFα, which is a canonical activator of NF-κB, may also be involved in the activation of the HIF pathway in ECs (Yao et al., 2015). Studies have shown that the anti-angiogenic activity of low-density lipoprotein (LDL) is focused on the reduction of HIF-1α and HIF-2α protein levels in ECs, and this is possibly related to the inactivation of NF-κB and down-regulation of HIF-1β (Yao et al., 2015). Inflammatory stimulants and other factors can increase the HIF-1 gene and protein level expressions by modulating NF-κB-dependent signaling (Xu et al., 2022). Other studies demonstrated that the NF-κB pathway can activate the expression of HIF-2α mRNA in osteoarthritis following the increased HIF-2α expression in mouse articular chondrocytes following the treatment with IL-1β, which is a stimulator of the NF-κB pathway (Yang et al., 2010). Additionally, icariin regulated the NF-κB/HIF-2α axis and reduced the inflammation in chondrocytes (Wang et al., 2020), and NF-κB signaling was found to stimulate the expression of HIF-1β (van Uden et al., 2011).

The ERK pathway represent another key pathway triggering the expression of HIF-1α by increasing the HIF-1α protein generation (Wan and Wu, 2016; Luo et al., 2022). It is important to note that the ERK pathway not only regulates the synthesis of HIF-1α but also phosphorylates the coactivator CBP/p300, thus enhancing the formation of the HIF-1α/p300 complex (Malekan et al., 2021). Hyperthermia induces the expression of HIF-1α in lung cancer through the AKT and ERK signaling pathways (Wan and Wu, 2016). In addition, it was demonstrated that photodynamic therapy (PDT) increases the expression of HIF-1α through the ROS-ERK axis, thereby enhancing the resistance to the treatment (Lamberti et al., 2017), indicating a regulation role of ERK signaling on the HIF-1α expression.

Besides the signaling pathways mentioned above, Wnt/β-catenin and Notch pathways are also involved in the modulation of HIF signaling. HIF-1α indeed acts downstream and upstream of the Wnt/β-catenin signaling pathway indicating their mutually regulating functions (Liu et al., 2015; Wu et al., 2015). Specifically, Wnt/β-catenin regulates the function of HIF-1α by initiating the PI3K/Akt signaling pathway (Laukoetter et al., 2008; Lau et al., 2011). The high expression of HIF-1α resulting from hypoxia in cells can activate the Wnt/β-catenin signaling pathway and thus increasing the β-catenin (Wu et al., 2015), while HIF-1α signal also regulates Wnt/β-catenin pathway via calreticulin (Liu et al., 2021). In turn, as an upstream pathway through related

signaling pathways, Wnt/β-catenin can indirectly regulate the HIF-1α expression. Recently, it was also demonstrated the role of Notch/HIF-1α signaling in different processes like liver regeneration, angiogenesis, and cancer epithelial-mesenchymal-transition (Li et al., 2020). It was reported that the downstream Janus kinase (JAK)-STAT3 signaling pathway is activated by IL-6, leading to an increase in HIF-α. Notably, this mechanism is consistent with the fact that mTORC1 phosphorylates STAT3, and is involved in the upregulation of HIF-1α mRNA expression (LaGory and Giaccia, 2016).

4. HIF and gut homeostasis

In the recent years, it was extensively described that the gut microbiota plays a crucial role for the balance between health status and disease development of host species. The microbiota can be considered as a distinct "human organ" (Shepherd et al., 2018; Marietta et al., 2019). The establishment, selection, and colonization of microbes are governed by a complex molecular network of host-gut microbiota interactions (Kumar et al., 2020). Specifically, several clinical studies showed that hypoxia and inflammation are present in the tissue microenvironment in several inflammatory diseases (Malkov et al., 2021). At a cellular level, the primary control underlying the tissue adaptation to hypoxia is through the HIF signaling pathway (Malkov et al., 2021), and in particular HIF-1α is considered a key regulator of hypoxic injury. In case of IBD, not only the entire mucosa can become even more hypoxic and the expression of HIF-1α and HIF-2α may also be elevated in intestinal surgical specimens (Eltzschig and Carmeliet, 2011). Intestinal epithelial cells (IECs) are thus exposed to the hypoxic environment within the intestinal lumen and represent a major site for host–microbe interactions in order to modulate physiological outcomes (Muenchau et al., 2019; Kumar et al., 2020). The intestinal epithelium not only is re-sponsible for the transfer of nutrients, water, and electrolytes from the lumen to the underlying tissues, but also plays a crucial role in maintaining gut homeostasis by serving as a physical and immunological barrier that prevents the entry of commensal bacteria and potentially harmful microorganisms (König et al., 2016). Altered intestinal barrier function is known to increase susceptibility to enteric infections and disrupt the physiological mechanisms responsible for maintaining tolerance to commensal microorganisms. These changes can ultimately lead to chronic gastrointestinal inflammation and the development of inflammatory bowel diseases, including Crohn's disease (CD) and ulcerative colitis (UC) (Konjar et al., 2021).

4.1. The effect of HIF on the intestinal barrier

Studies have found that HIF-1α may play a key role in maintaining intestinal homeostasis by regulating the integrity of the intestinal epithelial barrier while cultivating a suitable ecological niche (Kumar et al., 2020). Additionally, HIF-1α maintains the intestinal epithelial integrity by upregulating genes involved in the maintenance of the intestinal barrier integrity, such as muc2, ITF, cldn1 as well as other tight junction proteins (Figure 5) (Kumar et al., 2020). Given that HIF-1α plays a direct role in preserving the intestinal epithelial integrity by promoting barrier functionality (Muenchau et al., 2019), the increased integrity of the gut barrier may have immunosuppressive effects, as it seals paracellular pathways while inhibiting the immune cells activation (Chelakkot et al., 2018). Both HIF-1α and HIF-2α are expressed in the intestinal epithelial cells of ulcerative colitis and Crohn's disease patients and in mouse models of colitis (Xue et al., 2013). In a model of radiation-induced intestinal toxicity, HIF-2α restores epithelial integrity and reduces apoptosis by inducing angiogenic gene expression (Olcina and Giaccia, 2016). Following intestinal injury, HIF-2α directly regulates chemokine/cytokine networks to recruit neutrophils and multiple pro-inflammatory mediators to eliminate noxious stimuli and restore the mucosal barrier (Singhal and Shah, 2020). The differentiation of regulatory T (Treg) (Lei et al., 2015) cells plays a crucial role in the establishment and proliferation of the human gut microbiome (Luu et al., 2017). In particular, under cellular hypoxic conditions, HIF-1α promotes the differentiation of naive CD4 cells into regulatory T cells by inducing the transcription of FoxP3., and the anti-inflammatory cytokine IL-10 is produced, to downregulate the immune response, thereby reducing the colonic inflammation while promoting the immune tolerance (Luu et al., 2017). Extracellular ATP is subject to enzymatic hydrolysis by nucleoside triphosphate dephosphorylase (NTPDase or CD39) to generate AMP, which is in turn converted into adenosine by Ecto-5′-Nucleotidase (5-NT or CD73) (Allard et al., 2017). Adenosine exerts immune-modulatory effects and promotes the enhancement of the epithelial barrier by activating A2B adenosine receptors, that then trigger the phosphorylation of vasodilator-stimulated phosphoprotein (VASP) (Aherne et al., 2015). Elevated expression levels of HIF-1α were evidenced in patients with Crohn's disease and ulcerative colitis, indicating its protective role in mitigating inflammatory bowel disorders by improving the epithelial barrier functionality (Shah, 2016).

4.2. Interaction of NF-κB and HIF-1α in the intestine

The maintenance of gut homeostasis depends on an intricate functional crosstalk between HIF-1α and NF-κB signaling pathways. Specifically, HIF-1α inhibits TAK1, which in turn prevents the downstream activation of IKK, ultimately leading to downregulation of NF-κB activity and decreased inflammation (Liu et al., 2017). The transcription factor NF-κB is involved in the expression of numerous genes involved in immune response, serving as a key mediator of inflammatory responses. Specifically, NF-κB induces the expression of numerous pro-inflammatory genes, which encoding cytokines and chemokines, and also is involved in the regulation of inflammasomes (Cutolo et al., 2022). HIF-1α pathway indeed strongly affects the epithelial and immune system function and development during inflammation by activating an adaptive response in these cells (Taylor et al., 2016). The pro-inflammatory function of NF-κB has been widely investigated in macrophages, which are innate immune cells located across several tissues with defensive functions against infectious agents. Notably, the constitutive activation of NF-κB was detected in the inflamed colonic tissue of individuals diagnosed with IBD (Liu et al., 2017). NF-κB serves as a pivotal transcription factor in M1 macrophages and plays a crucial role in inducing a multitude of inflammatory genes, such as those encoding TNF-α, IL-1β, IL-6, IL-12p40, and cyclooxygenase-2 (Wang et al., 2014). A recent study employing dextran sulfate sodium (DSS)-induced colitis mouse model

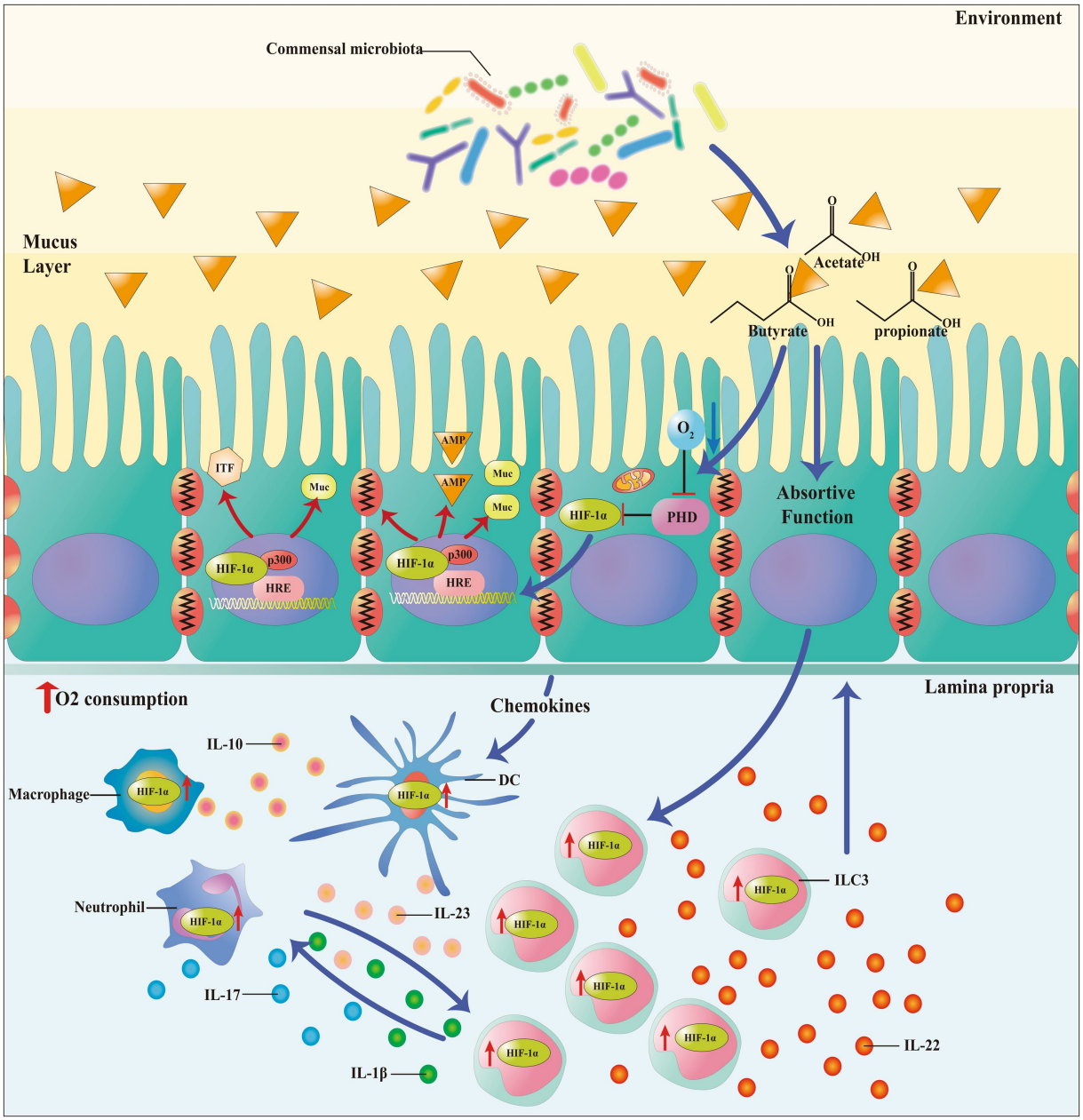

FIGURE 5
HIF-1α plays an important role in maintaining the intestinal homeostasis as it enhances the epithelial barrier integrity by upregulating the expression of genes, thereby reducing intestinal inflammation, and promoting microbial colonization. IEC and other cellular activation (such as ILC3) release anti-inflammation cytokines and increase the oxygen consumption to stabilize the expression of HIF-1α.

reported that the absence of HIF-1α in dendritic cells leads to intestinal inflammation through the upregulation IL-6 and IL-23 (Flück et al., 2016). Additionally, HIF-1α can activate NF-κB under hypoxic conditions and enhance the expression of TNF-α (Gerri et al., 2017; Gunton, 2020). Furthermore, HIF-1α-induced NF-κB pathway increases TLRs expression while inducing an inflammatory cascade leading to ECs injury (Groschwitz and Hogan, 2009; Gerri et al., 2017). In turn, downregulation of HIF-1α expression can effectively reduce the inflammation and oxidative stress-induced damage during EC stress in hyperglycemia-induced mice (Liu et al., 2018; Xie Y. et al., 2019). The stabilization of HIF-1α conferred protective effects by attenuating the NF-κB signaling pathway, and thus reducing the cellular inflammation (Bandarra et al., 2015). Given the potential therapeutic properties of HIF for inflammatory disorders, further investigations into the role of inflammatory cytokines in the HIF pathway may provide novel therapeutic insights into the management of inflammatory diseases.

4.3. Hypoxia-mediated effects of HIF-1α and SCFAs

Microbiota-derived short-chain fatty acids (SCFAs) have gained a growing attention in recent years. The microbial supply for SCFAs,

especially butyrate, is a recognized contributor to the gut homeostasis and disease resistance, while is used as a favorable energy source for enterocytes of the colon (Wang et al., 2021). The mucus layer, secreted by goblet cells, functions as a protective barrier against both endogenous and exogenous irritants, as well as microbial adhesion and invasion (Kang et al., 2022). As a vital element of the innate defense system, the mucosal barrier not only contributes significantly to mucosal repair but also safeguards the mucosal epithelium from a range of injuries within the gastrointestinal tract (Guo et al., 2023). SCFAs increase oxygen consumption by intestinal epithelial cells, reduce their availability in the intestine and lead to hypoxia (Bersten et al., 2013; Pral et al., 2021). The decrease in oxygen utilization stabilizes the expression of HIF-1α and translocates it to the nucleus, causing the transcriptional expression of multiple genes (Choudhry and Harris, 2018). Stable expression of HIF-1α upregulates the expression of related genes such as MUC2, MUC3, and intestinal trefoil factor (Guo et al., 2023). Furthermore, related studies have shown that mice lacking HIF-1α exhibit less organized and diffusible mucin granules, suggesting that HIF-1α is necessary for mucin processing and maintenance of mucosal integrity (Kumar et al., 2020). Fachi et al., demonstrated in a mouse model that butyrate increases the expression of claudin in a HIF-1α-dependent manner, leading to improved barrier integrity and reduced inflammation by inhibiting microbial translocation (Fachi et al., 2019; Muenchau et al., 2019), and HIF-1α was found to be required for butyrate protection of the intestinal epithelium in a mouse model of *Clostridium difficile* infection (CDI) (Fachi et al., 2019). Butyrate inhibits PHDs leading to the stabilization of HIF-1α, which in turn upregulates the expression of genes involved in intestinal barrier function, including muc2, ITF, cldn1, and other tight junction proteins (Kumar et al., 2020). Kelly et al. (2015) demonstrated that the treatment of Caco-2 cells with butyrate resulted in a decreased barrier permeability. Studies demonstrated that the knockdown of HIF-1β using specific shRNA in T84 and Caco-2 cells resulted in a decrease in the expression of claudin-1 at both mRNA and protein levels along with defects in barrier function and abnormal morphology of tight junctions (Muenchau et al., 2019). The researchers found that the gut barrier was weakened, HIF-1α was activated and the HIF-1αΔIEC phenotype was reversed during 2,4,6-trinitrobenzenesulfonic acid (TNBS) colitis in a mouse model (Karhausen et al., 2004; Holmquist-Mengelbier et al., 2006). Hirota et al., reported an alleviation of intestinal injury and inflammation induced by *C. difficile* in mice expressing HIF-1α in IECs compared to Hif1α-deficient mice (Hu et al., 2003; Hirota et al., 2010). Taken together, these findings suggest that SCFAs increase the oxygen consumption of intestinal epithelial cells, stabilize the expression of HIF-1α, and upregulate the gene expression of tight junction proteins and intestinal barrier function, maintaining mucosal and epithelial barrier integrity, thereby reducing intestinal inflammation and promoting Microbial colonization.

4.4. HIF-1α and SCFA-mediated regulation of ILC3s

Innate lymphoid cells (ILCs) play a crucial role in regulating mucosal immunity, inflammation, and tissue homeostasis." ILCs includes cytotoxic cells (NK cells) and "helper-like" ILCs, that are primarily tissue-resident cells and play a vital role in tissue homeostasis, inflammation, and mucosal immunity. These cells act rapidly in the immune response, responding to signals or inducer cytokines that are expressed by tis-sue-resident cells (Vivier et al., 2018). ILC3s are highly prevalent in mucosal tissues and play a crucial role in the innate immune response against extracellular bacteria and in the containment of intestinal commensals (Rankin et al., 2016). SCFAs regulates the number of ILC3s in peripheral tissues and attenuates rodent infection in mouse gut by promoting cytokine signaling and activating the mammalian target of rapamycin (mTOR) pathway (Sepahi et al., 2021). ILC3s produce the predominant homeostatic cytokine, IL-22, which is important for the maintenance of the intestinal homeostasis and proliferation of intestinal stem cells (Figure 5). Studies using antibodies against IL-22 and IL-22 knockout mice have demonstrated the role of this cytokine in alteration of the gut microbiota by stimulating the production of antimicrobial peptides (AMPs) such as the regenerating protein RegIIIγ (Lo et al., 2019). Additionally, activation of hypoxia/HIF-1α signaling was shown to enhance murine resistance to *C. difficile* infection. This was demonstrated by an improvement in clinical scores together with a reduction in intestinal bacterial translocation in infected wildtype (WT) mice com-pared to mice with a conditional RORyt-specific HIF-1α knockout (HIF-1αfl/flRORc-Cre) (Fachi et al., 2021). Several studies have also demonstrated that butyrate has anti-inflammatory effects on M1 macrophages stimulated with LPS, as it is capable of reducing the production of pro-inflammatory mediators such as NO and IL-6 (Chang et al., 2014). M2 macrophages are involved in the resolution of inflammation and tissue repair by producing anti-inflammatory cytokines such as IL-10 and IL-13, as well as growth factors and extracellular matrix proteins (Cekic and Linden, 2016; Liu et al., 2017). Commensal microbe-derived butyrate, as a novel effector molecule, can ameliorate DSS-induced colitis in mice by reducing the activation of M1 macrophages and promoting the differentiation of regulatory T cells. DCs treated with butyrate show less ability to stimulate T cells with a reduction in the production of the pro-inflammatory cytokines IL-12p40 and IFN-γ, while instead releasing greater amounts of the an-ti-inflammatory cytokine IL-10 (Liu et al., 2012).

4.5. HIF-1α and intestinal homeostasis mediate alcoholic liver disease

Previous research showed that alcoholic liver disease (ALD) is associated with gut dysbiosis and release of endotoxins (Shao et al., 2018). Shao et al. demonstrated that HIF-1α plays a critical role in regulating the expression of genes involved in maintaining intestinal homeostasis, including those involved in hepatic lipogenesis, maintenance of intestinal barrier function, antimicrobial defense, and the normal microbiome (Zhao et al., 2010). Goblet cells, a specialized cell type within the intestinal epithelium, are responsible for producing protective trefoil factors and mucins, which are heavily core glycosylated and can be found within the cell membrane or secreted into the lumen where they can form the mucus layer (Zhao et al., 2010; Shao et al., 2018), which is the first barrier encountered by bacteria and that needs to be penetrated in order to reach the epithelial cells (Johansson et al., 2008). In IEhif1α$^{-/-}$ mice subjected to alcohol exposure, there is a decrease in intestinal trefoil factor (ITF), claudin-1, and p-glycoprotein, leading to a compromised gut barrier

functionality. This leads to an increased concentration of lipopolysaccharide in the serum and *E. coli* protein in the liver (Shao et al., 2018). Long-term and excessive alcohol consumption can lead to intestinal dysbiosis, which leads to increased intestinal permeability and translocation of LPS into the blood. After LPS binds to TLR4 on hepatocytes (including hepatocytes and Kupffer cells), it triggers an inflammatory response and Leads to hepatic steatosis (fat buildup) and inflammation (Shao et al., 2018). Activation of HIF-1α can regulate the gut bacterial homeostasis by increasing the production of anti-microbial peptides. Additionally, HIF-1α stabilization leads to upregulation of P-glycoprotein and tight junction proteins, which help to maintain barrier functions (Chen et al., 2015). Therefore, ALD can be prevented/treated by developing dietary methods and drugs that specifically activate the intestinal HIF-1α.

5. Future perspectives

HIF-1α represents a crucial transcription factor produced under hypoxic conditions, and it plays a pivotal role in the regulation of several cellular processes, including angiogenesis, glucose metabolism, apoptosis, and autophagy. Moreover, HIF-1α is involved in the regulation of multiple signaling pathways, being essential during the body growth and development, as well as in several physiological and pathological processes. Diverse HIF isoforms are responsible for physiological and pathological processes, and HIF-1α may be involved in the development of diseases through the regulation of multiple target genes. Comprehensive investigations on HIF-1α better elucidated its regulatory roles in angiogenesis, glucose metabolism, apoptosis, autophagy, and several signaling pathways. It is well to point out that further advancements in HIF-1α-based therapeutic strategies and the related research will gain more attention, and it may lead to the development of potent HIF-1α inhibitors to use for clinical applications, allowing new discoveries and achievements in terms of disease prevention and treatment.

Significant differences in baseline oxygen tension between gastrointestinal mucosal tissues play unique roles in intestinal homeostasis and inflammation. With in-depth research, more and more evidence shows that the intestinal mucosal barrier, as an important component of intestinal immunity, not only serves as a medium for the absorption and exchange of substances between organisms and the environment, but also prevents external antigens from entering the body. The complete composition and function of the intestinal mucosal barrier function is critical for maintaining immune homeostasis. Once the intestinal barrier function is damaged under the action of multiple factors, immune homeostasis will be disrupted and inflammatory responses will be triggered. Hypoxia regulates the expression of hundreds of genes through HIF transcription factors, such as enhancing tight junctions and reducing intestinal permeability, as well as increasing mucus and AMP to protect mucosal integrity. HIF has been extensively studied in the areas of modulating intestinal tissue barrier function, metabolism, and inflammatory and immune responses. Many studies have highlighted the therapeutic potential of targeting hypoxic signaling pathways in intestinal diseases. Therefore, hypoxia and activation of the HIF pathway may be considered as putative therapeutic targets for the treatment of certain inflammatory and/or infectious diseases, particularly those affecting the gut such as CDI and IBD. Therefore, further studying the interaction between HIF and intestinal microorganisms may be a new strategy for preventing and treating different diseases in the future.

Author contributions

WL: Data curation, Investigation, Software, Writing – original draft. XF: Data curation, Software, Writing – original draft. BJ: Writing – original draft, Writing – review & editing. DW: Software, Writing – review & editing. HW: Data curation, Writing – review & editing. ZL: Conceptualization, Writing – review & editing. BL: Funding acquisition, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

Agani, F., and Jiang, B.-H. (2013). Oxygen-independent regulation of HIF-1: novel involvement of PI3K/ AKT/mTOR pathway in cancer. *Curr. Cancer Drug Targets* 13, 245–251. doi: 10.2174/1568009611313030003

Aherne, C. M., Saeedi, B., Collins, C. B., Masterson, J. C., McNamee, E. N., Perrenoud, L., et al. (2015). Epithelial-specific A2B adenosine receptor signaling protects the colonic epithelial barrier during acute colitis. *Mucosal Immunol.* 8, 1324–1338. doi: 10.1038/mi.2015.22

Aj, M., Wj, W., and Mc, S. (2010). Hypoxia-inducible factors and the response to hypoxic stress. *Mol. Cell* 40, 294–309. doi: 10.1016/j.molcel.2010.09.022

Allard, B., Longhi, M. S., Robson, S. C., and Stagg, J. (2017). The ectonucleotidases CD39 and CD73: novel checkpoint inhibitor targets. *Immunol. Rev.* 276, 121–144. doi: 10.1111/imr.12528

Bandarra, D., Biddlestone, J., Mudie, S., Müller, H.-A. J., and Rocha, S. (2015). HIF-1α restricts NF-κB-dependent gene expression to control innate immunity signals. *Dis. Model. Mech.* 8, 169–181. doi: 10.1242/dmm.017285

Barnabei, L., Laplantine, E., Mbongo, W., Rieux-Laucat, F., and Weil, R. (2021). NF-κB: at the Borders of autoimmunity and inflammation. *Front. Immunol.* 12:716469. doi: 10.3389/fimmu.2021.716469

Bersten, D. C., Sullivan, A. E., Peet, D. J., and Whitelaw, M. L. (2013). bHLH-PAS proteins in cancer. *Nat. Rev. Cancer* 13, 827–841. doi: 10.1038/nrc3621

Cekic, C., and Linden, J. (2016). Purinergic regulation of the immune system. *Nat. Rev. Immunol.* 16, 177–192. doi: 10.1038/nri.2016.4

Chang, P. V., Hao, L., Offermanns, S., and Medzhitov, R. (2014). The microbial metabolite butyrate regulates intestinal macrophage function via histone deacetylase inhibition. *Proc. Natl. Acad. Sci. U. S. A.* 111, 2247–2252. doi: 10.1073/pnas.1322269111

Chelakkot, C., Ghim, J., and Ryu, S. H. (2018). Mechanisms regulating intestinal barrier integrity and its pathological implications. *Exp. Mol. Med.* 50, 1–9. doi: 10.1038/s12276-018-0126-x

Chen, P., Miyamoto, Y., Mazagova, M., Lee, K.-C., Eckmann, L., and Schnabl, B. (2015). Microbiota protects mice against acute alcohol-induced liver injury. *Alcohol. Clin. Exp. Res.* 39, 2313–2323. doi: 10.1111/acer.12900

Choudhry, H., and Harris, A. L. (2018). Advances in hypoxia-inducible factor biology. *Cell Metab.* 27, 281–298. doi: 10.1016/j.cmet.2017.10.005

Colgan, S. P., and Eltzschig, H. K. (2012). Adenosine and hypoxia-inducible factor Signaling in intestinal injury and recovery. *Annu. Rev. Physiol.* 74, 153–175. doi: 10.1146/annurev-physiol-020911-153230

Cummins, E. P., Seeballuck, F., Keely, S. J., Mangan, N. E., Callanan, J. J., Fallon, P. G., et al. (2008). The hydroxylase inhibitor dimethyloxalylglycine is protective in a murine model of colitis. *Gastroenterology* 134, 156–165. doi: 10.1053/j.gastro.2007.10.012

Cutolo, M., Campitiello, R., Gotelli, E., and Soldano, S. (2022). The role of M1/M2 macrophage polarization in rheumatoid arthritis synovitis. *Front. Immunol.* 13:867260. doi: 10.3389/fimmu.2022.867260

Eltzschig, H. K., and Carmeliet, P. (2011). Hypoxia and inflammation. *N. Engl. J. Med.* 364, 656–665. doi: 10.1056/NEJMra0910283

Fachi, J. L., Felipe, J. D. S., Pral, L. P., Da Silva, B. K., Corrêa, R. O., De Andrade, M. C. P., et al. (2019). Butyrate protects mice from Clostridium difficile-induced colitis through an HIF-1-dependent mechanism. *Cell Rep.* 27, 750–761.e7. doi: 10.1016/j.celrep.2019.03.054

Fachi, J. L., Pral, L. P., Dos Santos, J. A. C., Codo, A. C., De Oliveira, S., Felipe, J. S., et al. (2021). Hypoxia enhances ILC3 responses through HIF-1α-dependent mechanism. *Mucosal Immunol.* 14, 828–841. doi: 10.1038/s41385-020-00371-6

Fan, D., Coughlin, L. A., Neubauer, M. M., Kim, J., Kim, M. S., Zhan, X., et al. (2015). Activation of HIF-1α and LL-37 by commensal bacteria inhibits Candida albicans colonization. *Nat. Med.* 21, 808–814. doi: 10.1038/nm.3871

Flück, K., Breves, G., Fandrey, J., and Winning, S. (2016). Hypoxia-inducible factor 1 in dendritic cells is crucial for the activation of protective regulatory T cells in murine colitis. *Mucosal Immunol.* 9, 379–390. doi: 10.1038/mi.2015.67

Gerri, C., Marín-Juez, R., Marass, M., Marks, A., Maischein, H.-M., and Stainier, D. Y. R. (2017). Hif-1α regulates macrophage-endothelial interactions during blood vessel development in zebrafish. *Nat. Commun.* 8:15492. doi: 10.1038/ncomms15492

Groschwitz, K. R., and Hogan, S. P. (2009). Intestinal barrier function: molecular regulation and disease pathogenesis. *J. Allergy Clin. Immunol.* 124, 3–20. doi: 10.1016/j.jaci.2009.05.038

Gunton, J. E. (2020). Hypoxia-inducible factors and diabetes. *J. Clin. Invest.* 130, 5063–5073. doi: 10.1172/JCI137556

Guo, Y., Liu, Y., Rui, B., Lei, Z., Ning, X., Liu, Y., et al. (2023). Crosstalk between the gut microbiota and innate lymphoid cells in intestinal mucosal immunity. *Front. Immunol.* 14:1171680. doi: 10.3389/fimmu.2023.1171680

Hankinson, O. (2008). Why does ARNT2 behave differently from ARNT? *Toxicol. Sci. Off. J. Soc. Toxicol.* 103, 1–3. doi: 10.1093/toxsci/kfn032

Hirota, S. A., Fines, K., Ng, J., Traboulsi, D., Lee, J., Ihara, E., et al. (2010). Hypoxia-inducible factor Signaling provides protection in Clostridium difficile-induced intestinal injury. *Gastroenterology* 139, 259–269.e3. doi: 10.1053/j.gastro.2010.03.045

Holmquist-Mengelbier, L., Fredlund, E., Löfstedt, T., Noguera, R., Navarro, S., Nilsson, H., et al. (2006). Recruitment of HIF-1alpha and HIF-2alpha to common target genes is differentially regulated in neuroblastoma: HIF-2alpha promotes an aggressive phenotype. *Cancer Cell* 10, 413–423. doi: 10.1016/j.ccr.2006.08.026

Hu, Y., Lu, H., Li, H., and Ge, J. (2022). Molecular basis and clinical implications of HIFs in cardiovascular diseases. *Trends Mol. Med.* 28, 916–938. doi: 10.1016/j.molmed.2022.09.004

Hu, C.-J., Wang, L.-Y., Chodosh, L. A., Keith, B., and Simon, M. C. (2003). Differential roles of hypoxia-inducible factor 1alpha (HIF-1alpha) and HIF-2alpha in hypoxic gene regulation. *Mol. Cell. Biol.* 23, 9361–9374. doi: 10.1128/MCB.23.24.9361-9374.2003

Jin, P., Shin, S.-H., Chun, Y.-S., Shin, H.-W., Shin, Y. J., Lee, Y., et al. (2018). Astrocyte-derived CCL20 reinforces HIF-1-mediated hypoxic responses in glioblastoma by stimulating the CCR6-NF-κB signaling pathway. *Oncogene* 37, 3070–3087. doi: 10.1038/s41388-018-0182-7

Jin, F., Zheng, X., Yang, Y., Yao, G., Ye, L., Doeppner, T. R., et al. (2019). Impairment of hypoxia-induced angiogenesis by LDL involves a HIF-centered signaling network linking inflammatory TNFα and angiogenic VEGF. *Aging* 11, 328–349. doi: 10.18632/aging.101726

Johansson, M. E. V., Phillipson, M., Petersson, J., Velcich, A., Holm, L., and Hansson, G. C. (2008). The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. *Proc. Natl. Acad. Sci. U. S. A.* 105, 15064–15069. doi: 10.1073/pnas.0803124105

Kang, Y., Park, H., Choe, B.-H., and Kang, B. (2022). The role and function of mucins and its relationship to inflammatory bowel disease. *Front. Med.* 9:848344. doi: 10.3389/fmed.2022.848344

Karhausen, J., Furuta, G. T., Tomaszewski, J. E., Johnson, R. S., Colgan, S. P., and Haase, V. H. (2004). Epithelial hypoxia-inducible factor-1 is protective in murine experimental colitis. *J. Clin. Invest.* 114, 1098–1106. doi: 10.1172/JCI200421086

Keith, B., Johnson, R. S., and Simon, M. C. (2011). HIF1α and HIF2α: sibling rivalry in hypoxic tumour growth and progression. *Nat. Rev. Cancer* 12, 9–22. doi: 10.1038/nrc3183

Kelly, C. J., Zheng, L., Campbell, E. L., Saeedi, B., Scholz, C. C., Bayless, A. J., et al. (2015). Crosstalk between microbiota-derived short-chain fatty acids and intestinal epithelial HIF augments tissue barrier function. *Cell Host Microbe* 17, 662–671. doi: 10.1016/j.chom.2015.03.005

Kobayashi, S., Yamashita, T., Ohneda, K., Nagano, M., Kimura, K., Nakai, H., et al. (2015). Hypoxia-inducible factor-3α promotes angiogenic activity of pulmonary endothelial cells by repressing the expression of the VE-cadherin gene. *Genes Cells Devoted Mol. Cell. Mech.* 20, 224–241. doi: 10.1111/gtc.12215

Koh, M. Y., Lemos, R., Liu, X., and Powis, G. (2011). The hypoxia-associated factor switches cells from HIF-1α- to HIF-2α-dependent signaling promoting stem cell characteristics, aggressive tumor growth and invasion. *Cancer Res.* 71, 4015–4027. doi: 10.1158/0008-5472.CAN-10-4142

Koh, M. Y., and Powis, G. (2012). Passing the baton: the HIF switch. *Trends Biochem. Sci.* 37, 364–372. doi: 10.1016/j.tibs.2012.06.004

König, J., Wells, J., Cani, P. D., García-Ródenas, C. L., MacDonald, T., Mercenier, A., et al. (2016). Human intestinal barrier function in health and disease. *Clin. Transl. Gastroenterol.* 7:e196. doi: 10.1038/ctg.2016.54

Konjar, Š., Pavšič, M., and Veldhoen, M. (2021). Regulation of oxygen homeostasis at the intestinal epithelial barrier site. *Int. J. Mol. Sci.* 22:9170. doi: 10.3390/ijms22179170

Kumar, T., Pandey, R., and Chauhan, N. S. (2020). Hypoxia inducible factor-1α: the curator of gut homeostasis. *Front. Cell. Infect. Microbiol.* 10:227. doi: 10.3389/fcimb.2020.00227

LaGory, E. L., and Giaccia, A. J. (2016). The ever-expanding role of HIF in tumour and stromal biology. *Nat. Cell Biol.* 18, 356–365. doi: 10.1038/ncb3330

Lamberti, M. J., Pansa, M. F., Vera, R. E., Fernández-Zapico, M. E., Rumie Vittar, N. B., and Rivarola, V. A. (2017). Transcriptional activation of HIF-1 by a ROS-ERK axis underlies the resistance to photodynamic therapy. *PLoS One* 12:e0177801. doi: 10.1371/journal.pone.0177801

Lau, M.-T., Klausen, C., and Leung, P. C. K. (2011). E-cadherin inhibits tumor cell growth by suppressing PI3K/Akt signaling via β-catenin-Egr1-mediated PTEN expression. *Oncogene* 30, 2753–2766. doi: 10.1038/onc.2011.6

Laukoetter, M. G., Nava, P., and Nusrat, A. (2008). Role of the intestinal barrier in inflammatory bowel disease. *World J. Gastroenterol.* 14, 401–407. doi: 10.3748/wjg.14.401

Lee, J. W., Ko, J., Ju, C., and Eltzschig, H. K. (2019). Hypoxia signaling in human diseases and therapeutic targets. *Exp. Mol. Med.* 51, 1–13. doi: 10.1038/s12276-019-0235-1

Lei, H., Schmidt-Bleek, K., Dienelt, A., Reinke, P., and Volk, H.-D. (2015). Regulatory T cell-mediated anti-inflammatory effects promote successful tissue repair in both indirect and direct manners. *Front. Pharmacol.* 6:184. doi: 10.3389/fphar.2015.00184

Li, X., Weintraub, D. S., and Fulton, D. J. R. (2017). Beyond impressions: how altered shear stress connects hypoxic Signaling to endothelial inflammation. *Arterioscler. Thromb. Vasc. Biol.* 37, 1987–1989. doi: 10.1161/ATVBAHA.117.310149

Li, Y., Xu, Y., Wang, R., Li, W., He, W., Luo, X., et al. (2020). Expression of notch-Hif-1α signaling pathway in liver regeneration of rats. *J. Int. Med. Res.* 48:300060520943790. doi: 10.1177/0300060520943790

Liu, B.-N., Han, B.-X., and Liu, F. (2014). Neuroprotective effect of pAkt and HIF-1 α on ischemia rats. *Asian Pac J Trop Med* 7, 221–225. doi: 10.1016/S1995-7645(14)60025-0

Liu, L., Li, L., Min, J., Wang, J., Wu, H., Zeng, Y., et al. (2012). Butyrate interferes with the differentiation and function of human monocyte-derived dendritic cells. *Cell. Immunol.* 277, 66–73. doi: 10.1016/j.cellimm.2012.05.011

Liu, H.-L., Liu, D., Ding, G.-R., Liao, P.-F., and Zhang, J.-W. (2015). Hypoxia-inducible factor-1α and Wnt/β-catenin signaling pathways promote the invasion of hypoxic gastric cancer cells. *Mol. Med. Rep.* 12, 3365–3373. doi: 10.3892/mmr.2015.3812

Liu, J., Wang, W., Wang, L., Chen, S., Tian, B., Huang, K., et al. (2018). IL-33 initiates vascular remodelling in hypoxic pulmonary hypertension by up-regulating HIF-1α and VEGF expression in vascular endothelial cells. *EBioMedicine* 33, 196–210. doi: 10.1016/j.ebiom.2018.06.003

Liu, X., Xie, P., Hao, N., Zhang, M., Liu, Y., Liu, P., et al. (2021). HIF-1-regulated expression of calreticulin promotes breast tumorigenesis and progression through Wnt/β-catenin pathway activation. *Proc. Natl. Acad. Sci. U. S. A.* 118:e2109144118. doi: 10.1073/pnas.2109144118

Liu, T., Zhang, L., Joo, D., and Sun, S.-C. (2017). NF-κB signaling in inflammation. *Signal Transduct. Target. Ther.* 2:17023. doi: 10.1038/sigtrans.2017.23

Lo, B. C., Shin, S. B., Canals Hernaez, D., Refaeli, I., Yu, H. B., Goebeler, V., et al. (2019). IL-22 preserves gut epithelial integrity and promotes disease remission during chronic salmonella infection. *J. Immunol. Baltim. Md* 1950, 956–965. doi: 10.4049/jimmunol.1801308

Luo, Y., Teng, X., Zhang, L., Chen, J., Liu, Z., Chen, X., et al. (2019). CD146-HIF-1α hypoxic reprogramming drives vascular remodeling and pulmonary arterial hypertension. *Nat. Commun.* 10:3551. doi: 10.1038/s41467-019-11500-6

Luo, Z., Tian, M., Yang, G., Tan, Q., Chen, Y., Li, G., et al. (2022). Hypoxia signaling in human health and diseases: implications and prospects for therapeutics. *Signal Transduct. Target. Ther.* 7:218. doi: 10.1038/s41392-022-01080-1

Luu, M., Steinhoff, U., and Visekruna, A. (2017). Functional heterogeneity of gut-resident regulatory T cells. *Clin. Transl. Immunol.* 6:e156. doi: 10.1038/cti.2017.39

Malekan, M., Ebrahimzadeh, M. A., and Sheida, F. (2021). The role of hypoxia-inducible factor-1alpha and its signaling in melanoma. *Biomed. Pharmacother.* 141:111873. doi: 10.1016/j.biopha.2021.111873

Malkov, M. I., Lee, C. T., and Taylor, C. T. (2021). Regulation of the hypoxia-inducible factor (HIF) by pro-inflammatory cytokines. *Cells* 10:2340. doi: 10.3390/cells10092340

Marietta, E., Horwath, I., Balakrishnan, B., and Taneja, V. (2019). Role of the intestinal microbiome in autoimmune diseases and its use in treatments. *Cell. Immunol.* 339, 50–58. doi: 10.1016/j.cellimm.2018.10.005

Muenchau, S., Deutsch, R., de Castro, I. J., Hielscher, T., Heber, N., Niesler, B., et al. (2019). Hypoxic environment promotes barrier formation in human intestinal epithelial cells through regulation of MicroRNA 320a expression. *Mol. Cell. Biol.* 39, e00553–e00518. doi: 10.1128/MCB.00553-18

Niu, G., Briggs, J., Deng, J., Ma, Y., Lee, H., Kortylewski, M., et al. (2008). Signal transducer and activator of transcription 3 is required for hypoxia-inducible factor-1alpha RNA expression in both tumor cells and tumor-associated myeloid cells. *Mol. Cancer Res. MCR* 6, 1099–1105. doi: 10.1158/1541-7786.MCR-07-2177

Oktay, Y., Dioum, E., Matsuzaki, S., Ding, K., Yan, L.-J., Haller, R. G., et al. (2007). Hypoxia-inducible factor 2alpha regulates expression of the mitochondrial aconitase chaperone protein frataxin. *J. Biol. Chem.* 282, 11750–11756. doi: 10.1074/jbc.M611133200

Olcina, M. M., and Giaccia, A. J. (2016). Reducing radiation-induced gastrointestinal toxicity - the role of the PHD/HIF axis. *J. Clin. Invest.* 126, 3708–3715. doi: 10.1172/JCI84432

Palazon, A., Tyrakis, P. A., Macias, D., Veliça, P., Rundqvist, H., Fitzpatrick, S., et al. (2017). An HIF-1α/VEGF-A Axis in cytotoxic T cells regulates tumor progression. Cancer cell 32, activation of HIF-1α and LL-37 by commensal bacteria inhibits Candida Albicans colonization. *Cancer Cell* 32, 669–683.e5. doi: 10.1016/j.ccell.2017.10.003

Prabhakar, N. R., and Semenza, G. L. (2012). Adaptive and maladaptive cardiorespiratory responses to continuous and intermittent hypoxia mediated by hypoxia-inducible factors 1 and 2. *Physiol. Rev.* 92, 967–1003. doi: 10.1152/physrev.00030.2011

Pral, L. P., Fachi, J. L., Corrêa, R. O., Colonna, M., and Vinolo, M. A. R. (2021). Hypoxia and HIF-1 as key regulators of gut microbiota and host interactions. *Trends Immunol.* 42, 604–621. doi: 10.1016/j.it.2021.05.004

Pugh, C. W., Tan, C. C., Jones, R. W., and Ratcliffe, P. J. (1991). Functional analysis of an oxygen-regulated transcriptional enhancer lying 3′ to the mouse erythropoietin gene. *Proc. Natl. Acad. Sci. U. S. A.* 88, 10553–10557. doi: 10.1073/pnas.88.23.10553

Rankin, L. C., Girard-Madoux, M. J. H., Seillet, C., Mielke, L. A., Kerdiles, Y., Fenis, A., et al. (2016). Complementarity and redundancy of IL-22-producing innate lymphoid cells. *Nat. Immunol.* 17, 179–186. doi: 10.1038/ni.3332

Ratcliffe, P. J. (2007). HIF-1 and HIF-2: working alone or together in hypoxia? *J. Clin. Invest.* 117, 862–865. doi: 10.1172/JCI31750

Ravenna, L., Salvatori, L., and Russo, M. A. (2016). HIF3α: the little we know. *FEBS J.* 283, 993–1003. doi: 10.1111/febs.13572

Rosenberger, C., Mandriota, S., Jürgensen, J. S., Wiesener, M. S., Hörstrup, J. H., Frei, U., et al. (2002). Expression of hypoxia-inducible factor-1alpha and -2alpha in hypoxic and ischemic rat kidneys. *Nephrol. Self Assess. Program* 13, 1721–1732. doi: 10.1097/01.asn.0000017223.49823.2a

Rr, R., Kw, L., Mg, T., Hm, S., Sj, M., Jl, L., et al. (2005). Contrasting properties of hypoxia-inducible factor 1 (HIF-1) and HIF-2 in von Hippel-Lindau-associated renal cell carcinoma. *Mol. Cell. Biol.* 25, 5675–5686. doi: 10.1128/MCB.25.13.5675-5686.2005

Salama, R., Masson, N., Simpson, P., Sciesielski, L. K., Sun, M., Tian, Y.-M., et al. (2015). Heterogeneous effects of direct hypoxia pathway activation in kidney cancer. *PLoS One* 10:e0134645. doi: 10.1371/journal.pone.0134645

Semenza, G. L. (2014). Hypoxia-inducible factor 1 and cardiovascular disease. *Annu. Rev. Physiol.* 76, 39–56. doi: 10.1146/annurev-physiol-021113-170322

Semenza, G. L., Nejfelt, M. K., Chi, S. M., and Antonarakis, S. E. (1991). Hypoxia-inducible nuclear factors bind to an enhancer element located 3′ to the human erythropoietin gene. *Proc. Natl. Acad. Sci. U. S. A.* 88, 5680–5684. doi: 10.1073/pnas.88.13.5680

Sepahi, A., Liu, Q., Friesen, L., and Kim, C. H. (2021). Dietary fiber metabolites regulate innate lymphoid cell responses. *Mucosal Immunol.* 14, 317–330. doi: 10.1038/s41385-020-0312-8

Shah, Y. M. (2016). The role of hypoxia in intestinal inflammation. *Mol. Cell. Pediatr.* 3:1. doi: 10.1186/s40348-016-0030-1

Shao, T., Zhao, C., Li, F., Gu, Z., Liu, L., Zhang, L., et al. (2018). Intestinal HIF-1α deletion exacerbates alcoholic liver disease by inducing intestinal dysbiosis and barrier dysfunction. *J. Hepatol.* 69, 886–895. doi: 10.1016/j.jhep.2018.05.021

Shen, C., Beroukhim, R., Schumacher, S. E., Zhou, J., Chang, M., Signoretti, S., et al. (2011). Genetic and functional studies implicate HIF1α as a 14q kidney cancer suppressor gene. *Cancer Discov.* 1, 222–235. doi: 10.1158/2159-8290.CD-11-0098

Shepherd, E. S., DeLoache, W. C., Pruss, K. M., Whitaker, W. R., and Sonnenburg, J. L. (2018). An exclusive metabolic niche enables strain engraftment in the gut microbiota. *Nature* 557, 434–438. doi: 10.1038/s41586-018-0092-4

Shu, S., Wang, Y., Zheng, M., Liu, Z., Cai, J., Tang, C., et al. (2019). Hypoxia and hypoxia-inducible factors in kidney injury and repair. *Cells* 8:E207. doi: 10.3390/cells8030207

Singh, L., Aldosary, S., Saeedan, A. S., Ansari, M. N., and Kaithwas, G. (2018). Prolyl hydroxylase 2: a promising target to inhibit hypoxia-induced cellular metabolism in cancer cells. *Drug Discov. Today* 23, 1873–1882. doi: 10.1016/j.drudis.2018.05.016

Singhal, R., and Shah, Y. M. (2020). Oxygen battle in the gut: hypoxia and hypoxia-inducible factors in metabolic and inflammatory responses in the intestine. *J. Biol. Chem.* 295, 10493–10505. doi: 10.1074/jbc.REV120.011188

Smythies, J. A., Sun, M., Masson, N., Salama, R., Simpson, P. D., Murray, E., et al. (2019). Inherent DNA-binding specificities of the HIF-1α and HIF-2α transcription factors in chromatin. *EMBO Rep.* 20:e46401. doi: 10.15252/embr.201846401

Sousa, F. M., Da, L., Abd Jamil, A. H., Stannard, G. A., and Heather, L. C. (2019). Hypoxia-inducible factor 1 signalling, metabolism and its therapeutic potential in cardiovascular disease. *Biochim. Biophys. Acta Mol. basis Dis.* 1865, 831–843. doi: 10.1016/j.bbadis.2018.09.024

Sun, M., Wu, W., Liu, Z., and Cong, Y. (2017). Microbiota metabolite short chain fatty acids, GPCR, and inflammatory bowel diseases. *J. Gastroenterol.* 52, 1–8. doi: 10.1007/s00535-016-1242-9

Takeda, N., O'Dea, E. L., Doedens, A., Kim, J., Weidemann, A., Stockmann, C., et al. (2010). Differential activation and antagonistic function of HIF-{alpha} isoforms in macrophages are essential for NO homeostasis. *Genes Dev.* 24, 491–501. doi: 10.1101/gad.1881410

Tanaka, T., Wiesener, M., Bernhardt, W., Eckardt, K.-U., and Warnecke, C. (2009). The human HIF (hypoxia-inducible factor)-3alpha gene is a HIF-1 target gene and may modulate hypoxic gene induction. *Biochem. J.* 424, 143–151. doi: 10.1042/BJ20090120

Taylor, C. T., Doherty, G., Fallon, P. G., and Cummins, E. P. (2016). Hypoxia-dependent regulation of inflammatory pathways in immune cells. *J. Clin. Invest.* 126, 3716–3724. doi: 10.1172/JCI84433

Tolonen, J.-P., Heikkilä, M., Malinen, M., Lee, H.-M., Palvimo, J. J., Wei, G.-H., et al. (2020). A long hypoxia-inducible factor 3 isoform 2 is a transcription activator that regulates erythropoietin. *Cell. Mol. Life Sci.* 77, 3627–3642. doi: 10.1007/s00018-019-03387-9

van Uden, P., Kenneth, N. S., Webster, R., Müller, H. A., Mudie, S., and Rocha, S. (2011). Evolutionary conserved regulation of HIF-1β by NF-κB. *PLoS Genet.* 7:e1001285. doi: 10.1371/journal.pgen.1001285

Vivier, E., Artis, D., Colonna, M., Diefenbach, A., Santo, J. P. D., Eberl, G., et al. (2018). Innate lymphoid cells: 10 years on. *Cells* 174, 1054–1066. doi: 10.1016/j.cell.2018.07.017

Wan, J., and Wu, W. (2016). Hyperthermia induced HIF-1a expression of lung cancer through AKT and ERK signaling pathways. *J. Exp. Clin. Cancer Res.* 35:119. doi: 10.1186/s13046-016-0399-7

Wang, R. X., Henen, M. A., Lee, J. S., Vögeli, B., and Colgan, S. P. (2021). Microbiota-derived butyrate is an endogenous HIF prolyl hydroxylase inhibitor. *Gut Microbes* 13:1938380. doi: 10.1080/19490976.2021.1938380

Wang, N., Liang, H., and Zen, K. (2014). Molecular mechanisms that influence the macrophage m1-m2 polarization balance. *Front. Immunol.* 5:614. doi: 10.3389/fimmu.2014.00614

Wang, P., Meng, Q., Wang, W., Zhang, S., Xiong, X., Qin, S., et al. (2020). Icariin inhibits the inflammation through down-regulating NF-κB/HIF-2α signal pathways in chondrocytes. *Biosci. Rep.* 40:11. doi: 10.1042/BSR20203107

Wu, C., Chen, J., Chen, C., Wang, W., Wen, L., Gao, K., et al. (2015). Wnt/β-catenin coupled with HIF-1α/VEGF signaling pathways involved in galangin neurovascular unit protection from focal cerebral ischemia. *Sci. Rep.* 5:16151. doi: 10.1038/srep16151

Wu, D., Huang, R.-T., Hamanaka, R. B., Krause, M., Oh, M.-J., Kuo, C.-H., et al. (2017). HIF-1α is required for disturbed flow-induced metabolic reprogramming in human and porcine vascular endothelium. *elife* 6:e25217. doi: 10.7554/eLife.25217

Wu, D., Su, X., Lu, J., Li, S., Hood, B. L., Vasile, S., et al. (2019). Bidirectional modulation of HIF-2 activity through chemical ligands. *Nat. Chem. Biol.* 15, 367–376. doi: 10.1038/s41589-019-0234-5

Wu, C., Yang, T., Liu, Y., Lu, Y., Yang, Y., Liu, X., et al. (2018). ARNT/HIF-1β links high-risk 1q21 gain and microenvironmental hypoxia to drug resistance and poor prognosis in multiple myeloma. *Cancer Med.* 7, 3899–3911. doi: 10.1002/cam4.1596

Xie, R.-Y., Fang, X.-L., Zheng, X.-B., Lv, W.-Z., Li, Y.-J., Ibrahim Rage, H., et al. (2019). Salidroside and FG-4592 ameliorate high glucose-induced glomerular endothelial cells injury via HIF upregulation. *Biomed. Pharmacother. Biomedecine Pharmacother.* 118:109175. doi: 10.1016/j.biopha.2019.109175

Xie, Y., Shi, X., Sheng, K., Han, G., Li, W., Zhao, Q., et al. (2019). PI3K/Akt signaling transduction pathway, erythropoiesis and glycolysis in hypoxia (review). *Mol. Med. Rep.* 19, 783–791. doi: 10.3892/mmr.2018.9713

Xu, R., Wang, F., Yang, H., and Wang, Z. (2022). Action sites and clinical application of HIF-1α inhibitors. *Mol. Basel Switz.* 27:3426. doi: 10.3390/molecules27113426

Xue, X., Ramakrishnan, S., Anderson, E., Taylor, M., Zimmermann, E. M., Spence, J. R., et al. (2013). Endothelial PAS domain protein 1 activates the inflammatory response in the intestinal epithelium to promote colitis in mice. *Gastroenterology* 145, 831–841. doi: 10.1053/j.gastro.2013.07.010

Yang, S., Kim, J., Ryu, J.-H., Oh, H., Chun, C.-H., Kim, B. J., et al. (2010). Hypoxia-inducible factor-2alpha is a catabolic regulator of osteoarthritic cartilage destruction. *Nat. Med.* 16, 687–693. doi: 10.1038/nm.2153

Yang, C., Zhong, Z.-F., Wang, S.-P., Vong, C.-T., Yu, B., and Wang, Y.-T. (2021). HIF-1: structure, biology and natural modulators. *Chin. J. Nat. Med.* 19, 521–527. doi: 10.1016/S1875-5364(21)60051-1

Yao, G., Zhang, Q., Doeppner, T. R., Niu, F., Li, Q., Yang, Y., et al. (2015). LDL suppresses angiogenesis through disruption of the HIF pathway via NF-κB inhibition which is reversed by the proteasome inhibitor BSc2118. *Oncotarget* 6:30251. doi: 10.18632/oncotarget.4943

Zhang, Q., Han, Z., Zhu, Y., Chen, J., and Li, W. (2021). Role of hypoxia inducible factor-1 in cancer stem cells (review). *Mol. Med. Rep.* 23:17. doi: 10.3892/mmr.2020.11655

Zhang, Z., Yao, L., Yang, J., Wang, Z., and Du, G. (2018). PI3K/Akt and HIF-1 signaling pathway in hypoxia-ischemia (review). *Mol. Med. Rep.* 18, 3547–3554. doi: 10.3892/mmr.2018.9375

Zhao, F., Edwards, R., Dizon, D., Afrasiabi, K., Mastroianni, J. R., Geyfman, M., et al. (2010). Disruption of Paneth and goblet cell homeostasis and increased endoplasmic reticulum stress in Agr2−/− mice. *Dev. Biol.* 338, 270–279. doi: 10.1016/j.ydbio.2009.12.008

Zheng, J., Chen, P., Zhong, J., Cheng, Y., Chen, H., He, Y., et al. (2021). HIF-1α in myocardial ischemia-reperfusion injury (review). *Mol. Med. Rep.* 23:352. doi: 10.3892/mmr.2021.11991

Zhong, J.-C., Li, X.-B., Lyu, W.-Y., Ye, W.-C., and Zhang, D.-M. (2020). Natural products as potent inhibitors of hypoxia-inducible factor-1α in cancer therapy. *Chin. J. Nat. Med.* 18, 696–703. doi: 10.1016/S1875-5364(20)60008-5

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Causal effects of gut microbiome on hypertension: a Mendelian randomization study

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Background: Previous observational studies have shown that there is an important relationship between gut microbiota and hypertension, we performed a two-sample Mendelian randomization analysis to examine whether the gut microbiota is causally related to hypertension in order to find a basis for potential diagnostic or intervention approaches for hypertension.

Methods: We obtained significant single nucleotide polymorphisms related to gut microbiota and hypertension from publicly available genome-wide association studies for a two-sample Mendelian randomization study. A total of 18,340 individual genome-wide genotype data were included from 24 population-based cohorts. The inverse-variance weighted meta-analysis is the main analytical method for evaluating causal relationships, and the Mendelian randomization research results have been validated through a series of sensitivity analyses.

Results: The inverse-variance weighted analysis results indicated that phylum *Verrucomicrobia* (OR:0.831, 95%CI: 0.710–0.972; $p = 0.021$), family *BacteroidalesS24.7group* (OR:0.672, 95%CI: 0.496–0.911; $p = 0.01$), family *Bifidobacteriaceae* (OR:0.709, 95%CI:0.569–0.884, $p = 0.002$), genus *Adlercreutzia* (OR: 0.991, 95%CI: 0.982–0.999, $p = 0.035$), genus *Phascolarctacterium* (OR:0.819, 95%CI:0.685–0.981; $p = 0.03$), genus *LachnospiraceaeNK4A136group* (OR:0.990, 95%CI:0.981–0.999; $p = 0.025$), and genus *Ruminococcus2* (OR:0.988, 95%CI: 0.979–0.997; $p = 0.008$) had protective causal effects on hypertension. The Family *Alcaliginaceae* (OR:1.011, 95%CI:1.000–1.021, $p = 0.04$), Genus *Anaerostipes* (OR:1.375, 95%CI:1.096–1.653; $p = 0.025$), Genus *Collinsella* (OR:1.899, 95%CI:1.361–2.348; $p = 0.02$), and Genus *Lachnospiraceae_UCG_010* (OR:1.536, 95%CI:1.072–2.202; $p = 0.019$) were associated with a higher risk of HTN. The reverse Mendelian randomization analysis results showed no reverse causal relationship between HTN and these bacterial taxa.

Conclusion: Our Mendelian randomization analysis results indicate a potential causal relationship between these bacterial taxa and hypertension, providing a new perspective for the treatment and prevention of hypertension.

KEYWORDS

hypertension, gut microbiota, Mendelian randomization, causal relationship, blood pressure

1 Introduction

As a global public health concern, Hypertension (HTN) is related to a significant global burden of cardiovascular disease and premature death (Mills et al., 2020). Hypertension is also a leading hazard factor for cerebrovascular, cardiovascular, and chronic kidney diseases

(Franklin and Wong, 2013; Mills et al., 2016; Kjeldsen, 2018). Globally, Compared to high-income countries, without effective intervention the increasing burden of hypertension in low- and middle-income countries will exacerbate the global epidemic of cardiovascular and kidney diseases. By 2010, more than 30% of the adult population (1.39 billion) suffered from hypertension, and hypertension is also recognized as the primary cause of global mortality (Zhou et al., 2021). The prevalence of hypertension is steadily rising worldwide due to factors such as an aging population and increased exposure to lifestyle risk factors, including unhealthy diets (such as high alcohol consumption, excessive sodium intake, and insufficient potassium intake) and lack of physical activity (Whelton, 2002; Louca et al., 2020). To effectively prevent and treat hypertension, it is crucial to gain a better understanding of the underlying mechanisms that contribute to its development. However, the exact cause of the increasing incidence rate of hypertension remains to be fully elucidated.

It is believed that the development process of hypertension is multifactorial, and one's predisposition to hypertension is influenced by bot genetic and environmental factors, and the interaction of the two. A multitude of environmental factors increase the risk for HTN, including unbalanced diet, lack of physical activity, overweight and obesity, smoking, and psychological stress (Louca et al., 2020; Tsao et al., 2023). Recently, accumulating evidence has indicated that gut microbiota (GM) composition is closely related to human health and cardiovascular disease, including hypertension, which was strongly supported by at least three systematic reviews (Tang et al., 2017; Muralitharan et al., 2020; Louca et al., 2021). Considerable attention has been paid to the potential role of the gut microbiome in altering the development of hypertension, obesity, type-2 diabetes, and atherosclerosis (Tilg and Kaser, 2011; Howitt and Garrett, 2012; Karlsson et al., 2012; Qin et al., 2012; Tang et al., 2013; Yan et al., 2017). Studies have consistently shown that patients with hypertension exhibit dysbiosis in their gut microbiota, including reduced microbial richness, evenness, and diversity, and an increase in the *Firmicutes*/*Bacteroidetes* ratio (Yang et al., 2015).

Compared with germ-free mice that received an FMT (fecal microbiota transplantation) from 2 normotensive donors, germ-free mice that received FMT from a hypertensive human donor developed a significant increase in diastolic and systolic blood pressure after 8 weeks (Li et al., 2017; Muralitharan et al., 2020). In addition, daily consumption of probiotics for more than 8 weeks can significantly reduce diastolic and systolic blood pressure in hypertensive patients (Khalesi et al., 2014). Oral medication (minocycline) can also regulate blood pressure and normalize the ratio of *Firmicutes* to *Bacteroidetes* in spontaneously hypertensive rats and angiotensin II-induced hypertensive rats (Yang et al., 2015). The observational study showed that the composition and abundance of intestinal microbiota in HTN patients had significant changes compared with the healthy control group. However, while all this research evidence emphasizes the correlation between gut microbiota and HTN, it is still unclear which specific bacterial taxa lead to population differences (Yang et al., 2015; Yan et al., 2017). Confirming whether the correlation between gut microbiota and hypertension is causal and which microbiota taxa are the most important for hypertension is of great significance for the clinical practice of HTN management. Further research on the causal relationship between hypertension and gut microbiota will provide new prospects and perspectives for the treatment and prevention of hypertension and related diseases.

The traditional observational study is vulnerable to the influence of many potential factors such as lifestyle, socioeconomic status, and so on in the implementation process, which is prone to prejudice. Large randomized controlled trials (RCT) or cohort studies for a specific gut microbiome taxa are expensive however, so a new strategy is needed to study the causal effect of gut microbiome taxa on hypertension.

Mendelian randomization (MR) studies use genetic variations associated with modifiable exposure, typically single nucleotide polymorphisms (SNPs), to statistically evaluate the causal relationship between exposure and outcomes, in order to reduce confounding factors (lifestyle, socio-economic factors) and potential biases in reverse causality (Skrivankova et al., 2021). At the same time, MR research can overcome the shortcomings of extrapolation differences and data acquisition difficulties of traditional observational epidemiological research results. The purpose of this study is to explore the causal effects of gut microbiota on hypertension, systolic blood pressure (SBP), and diastolic blood pressure (DBP) using the Genome-Wide Association Study (GWAS) dataset through MR studies.

2 Materials and methods

The summary-level data used in this study was obtained from publicly available GWAS studies. Each cohort involved in the GWAS study received ethical approval and participation consent from their respective institutions, and aggregated data was published for analysis. In short, the gut microbiota is exposure, while hypertension is the outcome. This study employed stringent inclusion and exclusion criteria to select single nucleotide polymorphisms (SNPs) that are strongly associated with specific gut microbiota taxa as instrumental variables (IVs). Sensitivity analyses were performed to assess the robustness of the observed correlations. Furthermore, a reverse Mendelian randomization (MR) analysis was conducted to address potential confounding effects of hypertension on the causal relationship between gut microbiota and health outcomes.

In addition, the MR analysis relies on three key assumptions: (1) the instrumental variables used should exhibit a significant correlation with the exposure of interest. The strength of this correlation is typically evaluated using F-statistics, with a value of $F \geq 10$ indicating no significant evidence of instrumental variable bias. If the F-statistic is less than 10, indicating a weak correlation, the corresponding instrumental variable is excluded. The formula for the F-statistic is F = (beta/se)^2. (2) The instrumental variables should be independent of confounding factors that may influence both the exposure and the outcomes. (3) There should be no horizontal pleiotropy, meaning that the instrumental variables only affect the outcomes through their impact on the exposure. Overall, the study employed rigorous methods to select instrumental variables and ensure the validity of the MR analysis.

2.1 Gut microbiota

The summary data of gut microbiota was obtained from a large-scale multi-ethnic GWAS coordinated by the MiBioGen consortium. As the largest human microbiome genetics study to date, a total of

18,340 individual genome-wide genotype data were included from 24 population-based cohorts (11 countries in Asia, Europe, North America, etc.) (Kurilshikov et al., 2021), and 22 cohorts are composed of adults or adolescents ($n = 16,632$), and two cohorts are composed of children ($n = 1708$). Among the 211 microbiome taxa, it includes five biological classifications: phylum, class, order, family, and genus. Five levels of IV of gut microbiome taxa were extracted from this large-scale GWAS to be applied in this study. The summary statistical data of the gut microbiota association research can be publicly available on the website www.mibiogen.org.

2.2 Hypertension

We obtained the outcome data (blood pressure) from the MR basic database, which is a well-planned database designed to ensure the effective implementation of the Mendelian randomization method. The MR-base database includes 1,674 GWAS datasets[1] (Hemani et al., 2018). To identify relevant studies, we searched for keywords such as "hypertension," "high blood pressure," "systolic blood pressure," and "diastolic blood pressure" in the MR-base database. We focused on studies conducted on the European population up to 2023.

Among the identified studies, we selected the one with the largest sample size as our outcome dataset. The selected dataset, with the ID "ukb-b-14177," is from the MRC Integrative Epidemiology Unit (MRC-IEU) consortium based on the UK Biobank. The UK Biobank is a large and detailed prospective research institute that recruited over 500,000 participants aged 40 to 69 globally between 2006 and 2010 (Sudlow et al., 2015). The "ukb-b-14177" dataset includes 46,188 participants, with 2,076 cases and 460,857 controls. This dataset provides information on the diagnosis of hypertension by doctors. For the outcomes of systolic and diastolic blood pressure, we selected the datasets "ieu-b-38" and "ieu-b-39," respectively. These datasets are based on the International Consortium for Blood Pressure (ICBP), which is a multi-stage design GWAS study on systolic and diastolic blood pressure for 200,000 Europeans (The International Consortium for Blood Pressure Genome-Wide Association Studies, 2011).[2] The "ieu-b-38" and "ieu-b-39" datasets include summary-level data from the ICBP study (Evangelou et al., 2018) (Supplementary Tables). Please note that Supplementary Tables are available for further details.

2.3 Statistical analysis

All statistical analyses in this study were conducted using R software (version 4.1.2). We utilized the R software package "TwoSampleMR" to perform MR analysis investigating the causal relationship between the GM classification group and hypertension. The evaluation indicators for assessing the magnitude of each specific microbiota effect in MR studies were odds ratio (OR) and 95% confidence interval (95% CI). A statistical significance level of $p < 0.05$ was considered as evidence of potential causal effects (Waters and Ley, 2019; Xiang et al., 2021).

To ensure the authenticity and accuracy of the causal relationship between gut microbiome and hypertension, we implemented quality control measures to eliminate the interference of strong linkage imbalance caused by SNPs. This was accomplished through a series of screening settings: (1) SNPs with a value of p threshold of $1 \times 10–5$ were identified based on the genetic group of 18,000 European individuals; (2) The clumping distance between two SNPs was set to 10,000 kb; (3) The correlation coefficient r2 threshold of linkage disequilibrium (LD) between genes is set to 0.001. (4) Palindrome SNPs were removed to prevent the influence of alleles on the causal relationship between gut microbiome taxa and hypertension. (5) In cases where there was no SNP associated with exposure in the outcome GWAS, a proxy SNP significantly associated with the variation of interest ($r^2 > 0.8$) was selected.

The primary analysis method used in this Mendelian randomization (MR) study was inverse variance weighted (IVW) (Burgess et al., 2013; Wang et al., 2021). IVW is a meta-analysis technique that combines ratio estimates with inverse variance weighting, ensuring the validity of each instrumental variable (IV) and accounting for SNP heterogeneity (Burgess et al., 2013; Bowden et al., 2017; Liu et al., 2022). The MR-Egger method, on the other hand, includes an intercept term in the weighted regression to assess horizontal pleiotropy among IVs (Burgess et al., 2017). The presence of a non-zero intercept suggests the presence of horizontal pleiotropy. While MR-Egger provides an estimate of the causal effect, it is less statistically efficient (Bowden et al., 2015). In contrast, the weighted median approach is able to provide consistent estimates of causal effects even when more than 50% of IVs are invalid (Hartwig et al., 2017). The weighted median method has advantages over MR-Egger in terms of result accuracy and maintaining a more precise causal effect estimate (Bowden et al., 2016; Xiang et al., 2021). Additionally, weighted mode and simple mode were used as additional methods for MR analysis (Hartwig et al., 2017; Wu et al., 2020).

To ensure the reliability and robustness of the causality assessment results, sensitivity analyses were performed. Cochrane's Q-test was used to assess heterogeneity among the selected SNPs associated with each bacterial taxa. A value of $p < 0.05$ indicated significant heterogeneity among the IVs. MR-Egger regression was used to test for horizontal pleiotropy among the included SNPs. Furthermore, a weighted median analysis was conducted, which is more robust to individual genetic variants with strong outlier causality estimates. To investigate the causal effect of hypertension (HTN) on the identified significant bacterial genus, a reverse MR analysis was performed (i.e., HTN as exposure and the identified causal bacterial genus as outcome) using SNPs associated with HTN as IVs.

3 Results

Table 1 shows the results of pleiotropy and heterogeneity tests for all bacterial taxa (phylum, order, family, genus) included in the study. In sensitivity analysis, we confirmed the impact of accurate MR results from one phylum, one order, three families, and seven genera on HTN.

Supplementary Tables show the relationship between 211 gut microbiome taxa and HTN; Figure 1 shows the results of Mendelian randomization analysis of three subtypes of hypertension and gut

1 https://gwas.Mrcieu.ac.uk/

2 www.ncbi.nlm.nih.gov/projects/gap/gibin/student.cgistudy_id=phs00585.v1.p1

TABLE 1 The results of pleiotropy and heterogeneity tests for all bacterial taxa.

Outcome	Exposure	Heterogeneity				Pleiotropy		MR-PRESSO	
		MR Egger		IVW		MR Egger		Global Test	
		Cochran's *Q*	*P*-value	Cochran's *Q*	*P*-value	Egger intercept	*P*-value	RSSobs	*P*-value
Systolic blood pressure	Family *BacteroidalesS24.7* group	6.417410448	0.267694319	6.589411516	0.360490811	−0.025533642	0.729287544	8.980260819	0.398
	Family *Prevotellaceae*	23.4754342	0.036312445	23.5033307	0.052556821	0.005568384	0.902986166	26.83959231	0.062
	Genus *Eubacteriumbrachy* group	20.2397522	0.00250993	20.25789365	0.005038763	0.008216542	0.943923509	26.52558077	0.02
	Genus *Collinsella*	4.715105303	0.580837017	5.02017508	0.657501049	0.027865662	0.600675254	6.568510054	0.662
	Genus *Lachnospiraceae UCG010*	6.342391837	0.385947644	8.085511126	0.325114084	−0.047622043	0.246446396	12.40471335	0.288
	Genus *Prevotella7*	29.91594433	0.000218733	30.02320844	0.00043475	0.023547219	0.869714619	37.10243428	<0.002
	Genus *Subdoligranulum*	20.54281123	0.014842612	22.04661013	0.014868904	−0.039179277	0.4379134	26.46365238	0.016
	Genus *Terrisporobacter*	12.78759604	0.005119199	12.97465967	0.011400277	0.020091871	0.847486003	18.86236513	0.104
Diastolic blood pressure	Family *Bifidobacteriaceae*	15.02526844	0.09024423	15.11111161	0.128063133	−0.006208448	0.825678433	17.6722745	0.178
	Family *Prevotellaceae*	17.95935564	0.159068812	17.97445667	0.207946692	0.002359752	0.918327686	20.44074244	0.264
	Genus *Anaerostipes*	13.47582365	0.142230941	14.19156678	0.164431622	−0.018258691	0.506762232	16.92058235	0.204
	Genus *Escherichia.Shigell*a	27.10840464	0.000318633	27.80392816	0.000513003	0.020538919	0.684435849	35.0228526	<0.002
	Genus *Phascolarctobacterium*	6.660894863	0.465019933	7.145382935	0.52103139	0.025052863	0.508840225	8.975238731	0.554
	Genus *Prevotella7*	46.40211668	1.99315E-07	46.40628768	5.06148E-07	−0.002663586	0.979263265	57.00583207	<0.002
	Genus *Terrisporobacter*	3.952220093	0.266669933	6.167996805	0.186948539	0.039684959	0.285404614	11.23244775	0.26
	Order *Bifidobacteriales*	15.02526844	0.09024423	15.11111161	0.128063133	−0.006208448	0.825678433	17.6722745	0.184
	Phylum *Verrucomicrobia*	10.8592147	0.368591242	11.07836421	0.436721724	0.009009972	0.662843233	13.07632936	0.49
Vascular/heart problems diagnosed by doctor: High blood pressure	Family *Alcaligenaceae*	8.984902097	0.533537023	9.632878335	0.563680602	−0.001199876	0.439556577	11.47265556	0.578
	Genus *Eubacteriumbrachy* group	7.982163269	0.435214413	11.08297392	0.270064365	0.00238492	0.116285204	13.57264127	0.32
	Genus *Adlercreutzia*	5.827899736	0.442742582	6.204478903	0.516086812	0.001102424	0.561967397	8.155085342	0.514
	Genus *Lachnospiraceae* NK4A136 group	17.81451668	0.164689992	19.12433776	0.160252445	−0.00068179	0.346083125	21.84675528	0.184
	Genus *Ruminococcus2*	17.04735469	0.197148326	17.12865265	0.24938676	0.000241436	0.807258568	19.56599246	0.254
	Genus *Terrisporobacter*	4.165436759	0.244145183	4.613481409	0.329302769	0.000927873	0.609733334	6.918175552	0.424
	Order *Burkholderiales*	17.01729425	0.048445838	18.49378295	0.047183938	−0.001440391	0.399862741	22.28472063	0.058

10.3389/fmicb.2023.1276050

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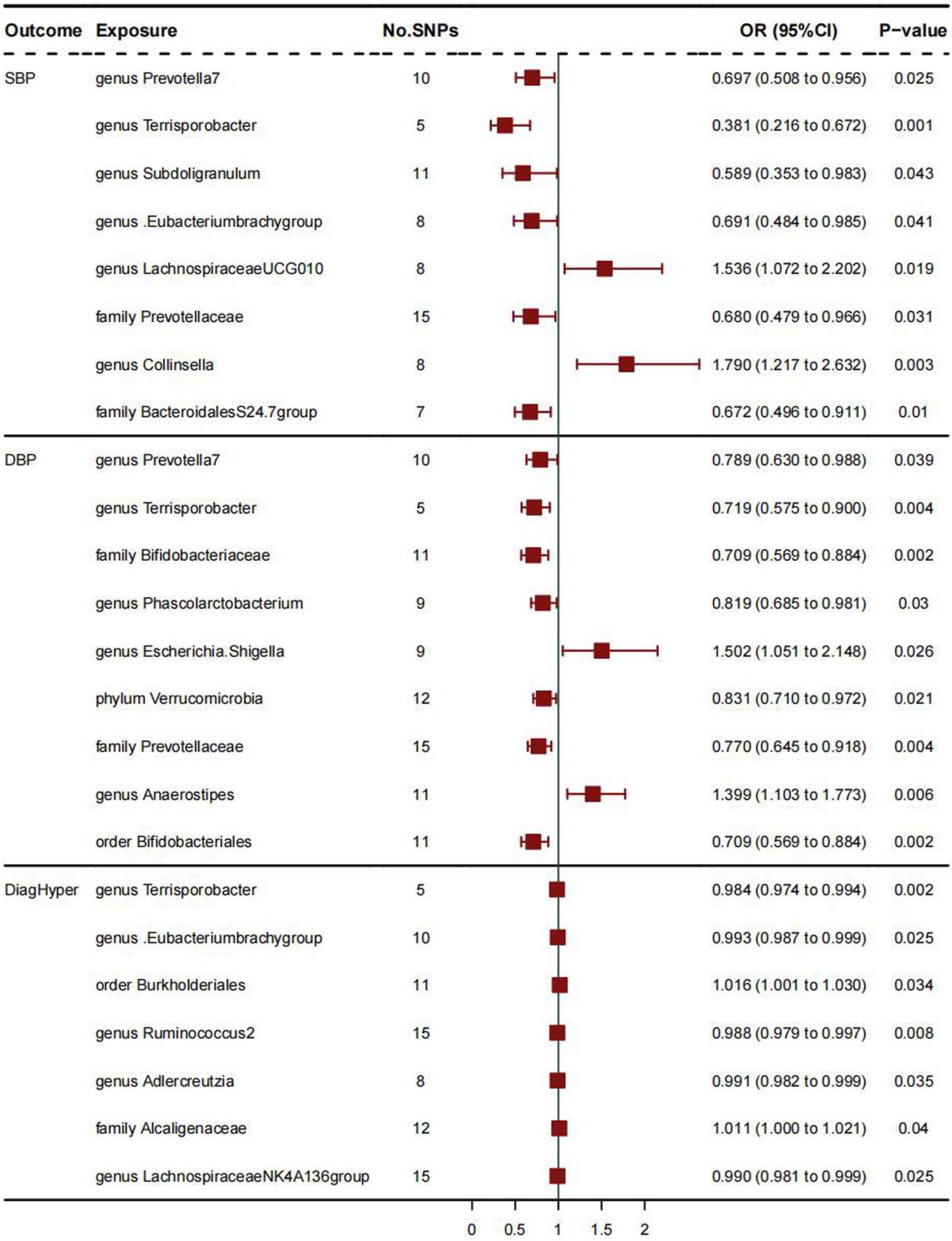

FIGURE 1
The results of Mendelian randomization analysis of three subtypes of hypertension and gut microbiota.

microbiota. For SBP, the IVW results showed a positive correlation between two bacterial traits: Genus *Collinsella* (OR:1.790, 95%CI:1.217–2.632; $p = 0.003$), Genus *Lachnospiraceae_UCG_010* (OR:1.536, 95%CI: 1.072–2.202; $p = 0.019$) and systolic blood pressure, indicating that these two bacteria are risk factors for hypertension. And family *BacteroidalesS24.7group* (OR:0.672, 95%CI:0.496–0.911; $p = 0.01$) is negatively correlated with systolic blood pressure, indicating that these bacterial traits are protective factors for

hypertension. The weighted median MR estimation also indicates that genus *Collinsella* (OR:1.899, 95%CI:1.361–2.348; $p = 0.02$) is a risk factor for systolic blood pressure.

As for DBP, IVW analysis results showed a positive correlation between genus *Anaerostipes* (OR:1.399, 95%CI:1.103–1.773; $p = 0.006$) and diastolic blood pressure, and these bacterial traits are a risk factor for diastolic blood pressure. The weighted mode MR analysis results of Genus *Anaerostipes* (OR:1.375, 95%CI:1.096–1.653; $p = 0.025$) also support this viewpoint. The IVW analysis results of bacterial traits: family *Bifidobacteriaceae* (OR:0.709, 95%CI:0.569–0.884, $p = 0.002$), genus *Phascolarctobacterium* (OR:0.819, 95%CI:0.685–0.981; $p = 0.03$), order *Bifidobacte- rials* (OR:0.709, 95%CI:0.569–0.884; p = 0.002), phylum *Verrucomimicrobia* (OR:0.831, 95%CI:0.710–0.972; $p = 0.021$) indicate that they are protective factors for diastolic blood pressure and negatively correlated with diastolic blood pressure. The MR analysis results of other weighted median values indicate that order *Bifidobacterials* (OR:0.738, 95%CI: 0.478–0.998; $p = 0.022$) is negatively correlated with diastolic blood pressure and has a protective effect on it.

As for DiagHyper (vascular/cardiac problem diagnosed by doctors: hypertension), IVW analysis results showed that family *Alcaligenaceae* (OR:1.011, 95%CI: 1.000–1.021, $p = 0.04$) was positively correlated with hypertension, indicating that these two bacteria are risk factors for hypertension, while genus *Adlercreutzia* (OR: 0.991, 95%CI:0.982–0.999, $p = 0.035$), genus *Lachnospiraceae NK4A136 group* (OR:0.990, 95%CI:0.981–0.999; $p = 0.025$), genus *Ruminococcus2* (OR:0.988, 95%CI:0.979–0.997; $p = 0.008$) were negatively correlated with hypertension, indicating that these bacteria traits are protective factors for hypertension.

4 Discussion

This Two-sample MR study is the first to analyze the causal relationship between gut microbiome taxa and hypertension through multiple datasets. After sensitivity analysis and reverse causality analysis, and the deletion of gut microbiota taxa lacking validity and reliability, The research results indicate that the levels of phylum *Verrucomimicrobia*, family *BacteroidalesS24.7group*, family *Bifidobacteriaceae*, genus *Adlercreutzia*, genus *Phascolarctacterium*, genus *Lachnospiraceae NK4A136 group*, and genus *Ruminococcus2* are negatively correlated with the risk of hypertension, and have a protective causal effect on the pathogenesis of HTN. Family *Alcaligenaceae*, Genus *Anaerostipes*, Genus *Collinsella*, and Genus *Lachnospiraceae- _UCG_010* may be risk factors for the onset of hypertension. The results were examined through some sensitivity analyses—MR-Egger analysis, IVW analysis, and MR-PRESSO Global Test analysis (Verbanck et al., 2018), which is consistent with our findings, and may promote the study of novel biomarkers in future HTN experiments. In the meantime, our results provide novel insights for future HTN prevention and therapeutic treatments: targeted regulation of dysbiosis of specific gut microbiome taxa to prevent and treat HTN.

The gut microbiota has the characteristic of diversity, it is mainly made up of 4 phyla: (1) *Firmicutes*, (2) *Bacteroidetes*, (3) *Actinobacteria*, and (4) *Proteobacteria*. The relative balance of gut microbiota composition plays a key role in maintaining intestinal immunity and systemic homeostasis; the imbalance of gut microbiota is often referred to as microecological imbalance, which is marked by the ratio of *Firmicutes* (F) to *Bacteroides* (B), compared to changes in the microbiota of healthy individuals (Guarner and Malagelada, 2003). Furthermore, some bacteria from the phylum *Firmicutes* are important producers of metabolic products that lower blood pressure, such as short-chain fatty acids (Petersen and Round, 2014). A multitude of studies indicated that there is an association between gut microbiota and hypertension (Yang et al., 2015; Li et al., 2017; Sun et al., 2019). The effect of gut microbiota on blood pressure regulation may be partially explained by the production of short-chain fatty acids (SCFAs) by gut bacteria, including beneficial SCFAs (acetate, butyrate, and propionate) and non-beneficial lactates. Meanwhile, Beli et al. suggested that gut microbiota interventions would be a new method for the prevention and treatment of HTN (Jose and Raj, 2015).

Considering the GM classification group at the phylum level, we found that Phylum *Verrucomimicrobia* is a protective factor for diastolic blood pressure. *Verrucomimicrobia* exists in the inner layer of the intestinal mucosa and is abundant in healthy individuals. They can decompose polysaccharides such as mucopolysaccharides and cellulose, providing energy and nutrients. The *Verrucomimicrobia* can also produce short-chain fatty acids, such as propionic acid and butyric acid, which play an important role in regulating intestinal health and the immune system (Schlesner et al., 2006). At the class level, we did not find a causal relationship between the GM taxa and HTN. It may be because refining the interactions between different taxonomic groups (such as the level of families and genera) can affect the observation results.

Furthermore, at the order level, we found that order *Bifidobacteriales* has a protective causal effect on diastolic blood pressure. Studies have shown that the abundance of *bifidobacteria* is higher in the healthy control group than in HTN patients (Peng et al., 2018). Short-chain fatty acids are produced in the process of fiber fermentation that are difficult to digest, and are one of the most characteristic microbial-derived metabolites. Acetate, propionate, and butyrate are three SCFAs with high abundance (Verhaar et al., 2020). The abundance of *bifidobacteria* in hypertension patients is lower, while *Bifidobacterium*, *Enterococcus,* and *Lactobacillus* are considered as probiotics. These three SCFA-producing microbes can produce SCFA and have multiple health benefits such as anti-inflammatory and beneficial metabolic effects (Hiippala et al., 2018; Parada Venegas et al., 2019). In addition, oral treatment of gut microbiota (specific *bifidobacteria*, *lactobacilli*, and SCFA-producing *Anaerobutyricum soehngenii* species that produce short-chain fatty acids) has a moderate antihypertensive effect on humans (Khalesi et al., 2014; Gilijamse et al., 2020).

At the family level, the family *BacteroidalesS24.7group* is a protective factor for systolic and diastolic blood pressure; research has shown a positive correlation between *Bacteroides* and blood pressure (Palmu et al., 2020). The family *Bifidobacteriaceae* belongs to the order *Bifidobacteriales*, and the analysis of the family *Bifidobacteriaceae* is as above. Family *Alcaligenaceae* is a risk factor for hypertension. Family *Alcaligenaceae*: In animal experiments, it was observed that after FMT transplantation in spontaneously hypertensive rats with normal blood pressure rats, the abundance of family *Alcaligenaceae* decreased in the gut (Adnan et al., 2017).

Unlike other GM and hypertension studies, we further identified three taxa at the genus level that increased the systolic blood pressure risk, five taxa at the genus level that increased the diastolic blood

pressure risk, and four taxa at the genus level that increased the hypertension risk. The research results also showed that the genus *adlercreutzia* is negatively associated with BP Indices (Dan et al., 2019). Palmu et al. (2020) found that Genu *Collinsella* is Positively Associated with BP Indices. Meanwhile, cross-sectional studies in humans showed that the gut microbiota of symptomatic atherosclerosis patients had a higher abundance of the *Collinsella* genus, *Enterobacteriaceae*, *Streptococcaceae*, and *Klebsiella* spp., and a lower abundance of bacteria *Eubacterium*, *Roseburia*, and *Ruminococcaceae* spp. that can produce short chain fatty acids compared with the healthy control group (Karlsson et al., 2012; Jie et al., 2017; Liu et al., 2019). In animal models of hypertension complications (acute myocardial infarction), the gut microbiome, especially the *Lachnospiraceae* family, *Syntrophomonadaceae* family, and *Tissierella soehngenia* genus, exhibit a higher trend (Wu et al., 2017). Cross-sectional studies on gut microbiota composition in hypertension in humans showed a lower abundance of genus *Anaerostipes* in HT. Salt intake in diets will affect the incidence rate of hypertension and the composition of intestinal microbiota. In animal trials, higher salt intake is associated with changes in microbial community composition, including an increase in *Ruminococcus* and *Lachnospiraceae*, as well as a decrease in *Lactobacillus* and *Oscillibacter* (Wilck et al., 2017; Bier et al., 2018). Butyrate is a kind of SCFA, and the microbial community that produces butyric acid includes bacteria from families *Ruminococcaceae* and *Lachnospiraceae*, as well as *Anaerobutyricum hallii* and *Anaerostipes* spp. Our research results also indicated a negative correlation between the *Lactobacillus* genus and some *Lachnospiraceae* genera and blood pressure. The reduction of butyric-acid-producing bacteria is related to inflammatory diseases (including diabetes, obesity, hypertension, and inflammatory bowel disease), which is because butyric acid has an anti-inflammatory effect (Bach Knudsen et al., 2018; Li et al., 2018). Previous studies have shown that butyrate, as the main energy source of colon cells, can regulate tight junction proteins and maintain intestinal barrier integrity (Wu et al., 2019). Onyszkiewicz et al. (2019) found that butyrate enters the bloodstream after passing through the intestinal vascular barrier, and has a vasodilation effect on the mesenteric artery. This process occurs after acting on the G-protein coupled receptor (GPR) (Onyszkiewicz et al., 2019). Wang et al. (2017) found that sodium-butyrate can also inhibit ANGII induced hypertension by inhibiting the renin-angiotensin system mediated by the renin-(pro) renin receptor. In short, butyric acid may play an important role as a differentially beneficial metabolite in the regulation of hypertension. When analyzing the microbiota composition, a negative correlation between the SCFA-producing taxa *Clostridiaceae*, *Ruminococcus*, and *Coprococcus* on women and systolic BP was observed (Durgan, 2017). Bacteria within the *Ruminococcaceae* family are the key SCFAs-producing bacteria, which also play a crucial role in maintaining homeostasis and gut development (Biddle et al., 2013).

The main advantage of our study is that it is the first to use MR analysis to examine the relationship between gut microbiota and hypertension. This approach reduces confounding factors and provides more reliable results compared to observational studies. Additionally, our findings highlight the potential role of *Verrucomimicrobia* in the development of hypertension, which has not been previously reported. This suggests that *Verrucomicronia* may serve as a new biomarker for hypertension. However, our study does have some limitations. First, in terms of sample size, the gut microbiota GWAS contains a relatively small number of samples. Second, the MR study cannot determine whether there is data overlap in the included GWAS summary data. Of course, we have minimized the bias of participant overlap using the F-statistic ($F > 10$). Third, in this MR analysis, we did not find a causal relationship with HTN at the class level. Researchers can expand the sample to explore the relationship between gut microbiome taxa and HTN at the class level in future research.

In summary, our study provides a comprehensive assessment of the causal relationship between gut microbiota and hypertension. We identified eight gut bacteria (phylum *Verrucomicrobia*, order *Bifidobacteriales*, family *BacteroidalesS24.7group*, family *Bifidobacteriaceae*, genus *Adlercreutzia*, genus *Phascolarctobacterium*, genus *Lachnospiraceae NK4A136 group*, and genus *Ruminococcus2*) that have a negative causal relationship with hypertension, making them potential protective factors. Additionally, we found four gut bacteria (family *Alcaligenaceae*, genus *Anaerostipes*, genus *Collinsella*, and genus *Lachnospiraceae_UCG_010*) that have a positive causal relationship with hypertension, indicating they are hazard factors. These strains may serve as new biomarkers for the treatment and prevention of hypertension, providing new insights into the mechanisms underlying gut microbiota-mediated hypertension.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Ethics statement

Each cohort involved in the GWAS study received ethical approval and participation consent from their respective institutions, and aggregated data was published for analysis.

Author contributions

GH: Conceptualization, Methodology, Writing – review & editing, Writing – original draft. YC: Conceptualization, Data curation, Investigation, Methodology, Software, Writing – original draft. HW: Supervision, Validation, Writing – review & editing. XL: Project administration, Resources, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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References

Adnan, S., Nelson, J. W., Ajami, N. J., Venna, V. R., Petrosino, J. F., Bryan, R. M., et al. (2017). Alterations in the gut microbiota can elicit hypertension in rats. *Physiol. Genomics* 49, 96–104. doi: 10.1152/physiolgenomics.00081.2016

Bach Knudsen, K., Lærke, H., Hedemann, M., Nielsen, T., Ingerslev, A., Gundelund Nielsen, D., et al. (2018). Impact of diet-modulated butyrate production on intestinal barrier function and inflammation. *Nutrients* 10:1499. doi: 10.3390/nu10101499

Biddle, A., Stewart, L., Blanchard, J., and Leschine, S. (2013). Untangling the genetic basis of Fibrolytic specialization by Lachnospiraceae and Ruminococcaceae in diverse gut communities. *Diversity* 5, 627–640. doi: 10.3390/d5030627

Bier, A., Braun, T., Khasbab, R., di Segni, A., Grossman, E., Haberman, Y., et al. (2018). A high salt diet modulates the gut microbiota and short chain fatty acids production in a salt-sensitive hypertension rat model. *Nutrients* 10:1154. doi: 10.3390/nu10091154

Bowden, J., Davey Smith, G., and Burgess, S. (2015). Mendelian randomization with invalid instruments: effect estimation and bias detection through Egger regression. *Int. J. Epidemiol.* 44, 512–525. doi: 10.1093/ije/dyv080

Bowden, J., Davey Smith, G., Haycock, P. C., and Burgess, S. (2016). Consistent estimation in Mendelian randomization with some invalid instruments using a weighted median estimator. *Genet. Epidemiol.* 40, 304–314. doi: 10.1002/gepi.21965

Bowden, J., Del Greco, M. F., Minelli, C., Davey Smith, G., Sheehan, N., and Thompson, J. (2017). A framework for the investigation of pleiotropy in two-sample summary data Mendelian randomization: a framework for two-sample summary data MR. *Statist Med.* 36, 1783–1802. doi: 10.1002/sim.7221

Burgess, S., Bowden, J., Fall, T., Ingelsson, E., and Thompson, S. G. (2017). Sensitivity analyses for robust causal inference from Mendelian randomization analyses with multiple genetic variants. *Epidemiology* 28, 30–42. doi: 10.1097/EDE.0000000000000559

Burgess, S., Butterworth, A., and Thompson, S. G. (2013). Mendelian randomization analysis with multiple genetic variants using summarized data. *Genet. Epidemiol.* 37, 658–665. doi: 10.1002/gepi.21758

Dan, X., Mushi, Z., Baili, W., Han, L., Enqi, W., Huanhu, Z., et al. (2019). Differential analysis of hypertension-associated intestinal microbiota. *Int. J. Med. Sci.* 16, 872–881. doi: 10.7150/ijms.29322

Durgan, D. J. (2017). Obstructive sleep apnea-induced hypertension: role of the gut microbiota. *Curr. Hypertens. Rep.* 19:35. doi: 10.1007/s11906-017-0732-3

Evangelou, E., Warren, H. R., Mosen-Ansorena, D., Mifsud, B., Pazoki, R., Gao, H., et al. (2018). Genetic analysis of over 1 million people identifies 535 new loci associated with blood pressure traits. *Nat. Genet.* 50, 1412–1425. doi: 10.1038/s41588-018-0205-x

Franklin, S. S., and Wong, N. D. (2013). Hypertension and cardiovascular disease: contributions of the Framingham heart study. *Gh* 8:49. doi: 10.1016/j.gheart.2012.12.004

Gilijamse, P. W., Hartstra, A. V., Levin, E., Wortelboer, K., Serlie, M. J., Ackermans, M. T., et al. (2020). Treatment with *Anaerobutyricum soehngenii*: a pilot study of safety and dose–response effects on glucose metabolism in human subjects with metabolic syndrome. *Npj Biofilms Microbiomes* 6:16. doi: 10.1038/s41522-020-0127-0

Guarner, F., and Malagelada, J.-R. (2003). Gut flora in health and disease. *Lancet* 361, 512–519. doi: 10.1016/S0140-6736(03)12489-0

Hartwig, F. P., Davey Smith, G., and Bowden, J. (2017). Robust inference in summary data Mendelian randomization via the zero modal pleiotropy assumption. *Int. J. Epidemiol.* 46, 1985–1998. doi: 10.1093/ije/dyx102

Hemani, G., Zheng, J., Elsworth, B., Wade, K. H., Haberland, V., Baird, D., et al. (2018). The MR-base platform supports systematic causal inference across the human phenome. *eLife* 7:e34408. doi: 10.7554/eLife.34408

Hiippala, K., Jouhten, H., Ronkainen, A., Hartikainen, A., Kainulainen, V., Jalanka, J., et al. (2018). The potential of gut commensals in reinforcing intestinal barrier function and alleviating inflammation. *Nutrients* 10:988. doi: 10.3390/nu10080988

Howitt, M. R., and Garrett, W. S. (2012). A complex microworld in the gut: gut microbiota and cardiovascular disease connectivity. *Nat. Med.* 18, 1188–1189. doi: 10.1038/nm.2895

Jie, Z., Xia, H., Zhong, S.-L., Feng, Q., Li, S., Liang, S., et al. (2017). The gut microbiome in atherosclerotic cardiovascular disease. *Nat. Commun.* 8:845. doi: 10.1038/s41467-017-00900-1

Jose, P. A., and Raj, D. (2015). Gut microbiota in hypertension. *Curr. Opin. Nephrol. Hypertens.* 24, 403–409. doi: 10.1097/MNH.0000000000000149

Karlsson, F. H., Fåk, F., Nookaew, I., Tremaroli, V., Fagerberg, B., Petranovic, D., et al. (2012). Symptomatic atherosclerosis is associated with an altered gut metagenome. *Nat. Commun.* 3:1245. doi: 10.1038/ncomms2266

Khalesi, S., Sun, J., Buys, N., and Jayasinghe, R. (2014). Effect of probiotics on blood pressure: a systematic review and Meta-analysis of randomized, controlled trials. *Hypertension* 64, 897–903. doi: 10.1161/HYPERTENSIONAHA.114.03469

Kjeldsen, S. E. (2018). Hypertension and cardiovascular risk: general aspects. *Pharmacol. Res.* 129, 95–99. doi: 10.1016/j.phrs.2017.11.003

Kurilshikov, A., Medina-Gomez, C., Bacigalupe, R., Radjabzadeh, D., Wang, J., Demirkan, A., et al. (2021). Large-scale association analyses identify host factors influencing human gut microbiome composition. *Nat. Genet.* 53, 156–165. doi: 10.1038/s41588-020-00763-1

Li, M., van Esch, B. C. A. M., Henricks, P. A. J., Folkerts, G., and Garssen, J. (2018). The anti-inflammatory effects of short chain fatty acids on lipopolysaccharide- or tumor necrosis factor α-stimulated endothelial cells via activation of GPR41/43 and inhibition of HDACs. *Front. Pharmacol.* 9:533. doi: 10.3389/fphar.2018.00533

Li, J., Zhao, F., Wang, Y., Chen, J., Tao, J., Tian, G., et al. (2017). Gut microbiota dysbiosis contributes to the development of hypertension. *Microbiome* 5:14. doi: 10.1186/s40168-016-0222-x

Liu, H., Chen, X., Hu, X., Niu, H., Tian, R., Wang, H., et al. (2019). Alterations in the gut microbiome and metabolism with coronary artery disease severity. *Microbiome* 7:68. doi: 10.1186/s40168-019-0683-9

Liu, K., Zou, J., Fan, H., Hu, H., and You, Z. (2022). Causal effects of gut microbiota on diabetic retinopathy: a Mendelian randomization study. *Front. Immunol.* 13:930318. doi: 10.3389/fimmu.2022.930318

Louca, P., Mompeo, O., Leeming, E. R., Berry, S. E., Mangino, M., Spector, T. D., et al. (2020). Dietary influence on systolic and diastolic blood pressure in the TwinsUK cohort. *Nutrients* 12:2130. doi: 10.3390/nu12072130

Louca, P., Nogal, A., Wells, P. M., Asnicar, F., Wolf, J., Steves, C. J., et al. (2021). Gut microbiome diversity and composition is associated with hypertension in women. *J. Hypertens.* 39, 1810–1816. doi: 10.1097/HJH.0000000000002878

Mills, K. T., Bundy, J. D., Kelly, T. N., Reed, J. E., Kearney, P. M., Reynolds, K., et al. (2016). Global disparities of hypertension prevalence and control: a systematic analysis of population-based studies from 90 countries. *Circulation* 134, 441–450. doi: 10.1161/CIRCULATIONAHA.115.018912

Mills, K. T., Stefanescu, A., and He, J. (2020). The global epidemiology of hypertension. *Nat. Rev. Nephrol.* 16, 223–237. doi: 10.1038/s41581-019-0244-2

Muralitharan, R. R., Jama, H. A., Xie, L., Peh, A., Snelson, M., and Marques, F. Z. (2020). Microbial Peer pressure: the role of the gut microbiota in hypertension and its complications. *Hypertension* 76, 1674–1687. doi: 10.1161/HYPERTENSIONAHA.120.14473

Onyszkiewicz, M., Gawrys-Kopczynska, M., Konopelski, P., Aleksandrowicz, M., Sawicka, A., Koźniewska, E., et al. (2019). Butyric acid, a gut bacteria metabolite, lowers arterial blood pressure via colon-vagus nerve signaling and GPR41/43 receptors. *Pflugers Arch. Eur. J. Physiol.* 471, 1441–1453. doi: 10.1007/s00424-019-02322-y

Palmu, J., Salosensaari, A., Havulinna, A. S., Cheng, S., Inouye, M., Jain, M., et al. (2020). Association between the gut microbiota and blood pressure in a population cohort of 6953 individuals. *JAHA* 9:e016641. doi: 10.1161/JAHA.120.016641

Parada Venegas, D., de la Fuente, M. K., Landskron, G., González, M. J., Quera, R., Dijkstra, G., et al. (2019). Short chain fatty acids (SCFAs)-mediated gut epithelial and immune regulation and its relevance for inflammatory bowel diseases. *Front. Immunol.* 10:277. doi: 10.3389/fimmu.2019.00277

Peng, J., Xiao, X., Hu, M., and Zhang, X. (2018). Interaction between gut microbiome and cardiovascular disease. *Life Sci.* 214, 153–157. doi: 10.1016/j.lfs.2018.10.063

Petersen, C., and Round, J. L. (2014). Defining dysbiosis and its influence on host immunity and disease. *Cell. Microbiol.* 16, 1024–1033. doi: 10.1111/cmi.12308

Qin, J., Li, Y., Cai, Z., Li, S., Zhu, J., Zhang, F., et al. (2012). A metagenome-wide association study of gut microbiota in type 2 diabetes. *Nature* 490, 55–60. doi: 10.1038/nature11450

Schlesner, H., Jenkins, C., and Staley, J. T. (2006). "The phylum Verrucomicrobia: a phylogenetically heterogeneous bacterial group" in *The prokaryotes*. eds. M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer and E. Stackebrandt (New York, NY: Springer New York)

Skrivankova, V. W., Richmond, R. C., Woolf, B. A. R., Yarmolinsky, J., Davies, N. M., Swanson, S. A., et al. (2021). Strengthening the reporting of observational studies in epidemiology using Mendelian randomization: the STROBE-MR statement. *JAMA* 326:1614. doi: 10.1001/jama.2021.18236

Sudlow, C., Gallacher, J., Allen, N., Beral, V., Burton, P., Danesh, J., et al. (2015). UK biobank: an open access resource for identifying the causes of a wide range of complex diseases of middle and old age. *PLoS Med.* 12:e1001779. doi: 10.1371/journal.pmed.1001779

Sun, S., Lulla, A., Sioda, M., Winglee, K., Wu, M. C., Jacobs, D. R., et al. (2019). Gut microbiota composition and blood pressure: the CARDIA study. *Hypertension* 73, 998–1006. doi: 10.1161/HYPERTENSIONAHA.118.12109

Tang, W. H. W., Kitai, T., and Hazen, S. L. (2017). Gut microbiota in cardiovascular health and disease. *Circ. Res.* 120, 1183–1196. doi: 10.1161/CIRCRESAHA.117.309715

Tang, W. H. W., Wang, Z., Levison, B. S., Koeth, R. A., Britt, E. B., Fu, X., et al. (2013). Intestinal microbial metabolism of phosphatidylcholine and cardiovascular risk. *N. Engl. J. Med.* 368, 1575–1584. doi: 10.1056/NEJMoa1109400

The International Consortium for Blood Pressure Genome-Wide Association Studies (2011). Genetic variants in novel pathways influence blood pressure and cardiovascular disease risk. *Nature* 478, 103–109. doi: 10.1038/nature10405

Tilg, H., and Kaser, A. (2011). Gut microbiome, obesity, and metabolic dysfunction. *J. Clin. Invest.* 121, 2126–2132. doi: 10.1172/JCI58109

Tsao, C. W., Aday, A. W., Almarzooq, Z. I., Anderson, C. A. M., Arora, P., Avery, C. L., et al. (2023). Heart disease and stroke statistics—2023 update: a report from the American Heart Association. *Circulation* 147, e93–e621. doi: 10.1161/CIR.0000000000001123

Verbanck, M., Chen, C.-Y., Neale, B., and Do, R. (2018). Detection of widespread horizontal pleiotropy in causal relationships inferred from Mendelian randomization between complex traits and diseases. *Nat. Genet.* 50, 693–698. doi: 10.1038/s41588-018-0099-7

Verhaar, B. J. H., Prodan, A., Nieuwdorp, M., and Muller, M. (2020). Gut microbiota in hypertension and atherosclerosis: a review. *Nutrients* 12:2982. doi: 10.3390/nu12102982

Wang, M., Jian, Z., Gao, X., Yuan, C., Jin, X., Li, H., et al. (2021). Causal associations between educational attainment and 14 urological and reproductive health outcomes: a Mendelian randomization study. *Front. Public Health* 9:742952. doi: 10.3389/fpubh.2021.742952

Wang, L., Zhu, Q., Lu, A., Liu, X., Zhang, L., Xu, C., et al. (2017). Sodium butyrate suppresses angiotensin II-induced hypertension by inhibition of renal (pro)renin receptor and intrarenal renin–angiotensin system. *J. Hypertens.* 35, 1899–1908. doi: 10.1097/HJH.0000000000001378

Waters, J. L., and Ley, R. E. (2019). The human gut bacteria Christensenellaceae are widespread, heritable, and associated with health. *BMC Biol.* 17:83. doi: 10.1186/s12915-019-0699-4

Whelton, P. K. (2002). Primary prevention of HypertensionClinical and public health advisory from the National High Blood Pressure Education Program. *JAMA* 288, 1882–1888. doi: 10.1001/jama.288.15.1882

Wilck, N., Matus, M. G., Kearney, S. M., Olesen, S. W., Forslund, K., Bartolomaeus, H., et al. (2017). Salt-responsive gut commensal modulates TH17 axis and disease. *Nature* 551, 585–589. doi: 10.1038/nature24628

Wu, F., Huang, Y., Hu, J., and Shao, Z. (2020). Mendelian randomization study of inflammatory bowel disease and bone mineral density. *BMC Med.* 18:312. doi: 10.1186/s12916-020-01778-5

Wu, Z.-X., Li, S.-F., Chen, H., Song, J.-X., Gao, Y.-F., Zhang, F., et al. (2017). The changes of gut microbiota after acute myocardial infarction in rats. *PLoS One* 12:e0180717. doi: 10.1371/journal.pone.0180717

Wu, D., Tang, X., Ding, L., Cui, J., Wang, P., du, X., et al. (2019). Candesartan attenuates hypertension-associated pathophysiological alterations in the gut. *Biomed. Pharmacother.* 116:109040. doi: 10.1016/j.biopha.2019.109040

Xiang, K., Wang, P., Xu, Z., Hu, Y.-Q., He, Y.-S., Chen, Y., et al. (2021). Causal effects of gut microbiome on systemic lupus erythematosus: a two-sample Mendelian randomization study. *Front. Immunol.* 12:667097. doi: 10.3389/fimmu.2021.667097

Yan, Q., Gu, Y., Li, X., Yang, W., Jia, L., Chen, C., et al. (2017). Alterations of the gut microbiome in hypertension. *Front. Cell. Infect. Microbiol.* 7:381. doi: 10.3389/fcimb.2017.00381

Yang, T., Santisteban, M. M., Rodriguez, V., Li, E., Ahmari, N., Carvajal, J. M., et al. (2015). Gut Dysbiosis is linked to hypertension. *Hypertension* 65, 1331–1340. doi: 10.1161/HYPERTENSIONAHA.115.05315

Zhou, B., Carrillo-Larco, R. M., Danaei, G., Riley, L. M., Paciorek, C. J., Stevens, G. A., et al. (2021). Worldwide trends in hypertension prevalence and progress in treatment and control from 1990 to 2019: a pooled analysis of 1201 population-representative studies with 104 million participants. *Lancet* 398, 957–980. doi: 10.1016/S0140-6736(21)01330-1

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Linkages between rumen microbiome, host, and environment in yaks, and their implications for understanding animal production and management

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Livestock on the Qinghai-Tibetan Plateau is of great importance for the livelihood of the local inhabitants and the ecosystem of the plateau. The natural, harsh environment has shaped the adaptations of local livestock while providing them with requisite eco-services. Over time, unique genes and metabolic mechanisms (nitrogen and energy) have evolved which enabled the yaks to adapt morphologically and physiologically to the Qinghai-Tibetan Plateau. The rumen microbiota has also co-evolved with the host and contributed to the host's adaptation to the environment. Understanding the complex linkages between the rumen microbiota, the host, and the environment is essential to optimizing the rumen function to meet the growing demands for animal products while minimizing the environmental impact of ruminant production. However, little is known about the mechanisms of host-rumen microbiome-environment linkages and how they ultimately benefit the animal in adapting to the environment. In this review, we pieced together the yak's adaptation to the Qinghai-Tibetan Plateau ecosystem by summarizing the natural selection and nutritional features of yaks and integrating the key aspects of its rumen microbiome with the host metabolic efficiency and homeostasis. We found that this homeostasis results in higher feed digestibility, higher rumen microbial protein production, higher short-chain fatty acid (SCFA) concentrations, and lower methane emissions in yaks when compared with other low-altitude ruminants. The rumen microbiome forms a multi-synergistic relationship among the rumen microbiota services, their communities, genes, and enzymes. The rumen microbial proteins and SCFAs act as precursors that directly impact the milk composition or adipose accumulation, improving the milk or meat quality, resulting in a higher protein and fat content in yak milk and a higher percentage of protein and abundant fatty acids in yak meat when compared to dairy cow or cattle. The hierarchical interactions between the climate, forage, rumen microorganisms, and host genes have

reshaped the animal's survival and performance. In this review, an integrating and interactive understanding of the host-rumen microbiome environment was established. The understanding of these concepts is valuable for agriculture and our environment. It also contributes to a better understanding of microbial ecology and evolution in anaerobic ecosystems and the host-environment linkages to improve animal production.

KEYWORDS

environmental adaptation, host metabolic regulations, rumen microbiome, host-rumen microbiome-environment linkages, management implications

1 Introduction

Ruminants play an essential role in global human societies due to their unique ability, via their rumen microbiome, to convert low-quality feedstuffs into valuable animal products such as milk and meat for human consumption. The rumen microbiota exerts a profound influence on dietary nutrient metabolism, the quality of animal products, animal production, and the environment (Mizrahi et al., 2021). Over the past decades, the rumen microbiome has emerged as central to tackling major challenges associated with the global demand for agriculture, rising animal protein demands (milk and meat products, 63% and 76%, respectively) (Alexandratos and Bruinsma, 2012; Huws et al., 2018), approximately 18% of total methane (CH_4) emissions from all anthropogenic sources (Mizrahi et al., 2021), and sustainable and efficient ruminant production along with land constraints (Huws et al., 2018).

Ruminant performance is not only affected by host genetics but also by the environment and the microorganisms that inhabit the rumen (Brito et al., 2020; Mizrahi et al., 2021). Genetics and environmental determinants and their interactions have guided empirical and theoretical research in animal production and ecology for decades (Brinks et al., 1962; Angilletta and Sears, 2011). Studies have reported that rumen microorganisms can provide more than 70% of the host's metabolic and protein requirements (Siciliano-Jones and Murphy, 1989; Bergman, 1990). The integrated interactions between the host, rumen microbiome, and the environment, therefore, mutually contribute to animal performance. Understanding the complex linkages between the rumen microbiota, the host, and the environment is essential to optimize rumen function to meet the growing demands for animal products while concurrently minimizing the environmental impact of ruminant production (Huws et al., 2018; Mizrahi et al., 2021). However, to date, little is known about the mechanisms related to host-rumen microbiome-environment linkages and how they ultimately benefit the animal in adapting to changes to optimize their performance.

More than 14 million yaks (*Bos grunniens*) are raised on the Qinghai-Tibetan Plateau. They are essential for the livelihood of the local inhabitants as they provide meat, milk, dung, fiber, and transport (Long et al., 1999a). Under the harsh environmental conditions, yaks have evolved and adapted themselves morphologically (Shao et al., 2010), physiologically (Ishizaki et al., 2005), and genetically (Qiu et al., 2012) to the severe Qinghai-Tibetan Plateau (QTP). Yaks are more efficient in utilizing the poor-quality, high-fiber forage that is mainly available for long periods on the QTP. Previous studies have reported that the rumen microbiota in yaks enables the host to survive the extreme environment (Zhang Z. et al., 2016; Mizrahi et al., 2021). In this review, we piece together the yak's unique adaptation to the QTP ecosystem by describing the rumen microbiome of yaks and the obligatory dependence of yaks on their microbes for the degradation and digestion of the plants they ingest. The linkages between host genes, metabolisms, and rumen microorganisms that coordinate and affect the quality of yak milk and meat are clarified. The review offers a systemic, integrated perspective on the host-rumen microbiome-environment linkages while attempting to decipher their key interactions for the purpose of understanding and regulating animal performance as a whole.

2 The harsh environment shapes the unique nutritional deprivation adaptation of yak

The Qinghai-Tibetan Plateau, regarded as the "Third Pole" and commonly referred to as the "Roof of the World," has an average elevation exceeding 4,000 m above sea level (m a.s.l.). The high-altitude Qinghai-Tibetan Plateau is characterized by severe cold, low atmospheric pressure, oxygen partial pressure, and intense ultraviolet light, resulting in a short forage growing season (Long et al., 2008). Winters are particularly severe for grazing livestock, as the availability of forage is frequently insufficient and of subpar quality. The cold season could last for 8 months, especially in the dry forage phase in autumn and winter, during which time the cellulose and lignin contents gradually increase while the crude protein content decreases (Figure 1) (Long et al., 1999a). Neutral detergent fiber (NDF) contents account for up to 65.7% and crude protein account for only 2.96%−6.81% of the pasture dry matter from November to April (Xie and Chai, 1996; Ding et al., 2014). This nutritional shortage could result in a body weight loss of 30% or could even cause the death of the livestock (Long et al., 1999b). Such harsh environmental conditions have shaped the yaks' extraordinary nutritional deprivation adaptations to the harsh QTP. Yaks have demonstrated distinct energy and protein metabolic adaptations and host gene regulation.

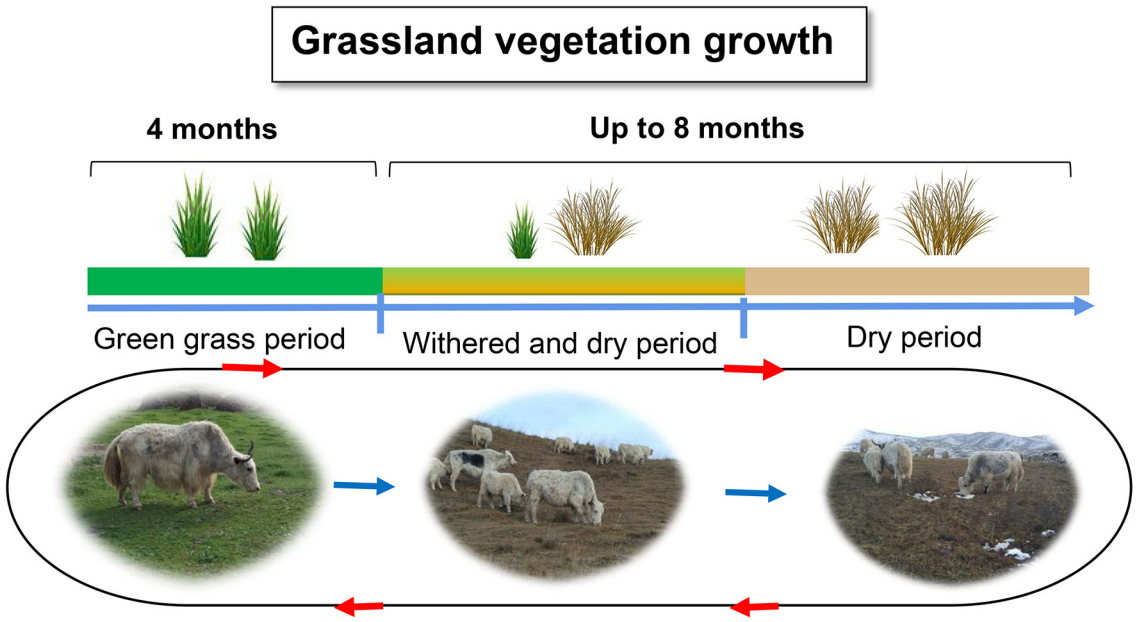

FIGURE 1
Yaks exposed to natural pastures of different qualities.

2.1 Energy metabolism

Basal energy metabolism is defined as the minimal level of heat production during complete rest in a thermoneutral environment. It was estimated that the maintenance energy requirement for yaks is 458 kJ/kg $BW^{0.75}$ (Han and Xie, 1991), and heat production scales to $BW^{0.52}$ rather than $BW^{0.75}$ (Hu, 1994; Hu et al., 2002). Diverging from other mammals, yaks exhibit a reduced metabolic rate as air temperature decreases, thereby employing a unique way of energy conservation (Han et al., 2002; Ding et al., 2014). At the lowest of the three elevations in the trials conducted by Hu (2001), the absolute fasting heat production of yaks exceeded that of yellow cattle (*Bos taurus*). However, such a distinction was not evident at the higher altitudes in their study. At 1–3 years of age, fasting heat production per day of yak at the altitudes of 3,250 m and 4,271 m were 329 kJ/kg$W^{0.75}$ and 281–376 kJ/kg$W^{0.75}$, respectively, compared to yellow cattle at 353–414 kJ/kg$W^{0.75}$ and 360–516 kJ/kg$W^{0.75}$, respectively (Hu and Xie, 1992; Han et al., 2002).

2.2 Nitrogen metabolism

Several studies have reported a higher efficiency of utilization of dietary nitrogen (N) in yaks than in lower-altitude cattle (Long et al., 1999c; Xue and Han, 2001; Wang et al., 2011). A few other studies reported the low N requirements for maintenance [0.40–0.53 g/(kg $W^{0.75}$·d)] by yaks (Hu, 2001; Long et al., 2004). For instance, Guo et al. (2012) found that 87% of the urea synthesized in the liver of yak could be recycled into the digestive tract, providing nitrogen for rumen microbes to synthesize microbial proteins. With low N intake, rumen microbes are almost the only source of digestible protein for the host (Ørskov, 1982). Under such conditions, yaks utilized urea-N to meet the requirements of nitrogen for nearly 6 months (Wang et al., 2002), which could well point to an adaptive response of the yak to life at high altitudes and to the nutritional deprivation that yaks experience in winter and early spring.

2.3 Genes' regulation

Gene families related to nutrition assimilation and utilization and energy metabolism have expanded dramatically in the yak genome (Qiu et al., 2012). Qiu et al. (2012) linked nutrition metabolism from the field of yak host genes in comparison with cattle. These authors' study revealed five genes that were involved in integrated nutrition pathways and positively selected in the yak lineage. Among the genes, *HSD17B12*, *GLUL*, *GCNT3*, and *WHSCL* play important roles in the metabolism of fatty acids, amino acids, and polysaccharides. *GLUL* may be vital for the high level of nitrogen utilization in yaks (Qiu et al., 2012). Correspondingly, in nitrogen cycling, the PepT1 expression was found to be enriched in the yak epithelium of the small intestine compared to cattle epithelium (Wang et al., 2016). The positively selected changes in *CAMK2B* play a regulatory role in the secretion of gastric acid in the rumen, thereby contributing to the assimilation of short-chain fatty acids (SCFAs) that provide 70% of metabolic energy for the host and are produced by ruminal fermentation (Bergman, 1990; Qiu et al., 2012). Through the transcriptome analysis of rumen wall epithelial cells in yak and cattle, it was further revealed that 36 genes associated with the energy (SCFAs) and translocation were upregulated in yak compared to cattle. The genes were the following: SCFA transport: *PLA2G5*, *FABP3*, *CLCN1*, *GABRA3*, *BEST1*, *SLC12A3*, *SLC4A11*, *P2RY4*, *P2RY6*, *SLC4A7*, *SLC20A2*,

SLC13A3, *SLC4A3*, *SLC13A5*, *SLC6A6*, and *SLC16A6*; fatty acid metabolic process: *ALOX5AP*, *SYK*, *ABCD1*, *CPT1C*, *PLA2G15*, *PTGES*, *LTC4S*, *PRKAA1*, *PRKAB2*, *BRCA1*, and *MCAT*; regulation of carbohydrate metabolic process: *IFNG*, *DYRK2*, and *SPDYA*; Glycolysis: *LDHC*, *PKLR*, and *HK3*; organic acid catabolic process: *DDO*; and pyruvate metabolic process: *PC* and *ENO2* (Zhang Z. et al., 2016).

3 Rumen microbiome services are synergetic linkages with nutritional deprivation

The host functions as an ecosystem within which microbial processes play out. The gut microbiota of importance in ruminants maintains a healthy state for the host, especially the rumen microbiota. The biomass of rumen microorganisms is comprised of protozoa (∼50%), fungi (∼8%), bacteria (largely distributed), archaea (0.3%−4%), and little proportion of as yet largely uncharacterized viruses, especially phages (Tapio et al., 2017; Gilbert et al., 2020; Lobo and Faciola, 2021). In comparison with cattle, rumen microbial activity provides the following services for yaks:

3.1 Lower methane emission

Methane (CH_4), a byproduct from ruminal fermentation by methanogens, results in energy loss, and the energy equivalent amounts to 2%−15% of gross energy intake (Moss et al., 2000; Shabat et al., 2016). Annual CH_4 emissions from ruminants are approximately 2.1 GtCO2e (gigatons of carbon dioxide equivalent), accounting for 20.2% of global CH_4 emissions (IPCC, 2019). Methane emission of yaks is less than other ruminants and has been confirmed through various approaches (mask, SF_6, model estimation, and *in vitro* gas technique) (Figures 2A, B, Supplementary Tables 1, 2), indicating that yaks are low-carbon emission animals and are proven to have high efficiency (Shabat et al., 2016). The rumen microbiome determines the amount of CH_4 production.

Studies of bacteria reported that S24-7, *Butyrivibrio*, *Shwartzia*, *Treponema*, *Clostridium*, RFP12, *Coriobacteriaceae*, and *Methanosphaera* reduce CH_4 emissions and improve animal production performance (Cunha et al., 2017). Under grazing conditions, the core genus (relative abundance >0.5%) of the yak is YRC22, with unidentifiable BS11 and BF311 and unidentified S24-7 and CF231 in *Bacteroidetes*; *Treponema* in *Spirochaetes*; *Clostridium sensustricto* in *Firmicutes*; and unrecognized RFP12 in *Verrucomicrobia*. *Treponema* and RFP12 are enriched in the grazing yak (Xue et al., 2017), which might be associated with the lower enteric CH_4 emission.

It has been reported that the percentage of archaea in the rumen microorganisms is only approximately 0.3%−4%, but the percentage is vital as hydrogen sinks in the rumen and for CH_4 emissions from ruminants. It is speculated that the low-methane emission trait may be due to the high diversity of archaea; the archaeal diversity in grazing yaks is higher than that in yellow cattle (Huang et al., 2016), which was also reported in another study (Xue et al., 2016). A high diversity in low CH_4 emitters has been confirmed by Auffret et al. (2017). *Methanobrevibacter* (>60%), *Methanomicrobium* (∼15%), and *Methanomassiliicoccales* (∼16%) are the abundant genera in the archaeal community (St-Pierre and Wright, 2012; Borrel et al., 2014), while *Methanobacteriales* and *Thermgymnomonas* have been reported as the dominant archaea in yaks (Huang et al., 2012, 2016; Mi, 2016; Wang et al., 2017). *Thermogymnomonas*, which belongs to Thermoplasmatales-affiliated Lineage C (TALC), has the highest abundance. *Methanobrevibacter*, composing only 25% (Wang et al., 2017), and a large number of unknown methanogen TALCs were found in yaks (Huang et al., 2012). *Thermoplasmatales*, belonging to the family *Methanomassiliicocaceae* (Rumen cluster C, RCC), is a major component of methanogens (Janssen and Kirs, 2008; Poulsen et al., 2013; Borrel et al., 2014) with a methylotrophic methanogenesis pathway; methylotrophic methanogens, belonging to the class Thermoplasmata, have been associated with decreased CH_4 production (Poulsen et al., 2013); and relative abundances are significantly higher in yaks than in cattle (*Bos taurus*) (Zhang Z. et al., 2016). Although the majority of methane is generated via the hydrogenotrophic methanogenesis pathway, i.e., utilizing H_2 and CO_2 or formate as substrates rather than acetate, the methylotrophic methanogenesis pathway, which employs methanol and methylamines as substrates, also contributes to a certain extent (Carberry et al., 2013), indicating that the methanogenesis pathway is unusual in yaks. *Methanomassiliicoccales* could also provide natural protection in the gut (Brugère et al., 2014) and tend to be more abundant in high-feed-efficiency ruminants (Li et al., 2016; Li and Guan, 2017). *Methanobrevibacter gottschalkii*, an archaea of higher abundance in cattle than yak (Zhang Z. et al., 2016), has also been correlated positively with CH_4 emission (Tapio et al., 2017).

The density of protozoa in the rumen is between 10^4 and 10^6/ml, and its biomass is large, accounting for more than half of the rumen microorganisms. The total number of ciliates ranges from 0.7 to 8.5 × 10^5/ml in yaks (Bi et al., 1989; Xie et al., 1989; Gui et al., 2000; Yao et al., 2002), which is lower than that in cattle (*Bos taurus*) and buffaloes (*Bubalus bubalis*) (Ito et al., 1994; Chaudhary et al., 2000). Rumens with fewer protozoa possess fewer methanogens, which may be one of the reasons for low CH_4 emissions in yaks. The functional genes of the protozoa, such as mcr A and fmd B genes, are also associated with CH_4 emissions (Roehe et al., 2016). The role of the protozoa has been reported as contributing to the maintenance of prokaryotic diversity in the rumen and potentially mitigating the impact of competitive exclusion among bacterial taxa (Solomon et al., 2022). However, the role of protozoa in yaks during methane production needs further research.

Sequencing of the rumen microbiome demonstrated that microbial genes are directly associated with CH_4 emissions. In yellow cattle, the gene enrichment included the CO_2/H_2 and methanogenic pathways (Zhang Z. et al., 2016), indicating the higher energy efficiency of yak ruminal microbes in utilizing crude feed. Similarly, in low-efficient dairy cows, the methanogenic metabolic pathway is enriched, while in the high-efficiency groups, the lactic acid-propionate conversion pathway is enriched (Shabat et al., 2016). In rumen anaerobic fermentation,

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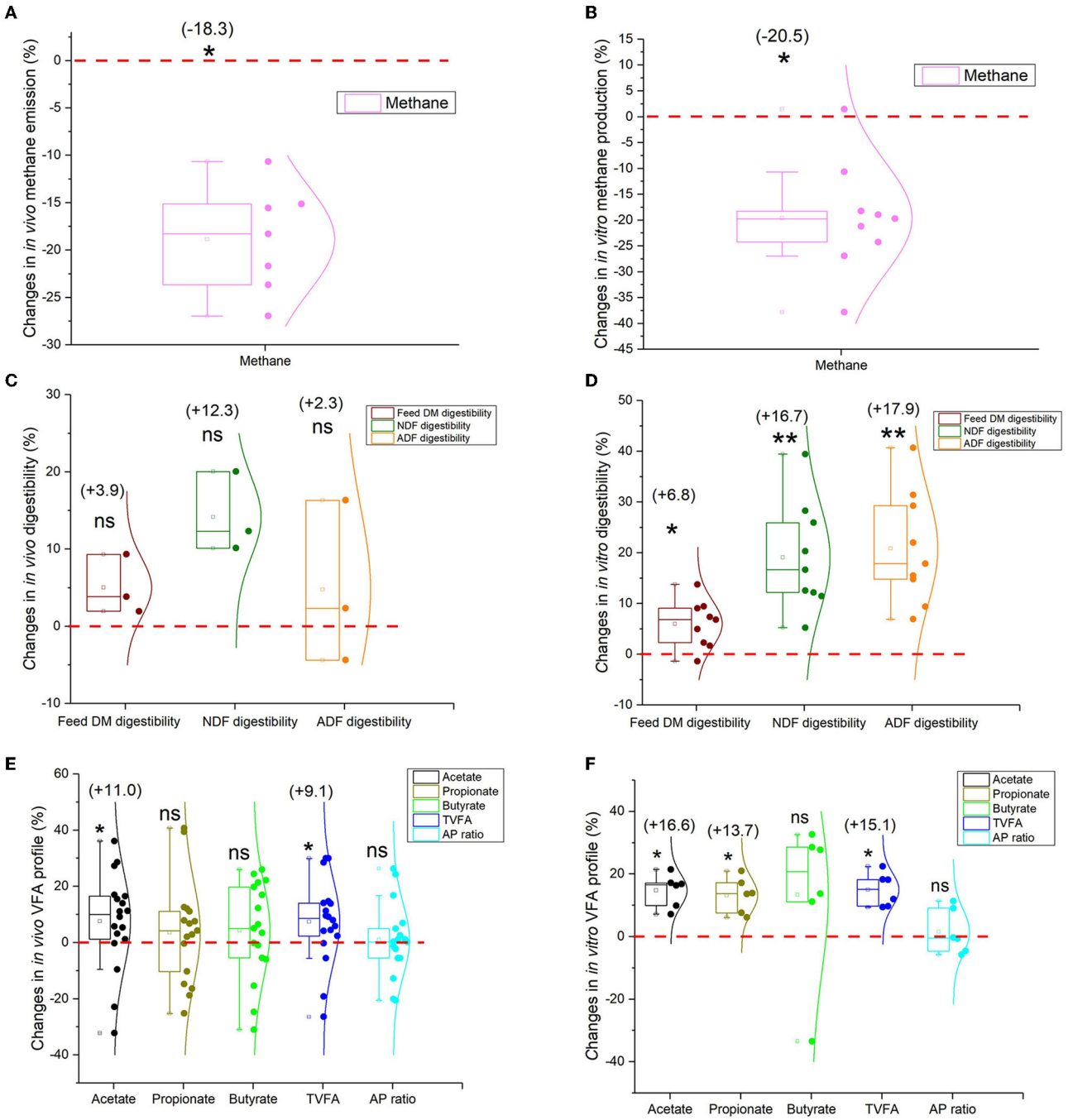

FIGURE 2
Box and whisker plots of rumen fermentation profiles comparing yaks and cattle. **(A)** The *in vivo* methane emission comparison ($n = 7$). **(B)** The *in vitro* methane production comparison ($n = 9$); **(C)** The *in vivo* comparison of feed digestibility ($n = 3$); **(D)** The *in vitro* comparison of feed digestibility ($n = 9$); **(E)** The rumen fermentation VFA profile (*in vivo*) (Acetate and TVFA: $n = 17$; propionate, butyrate, and AP ratio: $n = 16$); **(F)** The *in vitro* rumen fermentation VFA profile ($n = 6$) (see Supplementary Tables 1–5). Values in parentheses indicate the percentage from the statistical analysis comparing yaks with cattle (cattle represent baseline as zero). Wilcoxon Signed Rank test: $^{**}p < 0.01$; $^{*}p < 0.05$; ns = not significantly different from zero. TVFA, total volatile fatty acid; DM, dry matter; NDF, neutral detergent fiber; ADF, acid detergent fiber; AP ratio, acetate to propionate molar ratio.

stoichiometric laws of chemical balance are maintained between the amount of metabolic hydrogen released during carbohydrate oxidation and the amount of hydrogen incorporated into the reduced end products, namely, methane, propionate, and butyrate (Immig, 1996). Yaks have more unknown hydrogen sinks and fewer hydrogen sinks shifting to methanogenesis than other ruminants (Wang, 2020). An increase in the ratio of yak to cattle rumen inoculum decreased CH_4 production and increased fiber digestion and VFA profile *in vitro*. The reduced CH_4 production, possibly attributed to reductive acetogenesis competing for CO_2 and H_2

as intermediate, aligns with the decrease in metabolic hydrogen recovery ($[2H]_{recovery}$) as the yak rumen inoculum increases, which indicates that reductive acetogenesis may elucidate a portion of the unexplained metabolic hydrogen ([2H]) in the fermentation of the yak rumen inoculum. Reductive acetogenesis herein might be the case for yaks exerting more function in their digestive tract than other bovines (Joblin, 1999; Wang et al., 2020). *Streptococcus*, which is found to harbor hydrogenotrophic microbes, exhibited a significant correlation with the metabolism of hydrogen and carbon dioxide with higher relative abundances in the yak rumen inoculum compared with cattle (Godwin et al., 2014; Wang, 2020).

3.2 Higher feed digestibility

The ability to digest plant structural carbohydrates such as cellulose distinguishes ruminants from humankind. Yak rumen microbiota can digest more fibrous feed than cattle with higher feed digestibility (DM, NDF, and ADF digestibility) (Figure 2C, Supplementary Table 3); in particular, there are significant differences in their *in vitro* fermentation comparisons (Figure 2D, Supplementary Table 3). The core members of bacteria cellulose- and hemicellulose-degrading bacteria such as *Fibrobacter succinogenes*, *Ruminococcus albus*, and *Ruminococcus flavefaciens* are the main cellulose-degrading bacteria, and several bacterial species belonging to the genera *Prevotella*, *Butyrivibrio,* and *Pseudobutyrivibrio* demonstrate high efficiency in hemicellulose degradation (Perlman et al., 2021). A higher abundance of fiber-degrading bacteria is found in the yak than in cattle rumen under grazing conditions. These include *Ruminococcus*, *Fibrobacter*, *Clostridium*, *Butyrivibrio*, *Rumenococcus*, *Treponema,* cellulase-related (GH48, GH5, GH45), and hemicellulase-related (GH44, GH16, GH17, GH11) (Huang, 2013; Mi, 2016; Zhao et al., 2022). Ascomycota or Neocallimastigomycota is the most dominant fungi phylum in the rumen of yaks, regardless of dietary intake (Cao et al., 2010; Yan et al., 2018; Guo et al., 2020). The ability to decompose lignocellulose is enhanced when *Piromyces ruminosae* in yaks secreted polysaccharide hydrolase (xylanase) (Wei et al., 2016). This process may be associated with the fiber-digesting capacity, thus resulting in more structural carbohydrate fermentation and more acetate production when compared with cattle (*Bos taurus*). The carbohydrate-active enzymes (CAZymes) encoded by the microbiome in the rumen play a pivotal role in the digestion of feed in ruminants. Unlike cattle, yaks exhibit a higher relative abundance of CAZymes. Specifically, cellulase, hemicellulase, and PL families are significantly enriched in the rumen microbiome of yaks than in cattle (Zhao et al., 2022), which may help explain the improved fiber degradation in yaks.

3.3 Higher short-chain fatty acid concentrations

Higher feed digestibility (DM, NDF, and ADF digestibility) leads to higher energy SCFA concentrations. The rumen microbiome ferments plant materials anaerobically to produce metabolic end products, such as SCFAs while supporting complex food webs (Shabat et al., 2016). Many *in vivo* and *in vitro* studies suggest that yak rumen fermentation can produce more SCFAs (Figures 2E, F, Supplementary Tables 4, 5), especially more acetate and propionate SCFAs, than cattle under *in vitro* rumen fermentations to sustain themselves in harsh environments. *Prevotella* plays an important role in starch and protein degradation and hemicellulose utilization. A high relative abundance of *Prevotella* in the yak is associated with high propionate concentration (Zhang Z. et al., 2016). The increased succinate-producing and utilizing bacterial species such as *Prevotella albensis*, *Prevotella brevis*, *Prevotella bryantii*, *Fibrobacter succinogenes*, and *Succinimonas amylolytica* might also promote propionate concentration in yaks (Zhao et al., 2022). Metagenomic sequencing illustrated the enrichment of yaks' rumen microbial genes in the SCFA production pathways (such as the citrate cycle, TCA cycle, fructose, mannose metabolism, and carbon fixation pathways) (Zhang Z. et al., 2016). Similarly, increased gene and transcript abundances for propionate and butyrate were also observed in high-efficiency ruminants (Kamke et al., 2016). Moreover, yaks demonstrate a higher abundance of glycosyl transferases compared to cattle. The top four microbial KEGG pathways in yaks are pantothenate and CoA biosynthesis, lipopolysaccharide biosynthesis, cysteine and methionine metabolism, and biofilm formation—*Vibrio cholerae* (Zhao et al., 2022).

3.4 Higher rumen microbial protein production

Ammonia and amino acids serve as the sources of nitrogen in the rumen, which, in turn, is used by microbes to synthesize microbial proteins. The microbial proteins absorbed from the small intestine account for 40%−80% of the protein needs of the host (Owens and Bergen, 1983). Zhou et al. (2017) used purine derivative excretion estimation and nitrogen isotope techniques to study the differences in nitrogen (N) excretion and retention and urea N recycling in yaks and yellow cattle. The authors found that yaks had low urinary N excretion but higher N retention and urea N recycling to the gut. They also observed that recycled urea N captured by ruminal bacteria was higher in yaks, resulting in higher production of rumen microbial protein synthesis (MCP) than that of yellow cattle (*Bos taurus*) (Zhou et al., 2017, 2018) with the same dietary intake. In addition, *Streptococcus*, *Akkermansia*, and *uncultured Eubacterium WCHB141_ge* may also regulate the synthesis of MCP during rumen fermentation in yaks (Wang, 2020; Guo et al., 2021). Through the rumen metagenomic sequencing of yaks and cattle, the amino acid pathways (such as valine, leucine, and isoleucine biosynthesis, glycine, serine, and threonine metabolism) and nitrogen metabolism were enriched in the rumen microbiota of yaks compared with those of cattle (Zhang Z. et al., 2016; Zhao et al., 2022), which was likely related

to the higher MCP production in yaks (Zhou et al., 2017). Rumen microbial metabolic pathways and metabolites were different than in cattle, and mainly, amino acids were also confirmed later in yaks (Zhao et al., 2022). A total of 11% of amino acids absorbed by the small intestine were derived from protozoa (Shabi et al., 2000), and in the absence of nitrogen in the rumen, protozoa and bacteria synthesized and stored polysaccharides and used them when sufficient nitrogen was available (Dewhurst et al., 2000). Therefore, further research is needed to determine the function of protozoa in yaks during the MCP synthesis.

In total, the summarized research on yaks sheds light on the muti-dimensional and intricate rumen ecosystem. However, rumen microorganisms sustain their functionalities and homeostasis through a complex and coordinated process, which involves the establishment of successive food webs with cross-feeding interactions among different rumen microorganisms to provide synergetic services such as SCFAs and MCP for the host (Morais and Mizrahi, 2019). Homeostasis includes interactions between the number and diversity of species, hydrogen shifting, thermodynamics, and corresponding metabolisms of the microorganisms. Among them, microbial interactions were identified as key contributors to the formation of rumen community states, with niche modification emerging as a primary mechanism for their formation. The microbial metabolic cascades, thus, were carried out by the microbial community interactions and provided basic metabolites, which were the outcome of the establishment of numerous parallel trophic chains within each of these structured environments (Morais and Mizrahi, 2019; Mizrahi et al., 2021). To clarify the complex microbial community and its homeostasis, Morais and Mizrahi (2019) proposed categorizing the microbial community into functional groups that could streamline the comparative analysis of rumen communities. This approach is particularly valuable as functional groups have the potential to clarify taxonomic uncertainties resulting from functional redundancies and events of horizontal gene transfer. Mizrahi et al. (2021) additionally highlighted that rumen metabolism could be categorized into three trophic-like levels, representing the broad chemical transformations of plant fiber macromolecules and polymers in broad terms. The degradation and metabolism of cellulose and hemicellulose occurred in the first level. At the second level, in the process of utilizing hexoses and pentoses, specific transporters facilitated the import of soluble sugars into microbial cells. Subsequently, these sugars underwent metabolism through diverse pathways, including the pentose phosphate pathway and the Embden-Meyerhoff-Parnas pathway. In the third level, certain excreted metabolites, including hydrogen, carbon dioxide, lactate, and succinate, underwent additional transformations to yield methane, acetate, propionate, butyrate, and various other byproducts (Mizrahi et al., 2021). Therefore, in yaks, the rumen microbiota composition, the abundance of functional microbiota, and the gene expression were linked with rumen fermentation profiles, which are shown in Figures 3A–C. Although the rumen microbiome has been studied using metatranscriptomic, meta-proteomic, and metabolic methodologies, the study of metaproteome and virome behind the rumen is still in its infancy in yaks.

4 Host-rumen microbiome interacts with the feed efficiency-related traits

Evidence indicates a strong connection between host genetics and the microbiome present in the rumen, wherein the genetics of ruminants affects the rumen microbial community structure (Hernandez-Sanabria et al., 2013). The heritability of rumen microbiota has been investigated in ruminants (Li et al., 2019; Wang et al., 2023), and potentially heritable microorganisms could be linked to the phenotype of the host. Some microorganisms own moderate heritability estimates, and they were closely linked with feed efficiency and CH_4 emissions (Roehe et al., 2016; Difford et al., 2018; Li et al., 2019). For instance, rumen microbiota-associated single-nucleotide polymorphisms (SNPs) played an important role in contributing to the variability observed in feed efficiency traits within the beef cohort (Li et al., 2019), and the relative abundance of certain bacteria and archaea were heritable and exerted their association with CH_4 production (Difford et al., 2018). The genes annotated within specific genomic regions, such as chromosome 19 at position 3.0–4.0 Mb and chromosome 27 at position 3.0–4.0 Mb, also suggested that the observed associations were directed toward the selective absorption of SCFAs from the rumen, thereby increasing energy availability for the animal (Abbas et al., 2020). However, host genetics may outweigh rumen microorganisms in shaping the related heritable traits, as the variance in CH_4 production is attributable to both host genetics and the presence of rumen bacteria and archaea. While host genetics account for 21% of the variation, rumen microbes contribute 13% (Difford et al., 2018). Similarly, heritability for sheep body weight could be 39% in genetics, and rumen microbiota explain only 20% of the phenotypic variation (Wang et al., 2023). Although the coevolution of microorganisms with the host might be a mechanism that elucidates varying host genetic effects on distinct rumen microbial taxa, the related heritable rumen microbiota in yaks remains unknown. In addition, host microRNAs, a group of non-coding RNAs and potential molecules, might act as crucial regulators during the metabolic processes and interface the regulation of nutrition, genes, and gut microbes (Malmuthuge et al., 2019; Ojo and Kreuzer-Redmer, 2023), but further research is needed on yaks in this context (Figure 3D).

5 Host-rumen microbiome–environment interactions on the dependent animal productivity traits

Microbiome-, host-, and environment-dependent mechanisms contribute to varied performance in the milk and meat production quality of yaks. The microbial metabolite SCFAs, microbial proteins, and the host genes are key molecules that are involved in animal production.

5.1 Milk quality

Plants are degraded and fermented by rumen microbes, and the derived microbial metabolite SCFAs and microbial proteins

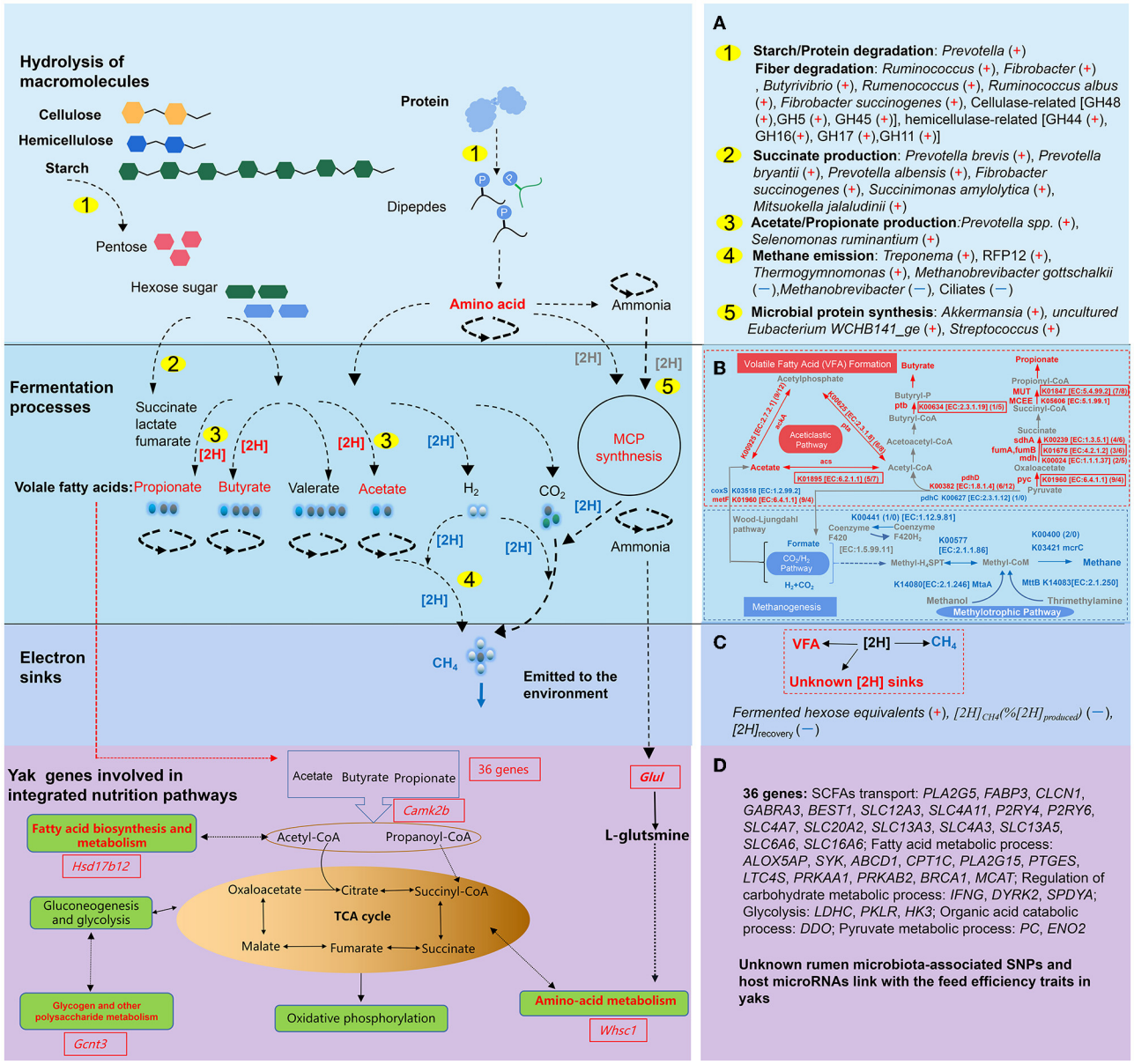

FIGURE 3
The summarized host gene, rumen microbiota, and the related microbial pathways and hydrogen balance response to the nutritional deprivation in yaks (adapted from Morais and Mizrahi, 2019). **(A)** The microbial community involved in the hydrolysis of macromolecules and fermentation processes. A "+" in the parentheses refers to higher relative abundance, a "−" in the parentheses refers to lower relative abundance in yaks; **(B)** Comparison of methanogenesis and volatile fatty acid (VFA) formation pathways between yaks and cattle (adapted from Zhang Z. et al., 2016). The annotation highlighted in red represents enrichment in the yaks and that in blue represents enrichment in cattle rumen microbiota fermentation. **(C)** Metabolic hydrogen ([2H]) shifting between yak and cattle *in vitro* inocula fermentation. In **(C)** (+) or (−) indicate the enrichment or reduction of the parameters in yaks. **(D)** Host and rumen microbiome interactions on regulating the VFA, microbial protein genesis, and their absorption (adapted from Qiu et al., 2012).

from microbial fermentation are precursors that directly impact the biosynthesis of milk (Flint and Bayer, 2008; Patil et al., 2018). Mostly, milk fatty acids (FAs) are synthesized by the rumen microbiota and exogenous uptake (Parodi, 2004). Acetate and β-hydroxybutyric acid are conveyed to the mammary gland for the synthesis of short and medium-chain FAs, while butyrate is assimilated and transformed into β-hydroxybutyric acid by rumen epithelial cells. The long-chain FAs primarily originate from dietary lipids and adipose tissue (Buitenhuis et al., 2019). The particular plateau environment and rumen microbial services, such as more ruminal SCFAs and more microbial proteins, contribute to the unique nutritional profile of yak milk. Yak milk is known as "natural milk concentrate," and its protein (4.0–5.9%), fat (5.3–8.8%), lactose (4.0–5.9%), total dry matter content, and ash are significantly higher than in milk of ruminants (Li et al., 2011, 2023).

The microbial metabolites greatly influence the milk quality of yaks. Compared to cow milk, yak milk exhibits considerably elevated levels of essential amino acids, immunoglobulin A (IgA),

IgG, and IgM. Immunoglobulin A and IgG concentrations could be approximately 1.5 times higher than those found in human milk (Li et al., 2023). Although the fat content ranges from approximately 5.3% to 8.8% and is the most variable component in yak milk (Ma et al., 2021a), yak milk possesses almost twice the amount of fat compared to Holstein milk (Li et al., 2011). Regarding the fatty acids (FAs) contained in yak milk, it has a reduced concentration of short and medium-chain FAs but an elevated level of long-chain FAs and unsaturated FAs in contrast to Holstein milk (Li et al., 2023). The concentration of conjugated linoleic acid (CLA) in yak milk is significantly greater than that found in cow milk (Zongo et al., 2021). In addition, due to the activity of yak rumen microorganisms and enzymes, numerous polyunsaturated FAs present in fresh forage are hydrogenated and eventually absorbed by the intestine in the form of saturated FAs and trans FAs, leading to their deposition in tissues affecting milk quality (Li et al., 2023). However, little is known about the microbiome-host-dependent mechanisms for yak milk containing high mineral and vitamin content. The main mineral and vitamin content in regular cow milk is lower than in yak milk (Dosek et al., 2007; Ma et al., 2017). The elevated levels of vitamin D in yak milk may be associated with strong ultraviolet radiation at high altitudes. Additionally, the abundant presence of vitamin C and vitamin E imparts strong antioxidant capabilities to yak milk, which may help mitigate oxidative damage induced by the high-altitude harsh environment (Dosek et al., 2007).

5.2 Meat quality

The fundamental health of livestock species and traits related to meat quality depend on the symbiotic interactions between the host and microbes (Yeoman and White, 2014). The fatty acid composition of meat can be influenced by rumen microbial fermentation, which supplies precursors for *de novo* fatty acid synthesis (Shingfield et al., 2013). The majority of fatty acids present in ruminant products primarily originate from the metabolism of fat in the rumen rather than from the diet (Toral et al., 2018). Short-chain fatty acids (SCFAs) improve meat quality traits and are conveyed through the host's systemic circulation, reaching extraintestinal organs and exerting broad-range impacts on the host (Tremaroli and Bäckhed, 2012; Koh et al., 2016). The conversion of SCFAs such as acetate, butyrate, and propionate into acetyl-CoA or propynyl-CoA occurs through pathways that include the acetyl-CoA carboxylase (ACSSs) and beta-oxidation. This process results in the generation of ATP, thereby sustaining cellular homeostasis (Dalile et al., 2019). Liu et al. (2021) have highlighted the favorable outcomes of SCFAs derived from the gut microbiota on both muscle and fat tissue, subsequently influencing meat quality. Altering the gut microbiota has the potential to regulate both intramuscular fat deposition and host immunity, contributing to the enhancement of meat quality. Given the overlap among numerous bacterial taxa associated with intramuscular and subcutaneous fat deposits that did not occur, the gut microbiota likely mainly influences adipose accumulation through separate adipogenic pathways (Krause et al., 2020). Therefore, understanding the interaction between the host and rumen microbiota is essential for developing knowledge-based strategies that improve both animal meat quality and host health. Concerning the quality of yak meat, it features a reduced fat yet a higher protein percentage and is abundant in essential amino acids, fatty acids, and minerals when compared to commercial beef meat from low-altitude regions (Yin et al., 2009; Wan et al., 2012).

5.3 Host genes are involved in milk and meat production

Yak milk is characterized by its elevated levels of fat and protein, and the gene expression patterns are also related to its synthesis. For instance, the genes associated with the uptake of fatty acids from the blood (*CD36* and *LPL*), intracellular fatty acid activation of long and short-chain fatty acids (*ACSL1*, *ACSS1*, and *ACSS2*), intracellular fatty acid transport (*FABP3*), triacylglycerol synthesis (*LPIN1*, *AGPAT6*, and *GPAM*), lipid droplet formation (*BTN1A1*, *PLIN2*, and *XDH*), desaturation (*SCD*), and ketone body utilization (*BDH1* and *OXCT1*) exhibit significant upregulation during yaks' lactation. In particular, compared to the upregulation levels in dairy cows, the processes of triacylglycerol synthesis (*GPAM*, *AGPAT6*, and *LPIN1*) and intracellular *de novo* fatty acid synthesis (*ACACA*, *ACSS2*, and *FABP3*), which potentially orchestrate as components within the gene network controlled by *SERBF1* during milk fat synthesis, exhibit a higher degree of activation (Lee et al., 2017). Moreover, in the lactation cycle of yaks, the highest expression of certain milk fat genes (such as *XDH* and *FABP3*) in mammary tissue occurs earlier than observed in dairy cows (Yuan et al., 2020). *FASN* is one of the genes with high expression levels in the yak mammary gland and subcutaneous fat and has the potential to be a genetic marker in breeding programs to enhance the milk fat content and total milk solid levels (Shi et al., 2019). As to the meat quality, under the nutritional deprivation environment, comparative transcriptomics of yak and cattle show that the genes differentially expressed in tissues, including skeletal muscles, are significantly enriched in the energy metabolism-related process (Tang et al., 2017; Ma et al., 2021b). Another comparative gene expression study performed based on subcutaneous adipose tissues showed that introducing yak genes into cattle breeds by hybridization dramatically changed the expression patterns of genes related to fatty acid biosynthesis and catabolism and improved the yield and quality of meat (Song et al., 2019), highlighting the unique genetic basis of nutrition accumulation in yaks. Similarly, unique patterns of adaptations related to meat production have also been revealed by population genetics in yak. Furthermore, the genomic copy number variations of the *CHKB* and *CHRM3* genes, which are detected in domesticated yak populations using whole genome resequencing data (Zhang X. et al., 2016), are significantly associated with improved growth traits such as higher body weight and greater chest girth (Goshu et al., 2019, 2020). Intriguingly, these copy number variations have not been detected in cattle breeds (Yue et al., 2014; Zhang et al., 2014), implying the distinct genetic basis of energy storage, growth, and development in yaks.

In summary, these findings provide insights into the rumen microbiome-dependent traits that interact with the metabolism,

environment, and animal production, demonstrating the host-rumen microbiome-environment as a whole in response to environmental stress. However, the interactions of host-rumen microbiome-environment on the yak productivity traits need to be clarified.

6 Implications

6.1 Integrating host-gut microbe-environment interactions into understanding animal performance and improving the systematic management

The proposal by Kohl (2018) to include host-microbe interactions in the established field of animal comparative physiology opened exciting research opportunities for both fields. However, our knowledge about the mechanistic bases of host-environment linkages and how they ultimately benefit the organism in adapting to changes to serve the ecosystem is limited (Hutchinson, 1957; Angilletta and Sears, 2011). The adaptation of yak to environmental stress sheds light on understanding some of these linkages. The yak's adaptation to the stress of nutritional shortage is directly related to environmental services (Figure 4). By coping with the harsh environment, the animals themselves show multi-faceted synergism. At the host level, yaks possess genes that are co-regulated, embodied in the regulation of traits ranging from molecules, cells, tissues, organs, and systems (Dalziel et al., 2009; Shao et al., 2010; Qiu et al., 2012; Jing et al., 2022). In addition to tissue morphology, the host's metabolism processes (energy metabolism, nitrogen metabolism, etc.) and gene regulation interact with each other (Qiu et al., 2012). Mediated by the host, gut microbes process their niches and reciprocally provide services to sustain a symbiotic relationship with the host (Figure 4). Adaptation, thus, is a consequence of the interactions at various hierarchical levels including climate pressure, nutritional pressure, host, and rumen microbiome and its services to the yaks. These selective pressures of the host combine with the environment to affect the gut microbe, which ultimately effectuates the adaptation of the microorganism-host symbionts to the environment.

From the ecological systematic balance, understanding the host-rumen-environment linkages is beneficial for further animal management. The host-environment linkages may provide a means for devising improved animal management strategies. For instance, under the stress of cold, with low temperature and insufficient forage on the Qinghai-Tibetan Plateau, the yak loses body weight (Long et al., 1999b) and increases forage intake from the grassland to avoid the nutritional deprivation, increasing the pressures on the grassland. Better management, therefore, would involve reducing the selective pressure of grassland to increase its biomass productivity or directly decreasing the number of grazing yaks. Artificial seeding or supplementary feeding of fodder for the animals are approaches to relieve the pressure. However, the animal management strategy should also focus on the forage (biomass, fatty acid content, richness and diversity, etc.) from the pasture. Since the forage from the pasture provides a certain level of nutrients, they can influence the quality of animal products. For instance, the concentrations of total conjugated linoleic acid (CLA), CLA isomer c9t11, and CLA isomer t10c12 in the milk of grazing yaks were significantly higher during the peak grass stage compared to the dry grass stages (Pan et al., 2021). Ruminants grazing artificial pasture led to an elevation in the levels of polyunsaturated fatty acids (PUFAs) in meat, particularly an increase in n3 PUFA concentrations and a decrease in the n6/n3 ratio (Wang et al., 2021). Ruminants fed with alfalfa (*Medicago sativa L.*) exhibited significantly higher contents of saturated fatty acids such as C14:0, C16:0, and C18:0 in meat (Wang et al., 2022). Therefore, maintaining the quality of the grass is also crucial for healthy animal products.

The gut-environment linkages are also vital in understanding adaptation (Boyce et al., 2020) and devising improved animal management strategies. Precise supplemental feeding methods for the yaks during warm and cold seasons are advisable since the rumen microbiota can utilize low levels of dietary nitrogen better than the high (Zhou et al., 2017; Hao, 2019), and excessively high concentrations of protein, which may result in unnecessary waste and environmental pollution. Moreover, the correlation between forage and milk fatty acids is significantly affected by the biohydrogenation occurring in the yak rumen alongside the particular community of rumen microorganisms (Li et al., 2023). The intestinal microbiota of yaks determine their feed quality, and studies have shown that yaks can selectively intake certain plants. Its consumption varies with different pastures and seasons (Ding, 2007; Guo et al., 2021), which is essential for regulating the flavor and quality of milk and animal products. From the gut-environment linkages, microbiota also provides regulation services that help to maintain a stable condition for the Qinghai-Tibetan Plateau and may prevent the plants from aggressive growth. Many plants produce compounds to deter herbivores and include chemical compounds such as alkaloids, glycosides, terpene, benzene, and some secondary metabolites such as essential oils, tannins, and nitrate compounds (Long et al., 1999a; Hart et al., 2008). Gut microbiota could detoxicate "biohazardous waste" that is poisonous or other undesirable ingesta. The long-term association between the gut microbiome of yaks and undesirable plants is perhaps indicative of the development of strategies adapted by yaks to benefit from these plants. It has been reported that yaks can digest toxic plants during harsh winters (Guo et al., 2021). The ingested plant tannins bind themselves to microbial proteins and prevent them from being degraded in the rumen, thereby forming more proteins for intestinal absorption. This may have helped yaks not suffer from nitrogen deficiency (Long et al., 2003). Studying such an adaptive behavior can suggest new prospects for improving animal production (for instance, incorporating host-gut-environment linkages into breeding strategies as a whole), understanding the role of rumen microbiota systematically that shape the feed efficiency and withstanding the body homeostasis or co-evolve with the host, and developing more tools to decipher and manipulate the microbes.

The latest "omics" techniques propose to integrate a database to optimize the microbiome fermentation traits, including flavonoids (Oh et al., 2017; Morales et al., 2018), essential oils (Cobellis et al., 2016), nitro-compounds (Latham et al., 2016), or

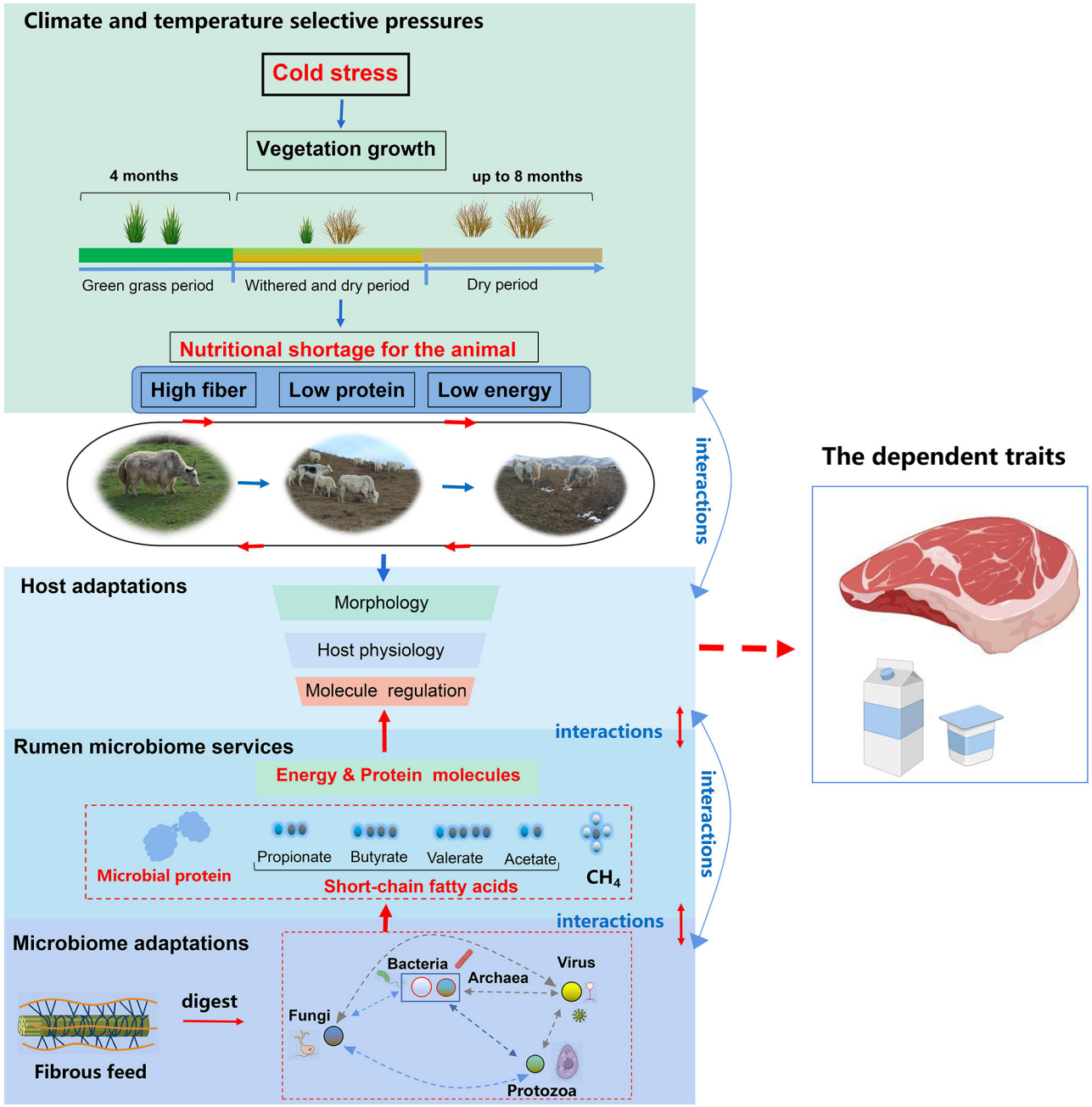

FIGURE 4
Hierarchical structure of the environment and host-gut microbe interactions.

other secondary metabolites fermentation. Although the rumen microbial community composition and fermentation profiles are attributable predominantly to diet, with the host having a lesser influence (Henderson et al., 2015), integrating host-gut microbe interactions into understanding the host-environment linkages might favor the establishment of a predictive theory of the niche in organismal biology. In this context, the yak is an excellent animal model to study the influences of environmental factors on the host and the relationship between the host and rumen microbiome.

6.2 The potential to understand the host-gut microbe-environment linkages

Understanding these linkages can augment our capacity to anticipate and predict relationships among hosts, gut microbes, and environments over space and time (Angilletta and Sears, 2011; Kohl, 2018). A modeling approach, thus, could advance prediction with a combination of experiments for validation by integrating and synthesizing biological principles from the bottom up. On the other hand, an experimental approach involving reduction

and analysis is from the top down. Generating such predictions necessitates a comprehensive collection of models that elucidate how hosts interact with their environments and the reasons behind their specific interactions. The poor coordination between theoretical and empirical activities and previous models fails to explain variations in fundamental ecological niches within and among organisms (Angilletta and Sears, 2011). Focusing on the ecological foundations of the rumen microbiota could lead to an enhanced understanding of both functions and unexplored fermentation pathways of the rumen in yaks (Huws et al., 2018; Solomon et al., 2022), improving the understanding and predicted linkages between host and environments and the integration of mathematical models and crucial experiments in a manner that has worked for biological disciplines (Gilarranz et al., 2017). With the development of research tools and methodology, mathematical models (Dalziel et al., 2009) such as network-based approaches (Dee et al., 2017; Huws et al., 2018) and deep learning algorithms. These are better choices for systematic processing and quantification, helping us to better understand complex systems and their related connections. Although comprehensive network-based approaches have clarified the connections in microbial communities (Adai et al., 2004), large-scale, integrative models have yet to be developed (Huws et al., 2018). A systematic analysis of animals (*in vivo* or between different host systems) and their living environment is essential to better manage the performance of animals and their habitats. This involves integrating various layers of interactive networks, including living organisms, vegetation, landscapes, soils, gut microbes, and other layers.

7 Conclusion

The host gene regulation, host gene metabolism, and the rumen microbial services of yaks to survive in an extreme environment provide a basic understanding of the animal adaptability mechanisms and performance. In this review, a hierarchical model of the adaptability between the host, the environment, and the related host-rumen microbiome-environment interactions was integrated and proposed. It offers more solutions for the regulation of rumen microorganisms and is mutually beneficial for the hosts as well as the microorganisms. To better understand the relationship between organisms and the environment, it is proposed that multi-level interactions and primary determinants be highlighted and clarified in systematic biology research. It would be beneficial for sustainable animal production management and systematized regulation, but further research is required.

Author contributions

WW: Writing – original draft, Conceptualization, Funding acquisition, Investigation, Methodology, Resources, Software. YD: Investigation, Writing – original draft. WG: Conceptualization, Writing – original draft. XZ: Investigation, Writing – original draft. AD: Writing – review & editing. SB: Investigation, Writing – original draft. LD: Investigation, Writing – review & editing. XC: Funding acquisition, Writing – review & editing, Conceptualization. RL: Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1301258/full#supplementary-material

References

Abbas, W., Howard, J. T., Paz, H. A., Hales, K. E., Wells, J. E., Kuehn, L. A., et al. (2020). Influence of host genetics in shaping the rumen bacterial community in beef cattle. *Sci. Rep.* 10:15101. doi: 10.1038/s41598-020-72011-9

Adai, A. T., Date, S. V., Wieland, S., and Marcotte, E. M. (2004). LGL: creating a map of protein function with an algorithm for visualizing very large biological networks. *J. Mol. Biol.* 340, 179–190. doi: 10.1016/j.jmb.2004.04.047

Alexandratos, N., and Bruinsma, J. (2012). *World Agriculture Towards 2030/2050: The 2012 Revision.* Rome: FAO, Food and Agriculture Organization of the United Nations.

Angilletta, J. M. J., and Sears, M. W. (2011). Coordinating theoretical and empirical efforts to understand the linkages between organisms and environments. *Integr. Comp. Biol.* 51, 653–661. doi: 10.1093/icb/icr091

Auffret, M. D., Stewart, R., Dewhurst, R. J., Duthie, C. A., Rooke, J., and Wallace, R. J. (2017). Identification, comparison and validation of robust rumen microbial biomarkers for methane emissions using diverse *Bos Taurus* breeds and basal diets. *Front. Microbiol.* 8:2642. doi: 10.3389/fmicb.2017.02642

Bergman, E. N. (1990). Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. *Physiol. Rev.* 70, 567–590. doi: 10.1152/physrev.1990.70.2.567

Bi, X. C., Xie, A. Y., Han, X. T., Zhuge, W. J., and Hu, L. H. (1989). Determination on degradability of several kinds of protein feeds in yak rumen (In Chinese). *J. Qinghai Anim. Husb. Vet. Med.* 19, 21–22.

Borrel, G., Parisot, N., Harris, H. M., Peyretaillade, E., Gaci, N., Tottey, W., et al. (2014). Comparative genomics highlights the unique biology of Methanomassiliicoccales, a Thermoplasmatales-related seventh order of methanogenic archaea that encodes pyrrolysine. *BMC Genomics* 15:679. doi: 10.1186/1471-2164-15-679

Boyce, W. T., Sokolowski, M. B., and Robinson, G. E. (2020). Genes and environments, development and time. *Proc. Natl. Acad. Sci. U.S.A.* 117, 23235–23241. doi: 10.1073/pnas.2016710117

Brinks, J. S., Clark, R. T., Kieffer, N. M., and Quesenberry, J. R. (1962). Genetic and environmental factors affecting performance traits of Hereford bulls. *J. Anim. Sci.* 21, 777–780. doi: 10.2527/jas1962.214777x

Brito, L. F., Oliveira, H. R., McConn, B. R., Schinckel, A. P., Arrazola, A., and Marchant-Forde, J. N. (2020). Large-scale phenotyping of livestock welfare in commercial production systems: a new frontier in animal breeding. *Front. Genet.* 11, 793. doi: 10.3389/fgene.2020.00793

Brugère, J. F., Borrel, G., Gaci, N., Tottey, W., O'Toole, P. W., and Malpuech-Brugère, C. (2014). Archaebiotics: proposed therapeutic use of archaea to prevent trimethylaminuria and cardiovascular disease. *Gut Microbes* 5, 5–10. doi: 10.4161/gmic.26749

Buitenhuis, B., Lassen, J., Noel, S. J., Plichta, D. R., Sørensen, P., Difford, G. F., et al. (2019). Impact of the rumen microbiome on milk fatty acid composition of Holstein cattle. *Genet. Sel. Evol.* 51, 23–29. doi: 10.1186/s12711-019-0464-8

Cao, Y. C., Yang, H. J., and Shen, B. T. (2010). Screening and identification of high activity cellulolytic anaerobic fungi from rumen of yak (In Chinese). *J. China Agric. Univ.* 15, 70–74.

Carberry, C. A., Waters, S. M., Kenny, D. A., and Creevey, C. J. (2013). Rumen Methanogenic genotypes differ in abundance according to host RFI phenotype and diet type. *Appl. Environ. Microbiol.* 80, 586–594. doi: 10.1128/AEM.03131-13

Chaudhary, L. C., Kamra, D. N., Singh, R., Agarwal, N., and Pathak, N. N. (2000). Effect of feeding bentonite on rumen fermentation, enzyme activity and protozoal population in adult buffaloes (*Bubalus bubalis*). *J. Buffalo.* 16, 73–79.

Cobellis, G., Trabalza-Marinucci, M., and Yu, Z. (2016). Critical evaluation of essential oils as rumen modifiers in ruminant nutrition: a review. *Sci. Total Environ.* 545, 556–568. doi: 10.1016/j.scitotenv.2015.12.103

Cunha, C. S., Veloso, C. M., Marcondes, M. I., Mantovani, H. C., Tomich, T. R., and Pereira, L. G. R. (2017). Assessing the impact of rumen microbial communities on methane emissions and production traits in Holstein cows in a tropical climate. *Syst. Appl. Microbiol.* 40, 492–499. doi: 10.1016/j.syapm.2017.07.008

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

Dalziel, A. C., Rogers, S. M., and Schulte, P. M. (2009). Linking genotypes to phenotypes and fitness: how mechanistic biology can inform molecular ecology. *Mol. Ecol.* 18, 4997–5017. doi: 10.1111/j.1365-294X.2009.04427.x

Dee, L. E., Allesina, S., Bonn, A., Eklöf, A., Gaines, S. D., and Hines, J. (2017). Operationalizing network theory for ecosystem service assessments. *Trends Ecol. Evol.* 32, 118–130. doi: 10.1016/j.tree.2016.10.011

Dewhurst, R. J., Davies, D. R., and Merry, R. J. (2000). Microbial protein supply from the rumen. *Anim. Feed Sci. Technol.* 85, 1–21. doi: 10.1016/S0377-8401(00)00139-5

Difford, G. F., Plichta, D. R., Løvendahl, P., Lassen, J., Noel, S. J., and Højberg, O. (2018). Host genetics and the rumen microbiome jointly associate with methane emissions in dairy cows. *PLoS Genet.* 14:e1007580. doi: 10.1371/journal.pgen.1007580

Ding, L. M. (2007). *Study on the ecology of grazing behaviour by yaks in Qinghai-Tibetan Plateau (In Chinese).* Ph.D. Dissertation. Xining: Northwest Institute of Plateau Biology, Chinese Academy of Sciences.

Ding, L. M., Wang, Y. P., Brosh, A., Chen, J. Q., Gibb, M. J., and Shang, Z. H. (2014). Seasonal heat production and energy balance of grazing yaks on the Qinghai-Tibetan plateau. *Anim. Feed Sci. Technol.* 198, 83–93. doi: 10.1016/j.anifeedsci.2014.09.022

Dosek, A., Ohno, H., Acs, Z., Taylor, A. W., and Radak, Z. (2007). High altitude and oxidative stress. *Respir. Physiol. Neurobiol.* 158, 128–131. doi: 10.1016/j.resp.2007.03.013

Flint, H. J., and Bayer, E. A. (2008). Plant cell wall breakdown by anaerobic microorganisms from the Mammalian digestive tract. *Ann. N. Y. Acad. Sci.* 1125, 280–288. doi: 10.1196/annals.1419.022

Gilarranz, L. J., Rayfield, B., Liñán-Cembrano, G., Bascompte, J., and Gonzalez, A. (2017). Effects of network modularity on the spread of perturbation impact in experimental metapopulations. *Science* 357, 199–201. doi: 10.1126/science.aal4122

Gilbert, R. A., Townsend, E. M., Crew, K. S., Hitch, T. C., Friedersdorff, J. C., and Creevey, C. J. (2020). Rumen virus populations: technological advances enhancing current understanding. *Front. Microbiol.* 11:450. doi: 10.3389/fmicb.2020.00450

Godwin, S., Kang, A., Gulino, L. M., Manefield, M., Gutierrez-Zamora, M. L., and Kienzle, M. (2014). Investigation of the microbial metabolism of carbon dioxide and hydrogen in the kangaroo foregut by stable isotope probing. *ISME J.* 8, 1855–1865. doi: 10.1038/ismej.2014.25

Goshu, H. A., Chu, M., Xiaoyun, W., Pengjia, B., Zhi, D. X., and Yan, P. (2019). Genomic copy number variation of the *CHKB* gene alters gene expression and affects growth traits of Chinese domestic yak (*Bos grunniens*) breeds. *Mol. Genet. Genom.* 294, 549–561. doi: 10.1007/s00438-018-01530-y

Goshu, H. A., Xiaoyun, W., Chu, M., Pengjia, B., Ding, X. Z., and Yan, P. (2020). Novel copy number variations of the *CHRM3* gene associated with gene expression and growth traits in Chinese Datong yak (*Bos grunniens*). *J. Appl. Anim. Res.* 48, 156–165. doi: 10.1080/09712119.2020.1753750

Gui, R., Su, N. R., Hua, Z. X., Se, Z., and Imai, S. (2000). Rumen ciliated protozoan fauna of the yak (*Bos grunniens*) in China with the description of *Entodinium monuon. sp. J. Eukaryot. Microbiol.* 47, 178–182. doi: 10.1111/j.1550-7408.2000.tb00029.x

Guo, N., Wu, Q., Shi, F., Niu, J., Zhang, T., Degen, A. A., et al. (2021). Seasonal dynamics of diet-gut microbiota interaction in adaptation of yaks to life at high altitude. *NPJ Biofilms Microb.* 7, 1–11. doi: 10.1038/s41522-021-00207-6

Guo, W., Wang, W., Bi, S., Long, R., Ullah, F., Shafiq, M., et al. (2020). Characterization of anaerobic rumen fungal community composition in yak, Tibetan sheep and small tail han sheep grazing on the Qinghai-Tibetan Plateau. *Animals.* 10:144. doi: 10.3390/ani10010144

Guo, X. S., Zhang, Y., Zhou, J. W., Long, R. J., Xin, G. S., Qi, B., et al. (2012). Nitrogen metabolism and recycling in yaks (*Bos grunniens*) offered a forage–concentrate diet differing in N concentration. *Anim. Prod. Sci.* 52, 287–296. doi: 10.1071/AN11208

Han, X. T., and Xie, A. Y. (1991). Verificial report on requirement of maintenance energy in growing yak (In Chinese). *Qinghai J. Anim. Husb. Vet. Med.* 21:10.

Han, X. T., Xie, A. Y., Bi, X. C., Liu, S. J., and Hu, L. H. (2002). Effects of high altitude and season on fasting heat production in the yak *Bos grunniens* or *Poephagus grunniens*. *Brit. J. Nutr.* 88, 189–197. doi: 10.1079/BJN2002610

Hao, L. Z. (2019). *Effect of supplementary feeding in warm season on meat quality of yaks and underlying mechanisms (In Chinese).* Ph.D Dissertation. Lanzhou: Lanzhou University.

Hart, K. J., Yanez-Ruiz, D. R., Duval, S. M., McEwan, N. R., and Newbold, C. J. (2008). Plant extracts to manipulate rumen fermentation. *Anim. Feed Sci. Technol.* 147, 8–35. doi: 10.1016/j.anifeedsci.2007.09.007

Henderson, G., Cox, F., Ganesh, S., Jonker, A., Young, W., and Abecia, L. (2015). Rumen microbial community composition varies with diet and host, but a core microbiome is found across a wide geographical range. *Sci. Rep.* 5:14567. doi: 10.1038/srep14567

Hernandez-Sanabria, E., Goonewardene, L. A., Wang, Z., Zhou, M., Moore, S. S., and Guan, L. L. (2013). Influence of sire breed on the interplay among rumen microbial populations inhabiting the rumen liquid of the progeny of beef cattle. *PLoS ONE* 8:e58461. doi: 10.1371/journal.pone.0058461

Hu, L. H. (1994). Study of energy metabolism and rumenal metabolism in growing yaks (In Chinese). *Gansu J. Agric. Univ.* 2, 188–195.

Hu, L. H. (2001). Chinese yak nutrition research advances (In Chinese). *Qinghai Sci. Technol.* 6, 37–39.

Hu, L. H., Liu, S. J., and Chai, S. T. (2002). "Advances in yak nutrition research", in *Yak production in central Asian highlands, Proceedings of the Third International*

Congress on Yak held in Lhasa, P.R. China,. eds H. Jianlin, C. Richard, O. Hanotte, C. McVeigh, and J. E. O. Rege (Nairobi, Kenya: International Livestock Research Institute), 237.

Hu, L. H., and Xie, A. Y. (1992). Study on metabolism of energy and rumen in growing yaks (In Chinese). *Qinghai J. Anim. Husb. Vet. Med.* 22:6.

Huang, X. (2013). *Rumen Microbial Diversity of Ruminants in Qinghai Tibetan Plateau (In Chinese)*. Ph.D Dissertation. Lanzhou: Lanzhou University.

Huang, X. D., Martinez-Fernandez, G., Padmanabha, J., Long, R., Denman, S. E., and McSweeney, C. S. (2016). Methanogen diversity in indigenous and introduced ruminant species on the Tibetan Plateau. *Archaea.* 2016:5916067. doi: 10.1155/2016/5916067

Huang, X. D., Tan, H. Y., Long, R., Liang, J. B., Wright, A. D. G., et al. (2012). Comparison of methanogen diversity of yak (*Bos grunniens*) and cattle (*Bos taurus*) from the Qinghai-Tibetan plateau, China. *BMC Microbiol.* 12:237. doi: 10.1186/1471-2180-12-237

Hutchinson, G. E. (1957). Concluding remarks. Cold Spring Harbor symposia on quantitative biology. *Cold Spring Harbor Labor. Press* 22, 415–427. doi: 10.1101/SQB.1957.022.01.039

Huws, S. A., Creevey, C. J., Oyama, L. B., Mizrahi, I., Denman, S. E., Popova, M., et al. (2018). Addressing global ruminant agricultural challenges through understanding the rumen microbiome: past, present, and future. *Front. Microbiol.* 9:2161. doi: 10.3389/fmicb.2018.02161

Immig, I. (1996). The rumen and hindgut as source of ruminant methanogenesis. *Environ. Monit. Assess*. 42, 57–72. doi: 10.1007/BF00394042

IPCC (2019). *Climate Change and Land 2019. An IPCC Special Report on Climate Change, Desertification, Land Degradation, Sustainable Land Management, Food Security, and Greenhouse Gas Fluxes in Terrestrial Ecosystems*. Accepted Draft. Intergov. Panel Climate Change, Geneva, Switzerland.

Ishizaki, T., Koizumi, T., Ruan, Z., Wang, Z., Chen, Q., and Sakai, A. (2005). Nitric oxide inhibitor altitude-dependently elevates pulmonary arterial pressure in high-altitude adapted yaks. *Respir. Physiol. Neurobiol.* 146, 225–230. doi: 10.1016/j.resp.2004.12.002

Ito, A., Imai, S., and Ogimoto, K. (1994). Rumen ciliate composition and diversity of Japanese beef black cattle in comparison with those of Holstein-Friesian cattle. *J. Vet. Med. Sci*. 56, 707–714. doi: 10.1292/jvms.56.707

Janssen, P. H., and Kirs, M. (2008). Structure of the archaeal community of the rumen. *Appl. Environ. Microbiol.* 74, 3619–3625. doi: 10.1128/AEM.02812-07

Jing, X., Ding, L., Zhou, J., Huang, X., Degen, A., and Long, R. (2022). The adaptive strategies of yaks to live in the Asian highlands. *Anim. Nutr.* 9, 249–258. doi: 10.1016/j.aninu.2022.02.002

Joblin, K. N. (1999). Ruminal acetogens and their potential to lower ruminant methane emissions. *Aust. J. Agric. Res*. 50, 1307–1314. doi: 10.1071/AR99004

Kamke, J., Kittelmann, S., Soni, P., Li, Y., Tavendale, M., and Ganesh, S. (2016). Rumen metagenome and metatranscriptome analyses of low methane yield sheep reveals a Sharpea-enriched microbiome characterised by lactic acid formation and utilisation. *Microbiome* 4:56. doi: 10.1186/s40168-016-0201-2

Koh, A., De Vadder, F., Kovatcheva-Datchary, P., and Bäckhed, F. (2016). From dietary fiber to host physiology: short-chain fatty acids as key bacterial metabolites. *Cell.* 165, 1332–1345. doi: 10.1016/j.cell.2016.05.041

Kohl, K. D. (2018). A microbial perspective on the grand challenges in comparative animal physiology. *Msystems* 3, e00146–e00117. doi: 10.1128/mSystems.00146-17

Krause, T. R., Lourenco, J. M., Welch, C. B., Rothrock, M. J., Callaway, T. R., and Pringle, T. D. (2020). The relationship between the rumen microbiome and carcass merit in Angus steers. *J. Anim. Sci.* 98:skaa287. doi: 10.1093/jas/skaa287

Latham, E. A., Anderson, R. C., Pinchak, W. E., and Nisbet, D. J. (2016). Insights on alterations to the rumen ecosystem by nitrate and nitrocompounds. *Front. Microbiol.* 7:228. doi: 10.3389/fmicb.2016.00228

Lee, J. N., Wang, Y., Xu, Y. O., Li, Y. C., Tian, F., and Jiang, M. F. (2017). Characterisation of gene expression related to milk fat synthesis in the mammary tissue of lactating yaks. *J. Dairy Res.* 84, 283–288. doi: 10.1017/S0022029917000413

Li, A., Liu, C., Han, X., Zheng, J., Zhang, G., Qi, X., et al. (2023). Tibetan Plateau yak milk: A comprehensive review of nutritional values, health benefits, and processing technology. *Food Chem.* 10:100919. doi: 10.1016/j.fochx.2023.100919

Li, F., and Guan, L. L. (2017). Metatranscriptomic profiling reveals linkages between the active rumen microbiome and feed efficiency in beef cattle. *Appl. Environ. Microbiol.* 83, e00061–e00017. doi: 10.1128/AEM.00061-17

Li, F., Li, C., Chen, Y., Liu, J., Zhang, C., Irving, B., et al. (2019). Host genetics influence the rumen microbiota and heritable rumen microbial features associate with feed efficiency in cattle. *Microbiome.* 7:92. doi: 10.1186/s40168-019-0699-1

Li, F., Zhou, M., Ominski, K., and Guan, L. L. (2016). Does the rumen microbiome play a role in feed efficiency of beef cattle? *J. Anim. Sci.* 94, 44–48. doi: 10.2527/jas.2016-0524

Li, H., Ma, Y., Li, Q., Wang, J., Cheng, J., Xue, J., et al. (2011). The chemical composition and nitrogen distribution of Chinese Yak (Maiwa) milk. *Int. J. Mol. Sci.* 12, 4885–4895. doi: 10.3390/ijms12084885

Liu, C., Cheung, W. H., Li, J., Chow, S. K., Yu, J., Wong, S. H., et al. (2021). Understanding the gut microbiota and sarcopenia: a systematic review. *J. Cachexia Sarcopenia Muscle.* 12, 1393–1407. doi: 10.1002/jcsm.12784

Lobo, R. R., and Faciola, A. P. (2021). Ruminal phages–a review. *Front. Microbiol.* 12:763416. doi: 10.3389/fmicb.2021.763416

Long, R., Wiener, G., and Han, J. L. (2003). "Yak nutrition-a scientific basis," in *The Yak* (Thailand: RAP Publication) 389–409.

Long, R. J., Apori, S. O., Castro, F. B., and Ørskov, E. R. (1999a). Feed value of native forages of the Tibetan Plateau of China. *Anim. Feed Sci. Technol.* 80, 101–113. doi: 10.1016/S0377-8401(99)00057-7

Long, R. J., Ding, L. M., Shang, Z. H., and Guo, X. H. (2008). The yak grazing system on the Qinghai-Tibetan plateau and its status. *Rangel. J.* 30, 241–246. doi: 10.1071/RJ08012

Long, R. J., Dong, S. K., Chen, X. B., Ørskov, E. R., and Hu, Z. Z. (1999c). Preliminary studies on urinary excretion of purine derivatives and creatinine in yaks. *J. Agric. Sci.* 133, 427–431. doi: 10.1017/S0021859699007169

Long, R. J., Dong, S. K., Hu, Z. Z., Shi, J. J., Dong, Q. M., and Han, X. T. (2004). Digestibility, nutrient balance and urinary purine derivative excretion in dry yak cows fed oat hay at different levels of intake. *Livest. Sci.* 88, 27–32. doi: 10.1016/j.livprodsci.2003.11.004

Long, R. J., Zhang, D. G., Wang, X., Hu, Z. Z., and Dong, S. K. (1999b). Effect of strategic feed supplementation on productive and reproductive performance in yak cows. *Prev. Vet. Med*. 38, 195–206. doi: 10.1016/S0167-5877(98)00125-1

Ma, J., Chai, S. T., Sun, L., and Liu, S. J. (2021a). Correlation analysis between natural pasture grasses at different altitudes and routine nutritional indexes of yak milk in the Qinghai region (In Chinese). *Chin. J. Anim. Nutr.* 33, 4502–4510. doi: 10.3969/j.issn.1006-267x.2021.08.030

Ma, J., Zhang, T., Wang, W., Chen, Y., Cai, W., and Zhu, B. (2021b). Comparative transcriptome analysis of gayal (*Bos frontalis*), yak (*Bos grunniens*), and cattle (*Bos taurus*) reveal the high-altitude adaptation. *Front. Genet.* 12:778788. doi: 10.3389/fgene.2021.778788

Ma, Y., He, S. H., and Park, Y. W. (2017). "Yak milk", in: *Handbook of Milk of Non-Bovine Mammals*, eds. Y. W., Park, D., Min, G., Haenlein (New York: John Wiley and Sons), 481–513. doi: 10.1002/9781119110316.ch7

Malmuthuge, N., Liang, G., and Guan, L. L. (2019). Regulation of rumen development in neonatal ruminants through microbial metagenomes and host transcriptomes. *Genome Biol.* 20, 1–16. doi: 10.1186/s13059-019-1786-0

Mi, J. D. (2016). *Dynamics in rumen and distribution along gastrointestinal tracts of bacteria and methanogen in yak (In Chinese)*. Ph.D Dissertation. Lanzhou: Lanzhou University.

Mizrahi, I., Wallace, R. J., and Moraïs, S. (2021). The rumen microbiome: balancing food security and environmental impacts. *Nat. Rev. Microbiol.* 19, 553–566. doi: 10.1038/s41579-021-00543-6

Morais, S., and Mizrahi, I. (2019). The road not taken: the rumen microbiome, functional groups, and community states. *Trends Microbiol.* 27, 538–549. doi: 10.1016/j.tim.2018.12.011

Morales, E. R., Rossi, G., Cattin, M., Jones, E., Braganca, R., and Newbold, C. J. (2018). The effect of an isoflavonid-rich liquorice extract on fermentation, methanogenesis and the microbiome in the Rumen Simulation Technique. *FEMS Microbiol. Ecol.* 94:fiy009. doi: 10.1093/femsec/fiy009

Moss, A. R., Jouany, J. P., and Newbold, J. (2000). "Methane production by ruminants: its contribution to global warming," in *Annales de zootechnie* (EDP Sciences) 231–253. doi: 10.1051/animres:2000119

Oh, S., Shintani, R., Koike, S., and Kobayashi, Y. (2017). Ginkgo fruit extract as an additive to modify rumen microbiota and fermentation and to mitigate methane production. *J. Dairy Sci.* 100, 1923–1934. doi: 10.3168/jds.2016-11928

Ojo, O. E., and Kreuzer-Redmer, S. (2023). MicroRNAs in ruminants and their potential role in nutrition and physiology. *Vet. Sci.* 10:57. doi: 10.3390/vetsci10010057

Ørskov, E. R. (1982). *Protein Nutrition in Ruminants*. London: Academic Press Inc.

Owens, F. N., and Bergen, W. G. (1983). Nitrogen metabolism of ruminant animals: historical perspective, current understanding and future implications1, 2. *J. Anim. Sci.* 57, 498–518.

Pan, F. M., Chang, S. H., Wang, G. D., Hao, S. Y., Liu, J., Zhang, H. Y., et al. (2021). Effects of phenological period on the composition of fatty acids and conjugated linoleic acids in rumen fluid, forage and milk fat of grazing yak and their correlation analysis. *Acta Pratac. Sin.* 30:110.

Parodi, P. W. (2004). Milk fat in human nutrition. *Aust. J. Dairy Technol.* 59, 53–59.

Patil, R. D., Ellison, M. J., Wolff, S. M., Shearer, C., Wright, A. M., Cockrum, R. R., et al. (2018). Poor feed efficiency in sheep is associated with several structural abnormalities in the community metabolic network of their ruminal microbes. *J. Anim. Sci.* 96:2113. doi: 10.1093/jas/sky096

Perlman, D., Martínez-Álvaro, M., Moraïs, S., Altshuler, I., Hagen, L. H., and Jami, E. (2021). Concepts and consequences of a core gut microbiota for animal growth and development. *Annu. Rev. Anim. Biosci.* 10, 177–201. doi: 10.1146/annurev-animal-013020-020412

Poulsen, M., Schwab, C., Jensen, B. B., Engberg, R. M., Spang, A., Canibe, N., et al. (2013). Methylotrophic methanogenic Thermoplasmata implicated in reduced methane emissions from bovine rumen. *Nat. Commun.* 4:1428. doi: 10.1038/ncomms2847

Qiu, Q., Zhang, G., Ma, T., Qian, W., Wang, J., Ye, Z., et al. (2012). The yak genome and adaptation to life at high altitude. *Nat. Genet.* 44:946. doi: 10.1038/ng.2343

Roehe, R., Dewhurst, R. J., Duthie, C. A., Rooke, J. A., McKain, N., Ross, D. W., et al. (2016). Bovine host genetic variation influences rumen microbial methane production with best selection criterion for low methane emitting and efficiently feed converting hosts based on metagenomic gene abundance. *PLoS Genet.* 12:e1005846. doi: 10.1371/journal.pgen.1005846

Shabat, S. K. B., Sasson, G., Doron-Faigenboim, A., Durman, T., Yaacoby, S., Miller, M. E. B., et al. (2016). Specific microbiome-dependent mechanisms underlie the energy harvest efficiency of ruminants. *ISME J.* 10:2958. doi: 10.1038/ismej.2016.62

Shabi, Z., Tagari, H., Murphy, M. R., Bruckental, I., Mabjeesh, S. J., Zamwel, S., et al. (2000). Partitioning of amino acids flowing to the abomasum into feed, bacterial, protozoal, and endogenous fractions. *J. Dairy Sci.* 83, 2326–2334. doi: 10.3168/jds.S0022-0302(00)75120-4

Shao, B., Long, R., Ding, Y., Wang, J., Ding, L., Wang, H., et al. (2010). Morphological adaptations of yak (*Bos grunniens*) tongue to the foraging environment of the Qinghai-Tibetan Plateau 1. *J. Anim. Sci.* 88, 2594–2603. doi: 10.2527/jas.2009-2398

Shi, B., Jiang, Y., Chen, Y., Zhao, Z., Zhou, H., Luo, Y., et al. (2019). Variation in the fatty acid synthase gene (*FASN*) and its association with milk traits in Gannan yaks. *Animals.* 9:613. doi: 10.3390/ani9090613

Shingfield, K. J., Bonnet, M., and Scollan, N. D. (2013). Recent developments in altering the fatty acid composition of ruminant-derived foods. *Animal* 7, 132–162. doi: 10.1017/S1751731112001681

Siciliano-Jones, J., and Murphy, M. R. (1989). Production of volatile fatty acids in the rumen and cecum-colon of steers as affected by forage: concentrate and forage physical form. *J. Dairy Sci.* 72, 485–492. doi: 10.3168/jds.S0022-0302(89)79130-X

Solomon, R., Wein, T., Levy, B., Eshed, S., Dror, R., Reiss, V., et al. (2022). Protozoa populations are ecosystem engineers that shape prokaryotic community structure and function of the rumen microbial ecosystem. *ISME J.* 16, 1187–1197. doi: 10.1038/s41396-021-01170-y

Song, C., Huang, Y., Yang, Z., Ma, Y., Chaogetu, B., and Zhuoma, Z. (2019). RNA-Seq analysis identifies differentially expressed genes insubcutaneous adipose tissuein qaidamford cattle, cattle-yak, and angus cattle. *Animals.* 9:1077. doi: 10.3390/ani9121077

St-Pierre, B., and Wright, A. D. G. (2012). Molecular analysis of methanogenic archaea in the forestomach of the alpaca (*Vicugna pacos*). *BMC Microbiol.* 12:1. doi: 10.1186/1471-2180-12-1

Tang, Q., Gu, Y., Zhou, X., Jin, L., Guan, J., and Liu, R. (2017). Comparative transcriptomics of 5 high-altitude vertebrates and their low-altitude relatives. *Gigascience,* 6:gix105. doi: 10.1093/gigascience/gix105

Tapio, I., Snelling, T. J., and Strozzi, F. (2017). The ruminal microbiome associated with methane emissions from ruminant livestock. *J. Anim. Sci. Biotechnol.* 8, 1–11. doi: 10.1186/s40104-017-0141-0

Toral, P. G., Monahan, F. J., Hervás, G., Frutos, P., and Moloney, A. P. (2018). Modulating ruminal lipid metabolism to improve the fatty acid composition of meat and milk. Challenges and opportunities. *Animal* 12, s272–s281. doi: 10.1017/S1751731118001994

Tremaroli, V., and Bäckhed, F. (2012). Functional interactions between the gut microbiota and host metabolism. *Nature.* 489, 242–249. doi: 10.1038/nature11552

Wan, H. L., Luo, L. T., and Wu, J. P. (2012). Research advances in yak meat quality characteristics (In Chinese). *J. Anim. Sci. Vet. Med.* 31, 36–40.

Wang, B., Wang, Y., Zuo, S., Peng, S., Wang, Z., Zhang, Y., et al. (2021). Untargeted and targeted metabolomics profiling of muscle reveals enhanced meat quality in artificial pasture grazing tan lambs via rescheduling the rumen bacterial community. *J. Agric. Food Chem.* 69, 846–858. doi: 10.1021/acs.jafc.0c06427

Wang, H. C., Long, R. J., Liang, J. B., Guo, X. S., Ding, L. M., and Shang, Z. H. (2011). Comparison of nitrogen metabolism in Yak (*Bos grunniens*) and indigenous cattle (*Bos taurus*) on the Qinghai-Tibetan Plateau. *Asian Austral. J. Anim. Sci.* 24, 766–773. doi: 10.5713/ajas.2011.10350

Wang, H. C., Shi, F. Y., Hou, M. J., Fu, X. Y., and Long, R. J. (2016). Cloning of oligopeptide transport carrier PepT1 and comparative analysis of PepT1 messenger ribonucleic acid expression in response to dietary nitrogen levels in yak (*Bos grunniens*) and indigenous cattle (*Bos taurus*) on the Qinghai–Tibetan plateau. *J. Anim. Sci.* 94, 3431–3440. doi: 10.2527/jas.2016-0501

Wang, L. Z., Wang, Z. S., Xue, B., Wu, D., and Peng, Q. H. (2017). Comparison of rumen archaeal diversity in adult and elderly yaks (*Bos grunniens*) using 16S rRNA gene high-throughput sequencing. *J. Integr. Agric.* 16, 1130–1137. doi: 10.1016/S2095-3119(16)61454-5

Wang, M. Q., Li, P. L., Tian, Y. Q., Chen, G. H., and Yuan, X. F. (2002). Dynamics of serum metabolite concentrations of growing yak (In Chinese). *Yangzhou J. Univ.* 23, 27–30.

Wang, W., Zhang, Y., Zhang, X., Li, C., Yuan, L., Zhang, D., et al. (2023). Heritability and recursive influence of host genetics on the rumen microbiota drive body weight variance in male Hu sheep lambs. *Microbiome.* 11:197. doi: 10.1186/s40168-023-01642-7

Wang, W. W. (2020). *Study on characteristics and meachanism of the cross-incubation of rumen inoculum from yak-cattle and Tibetan sheep-Small tailed (In Chinese).* Ph.D Dissertation. Lanzhou: Lanzhou University.

Wang, W. W., Ungerfeld, E. M., Degen, A. A., Jing, X., Guo, W., Zhou, J., et al. (2020). Ratios of rumen inoculum from tibetan and small-tailed han sheep influenced in vitro fermentation and digestibility. *Anim. Feed Sci. Technol.* 267:114562. doi: 10.1016/j.anifeedsci.2020.114562

Wang, Y., Li, T., Chen, X., Liu, C., Jin, X., Tan, H., et al. (2022). Preliminary investigation of mixed orchard hays on the meat quality, fatty acid profile, and gastrointestinal microbiota in goat kids. *Animals* 12:780. doi: 10.3390/ani12060780

Wei, Y. Q., Yang, H. J., Luan, Y., Long, R. J., Wu, Y. J., Wang, Z. Y., et al. (2016). Isolation, identification and fibrolytic characteristics of rumen fungi grown with indigenous methanogen from yaks (*Bos grunniens*) grazing on the Qinghai-Tibetan Plateau. *J. Appl. Microbiol.* 120, 571–587. doi: 10.1111/jam.13035

Xie, A. Y., Bi, X. C., Zhuge, W. J., Han, X. T., and Hu, L. H. (1989). Study on ruminal digestible metabolism in growing yak under barn feeding conditions (In Chinese). *Qinghai J. Anim. Husb. Vet. Med.* 19, 2–5.

Xie, A. Y., and Chai, S. T. (1996). The herbage yield and the nutrient variation in mountain meadow (In Chinese). *Qinghai J. Anim. Husb. Vet. Med.* 26:3.

Xue, B., and Han, X. T. (2001). Differences of ruminal dietary protein degradation between yaks and holsteins (In Chinese). *China Herbivores* 3, 3–8.

Xue, D., Chen, H., Chen, F., He, Y., Zhao, C., and Zhu, D. (2016). Analysis of the rumen bacteria and methanogenic archaea of yak (*Bos grunniens*) steers grazing on the Qinghai-Tibetan Plateau. *Livest. Sci.* 188, 61–71. doi: 10.1016/j.livsci.2016.04.009

Xue, D., Chen, H., Zhao, X., Xu, S., Hu, L., Xu, T., et al. (2017). Rumen prokaryotic communities of ruminants under different feeding paradigms on the Qinghai-Tibetan Plateau. *Syst. Appl. Microbiol.* 40, 227–236. doi: 10.1016/j.syapm.2017.03.006

Yan, X. T., Yan, B. Y., Ren, Q. M., Dou, J. J., Wang, W. W., Zhang, J. J., et al. (2018). Effect of slow-release urea on the composition of ruminal bacteria and fungi communities in yak. *Anim. Feed Sci. Technol.* 244, 18–27. doi: 10.1016/j.anifeedsci.2018.07.016

Yao, J., Guo, J., Zhao, J. J., Yang, S. Z., and Niu, C. E. (2002). Seasonal dynamics of rumen protozoa population in yak and Tibetan sheep (In Chinese). *China Herbivores* 22, 17–18.

Yeoman, C. J., and White, B. A. (2014). Gastrointestinal tract microbiota and probiotics in production animals. *Annu. Rev. Anim. Biosci.* 2, 469–486. doi: 10.1146/annurev-animal-022513-114149

Yin, R. H., Bai, W. L., Wang, J. M., Wu, C. D., Dou, Q. L., Yin, R. L., et al. (2009). Development of an assay for rapid identification of meat from yak and cattle using polymerase chain reaction technique. *Meat Sci.* 83, 38–44. doi: 10.1016/j.meatsci.2009.03.008

Yuan, M., Xia, W., Zhang, X., Liu, Y., and Jiang, M. (2020). Identification and verification of differentially expressed genes in yak mammary tissue during the lactation cycle. *J. Dairy Res.* 87, 158–165. doi: 10.1017/S0022029919001006

Yue, X. P., Dechow, C., Chang, T. C., DeJarnette, J. M., Marshall, C. E., and Lei, C. Z. (2014). Copy number variations of the extensively amplified Y-linked genes, HSFY and ZNF280BY, in cattle and their association with male reproductive traits in Holstein bulls. *BMC Genom.* 15, 1–12. doi: 10.1186/1471-2164-15-113

Zhang, L., Jia, S., Yang, M., Xu, Y., Li, C., and Sun, J. (2014). Detection of copy number variations and their effects in Chinese bulls. *BMC Genom.* 15, 1–9. doi: 10.1186/1471-2164-15-480

Zhang, X., Wang, K., Wang, L., Yang, Y., Ni, Z., and Xie, X. (2016). Genome-wide patterns of copy number variation in the Chinese yak genome. *BMC Genom.* 17, 1–12. doi: 10.1186/s12864-016-2702-6

Zhang, Z., Xu, D., Wang, L., Hao, J., Wang, J., Zhou, X., et al. (2016). Convergent evolution of rumen microbiomes in high-altitude mammals. *Curr. Biol.* 26, 1873–1879. doi: 10.1016/j.cub.2016.05.012

Zhao, C., Wang, L., Ke, S., Chen, X., Kenéz, A. and Xu, W., et al. (2022). Yak rumen microbiome elevates fiber degradation ability and alters rumen fermentation pattern to increase feed efficiency. *Anim. Nutr.* 11, 201–214. doi: 10.1016/j.aninu.2022.07.014

Zhou, J. W., Liu, H., Zhong, C. L., Degen, A. A., Yang, G., Zhang, Y., et al. (2018). Apparent digestibility, rumen fermentation, digestive enzymes and urinary purine derivatives in yaks and Qaidam cattle offered forage-concentrate diets differing in nitrogen concentration. *Livest. Sci.* 208, 14–21. doi: 10.1016/j.livsci.2017.11.020

Zhou, J. W., Zhong, C. L., Liu, H., Degen, A. A., Titgemeyer, E. C., Ding, L. M., et al. (2017). Comparison of nitrogen utilization and urea kinetics between yaks (*Bos grunniens*) and indigenous cattle (*Bos taurus*). *J. Anim. Sci.* 95, 4600–4612. doi: 10.2527/jas2017.1428

Zongo, K., Krishnamoorthy, S., Moses, J. A., Yazici, F., Con, A. H., and Anandharamakrishnan, C. (2021). Total conjugated linoleic acid content of ruminant milk: The world status insights. *Food Chem.* 334:127555. doi: 10.1016/j.foodchem.2020.127555

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