



RESEARCH ARTICLE

Gut Microbiome and Metabolism Reveal Livestock Farm Wastewater Discharge Perturbed Intestinal Homeostasis in Ducks

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ABSTRACT

The livestock industry has experienced explosive growth over the past few decades, owing to a huge population, which effectively alleviated protein shortage. However, livestock farm wastewater containing heavy metals, antibiotics, and pathogenic bacteria caused serious environmental pollution. Ducks, as an important waterfowl, are inevitably exposed to sewage contaminated by livestock farm wastewater, but information regarding their influence on ducks remains scarce. In this study, we investigated the effects of sewage contaminated by livestock farm wastewater on growth parameters, intestinal microbiota, and metabolism of ducks. Sewage administration not only decreased growth performance and liver index but also resulted in intestinal, liver, and kidney damage in ducks. Amplicon sequencing indicated 1084 OTUs across the two groups, and *Firmicutes*, *Proteobacteria*, *Actinobacteria*, and *Bacteroidetes* were the most preponderant phyla in the ducks regardless of treatment. Interestingly, although sewage administration did not alter the gut microbial diversity, the proportion of some intestinal bacteria altered dramatically. Bacterial taxonomic analysis indicated that 24 discriminatory genera were recognized for the gut microbiota between both groups, where 20 bacterial genera decreased significantly, and 4 bacterial genera increased significantly during sewage exposure. Metabolomics analysis revealed that 37 differential metabolites were totally recognized, mainly involved in 8 metabolic pathways. In summary, this study showed that sewage exposure significantly changed the intestinal microbiota and metabolism and resulted in negative health outcomes for ducks. This research has served as motivation for environmental agencies globally to regulate livestock farm wastewater discharge and reduce environmental contamination.

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INTRODUCTION

Growing population size, income levels, and nutritional requirements all contribute to increased demand for protein and thus for livestock. However, the rapid expansion of the livestock industry has caused serious environmental pollution. Veterinary antibiotics and feed additives containing heavy metals were widely used in

intensive livestock farming to improve growth performance and prevent diseases (Li *et al.*, 2022). However, most antibiotics used in livestock breeding can be released through feces and urine due to the low absorption and metabolism of antibiotics by the animal intestine (Mustafa *et al.*, 2021). The persistent exposure of residual antibiotics in animal wastes could cause the spread of antibiotic resistance genes and antibiotic-resistant

bacteria around the livestock farm environment, posing potential risks to surrounding water quality, aquatic animals, terrestrial animals, and humans. Moreover, heavy metal and microbial contamination from animal excrement could reach aquatic environments through multiple pathways such as direct outflow of animal excrement, wastewater treatment systems, agricultural fertilizer infiltration, and storm water runoff, which seriously threatens the health of aquatic animals and waterfowl (Zhang *et al.*, 2021). Currently, gastrointestinal tract and skin diseases caused by exposure to or direct consumption of polluted water are gradually increasing.

The intestine is the primary organ responsible for digestion and absorption, inhabited by trillions of microorganisms, including bacteria, fungi, and viruses (Liu *et al.*, 2021; Bian *et al.*, 2025). There is growing evidence suggesting that the gut microbiota plays a significant role in maintaining intestinal balance and overall host health (Zhao *et al.*, 2022). Moreover, gut microbiota has also been shown to function in host metabolism, immunity, and intestinal mucosal barrier maintenance (Dong *et al.*, 2022). However, several factors, such as dietary changes, residential environment, antibiotics administration, and heavy metals, can directly or indirectly affect intestinal homeostasis, thereby inducing gut microbial dysbiosis and many diseases (Li *et al.*, 2021). Currently, numerous investigations on gut microbiota have shown a strong connection between gut microbial dysbiosis and many gastrointestinal diseases, including colorectal cancer, constipation, and diarrhea (Wu *et al.*, 2022). Importantly, although these microbial populations reside in the intestine, they can affect both nearby organs and distant organ systems, resulting in systemic effects (Yang *et al.*, 2020). Moreover, crosstalk and interactions between the intestine and other organs, including the liver, brain, and kidney, are also primarily through the metabolites and molecular patterns associated with gut microbiota, which connect the gut microbial community with other organ systems via serving as signaling molecules with immune-modulating function.

Recently, amplicon sequencing and metabolomics techniques based on high-throughput sequencing have been successfully developed and used to investigate the complicated intestinal ecosystem and gut microbiota-host interaction research because of their high sensitivity and specificity (Dong *et al.*, 2022). By profoundly comparing and analyzing the data acquired, the host health status and mechanisms causing ill-health can be further understood, and effective measures can be developed to minimize collateral damage. Increasing studies on the effects of sewage on animals indicated that environmental pollutants, including heavy metals, antibiotics, and fecal microorganisms, can disturb intestinal physiology and metabolic homeostasis (Li *et al.*, 2021). As an important waterfowl, ducks may be chronically exposed to a river contaminated by animal waste, but studies regarding the influence of sewage on intestinal microbiota and metabolism in ducks are still insufficient to report. Here, we investigated the alterations of intestinal microbiota and metabolism in ducks exposed to sewage contaminated with animal excrement.

MATERIALS AND METHODS

Animal experiments: A group of 60 1-day-old ducks (with similar original weight and regardless of sex) were purchased from a market (Hefei, China). After acclimatization for three days, the selected ducks were randomly divided into control (Con) and sewage (Sew) groups, with 30 ducks per group. Ducks in the Sew group were raised in sewage contaminated by livestock farm wastewater, whereas ducks in the Con group were raised in normal waters. Feed was provided *ad-libitum* for ducks in both groups throughout the trial. The average daily feed intake and body weight of each duck were recorded prior to morning feeding from days 1 to 14. After 14 consecutive days, all subjects were euthanized under the state of anesthesia, and then the intestinal tissue and its contents. After 14 consecutive days, all subjects were euthanized with pentobarbital (25mg/kg), and then the intestinal tissue and its contents. Meanwhile, the heart, kidney, liver, spleen, bursa of fabricius and thymus were removed from all ducks. The weight of the collected organs was measured using an electronic balance. The organ index was calculated by dividing the weight of each organ by the body weight. Some collected organs along with intestinal samples were soaked in 4% paraformaldehyde, and others were quick-frozen with liquid nitrogen and stored at -80°C for further study.

Histological observations: Formalin-soaked tissues, including liver, kidney, duodenum, and ileum, were subjected to hematoxylin and eosin (H&E) stains, and their specific methods and details were based on previous studies (Zhang *et al.*, 2018).

Analysis of amplicon sequencing and metabolome: The specific steps and methods of gut microbiota sequencing and metabolomics are based on previous studies (Lin *et al.*, 2020; Li *et al.*, 2021).

RESULTS

Sewage administration decreased the performance parameters of ducks: Results revealed that the body weight was dramatically decreased, whereas the average daily feed intake did not change in ducks exposed to sewage as compared to controls (Fig. 1A, B). Moreover, the organ parameters analysis indicated that sewage administration resulted in a significant decrease of liver index, but had no effect on cardiac, kidney, spleen, bursa of fabricius and thymus indices (Fig. 1C-H).

Histological observations: Histopathological changes in duodenum, ileum, liver and kidney are illustrated in Fig. 2. Results showed that the duodenal and ileal structures in the control group had clear borders and were well-integrated, while those in ducks exposed to sewage were loosely arranged, irregular, and disordered (Fig. 2A1, A2, B1, B2, C1, C2, D1, D2). Moreover, the liver and kidney tissues in the Con group showed regular and normal structures (Fig. 2E1, E2, G1, G2). However, sewage exposure caused a decrease in glycogen of hepatocytes, accompanied by a reduction in hepatocyte volume (Fig. 2F1, F2). Sewage exposure also resulted in pigment deposition and lymphocyte infiltration in renal tubular epithelial cells (Fig. 2H1, H2).

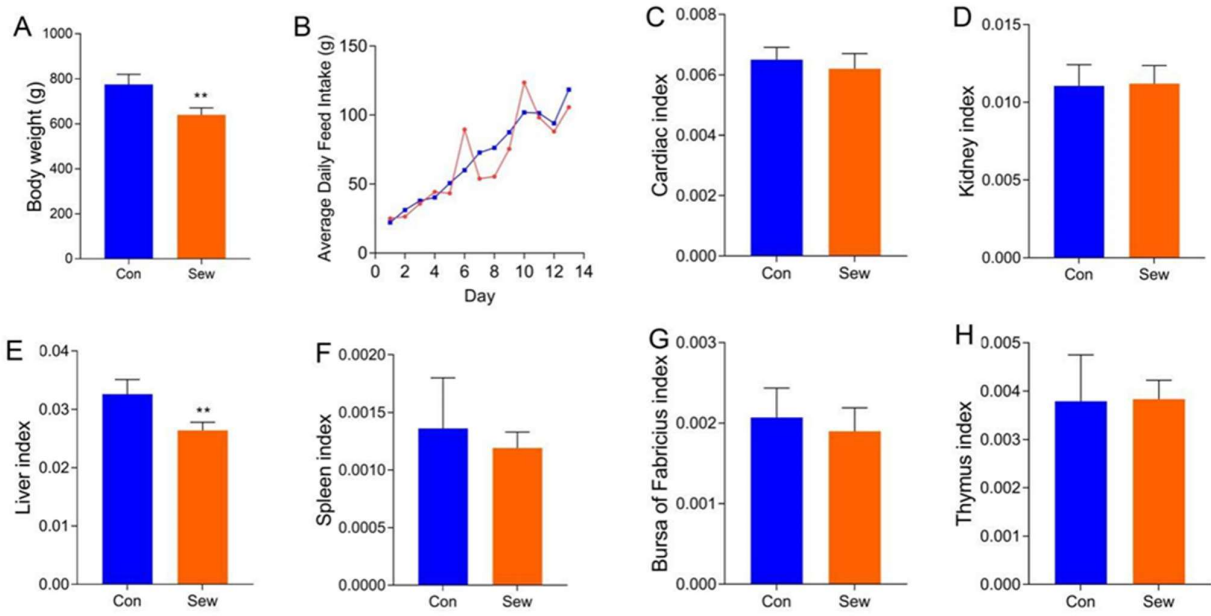


Fig. 1: Sewage administration decreases the overall performance parameters of ducks. A-H represent body weight, average daily feed intake, cardiac, kidney, liver, spleen, bursa of fabricius and thymus indices, respectively. The data were expressed as means±SD. ** indicates the difference between control and sewage exposure groups, **P<0.01. Con: control group. Sew: sewage exposure group.

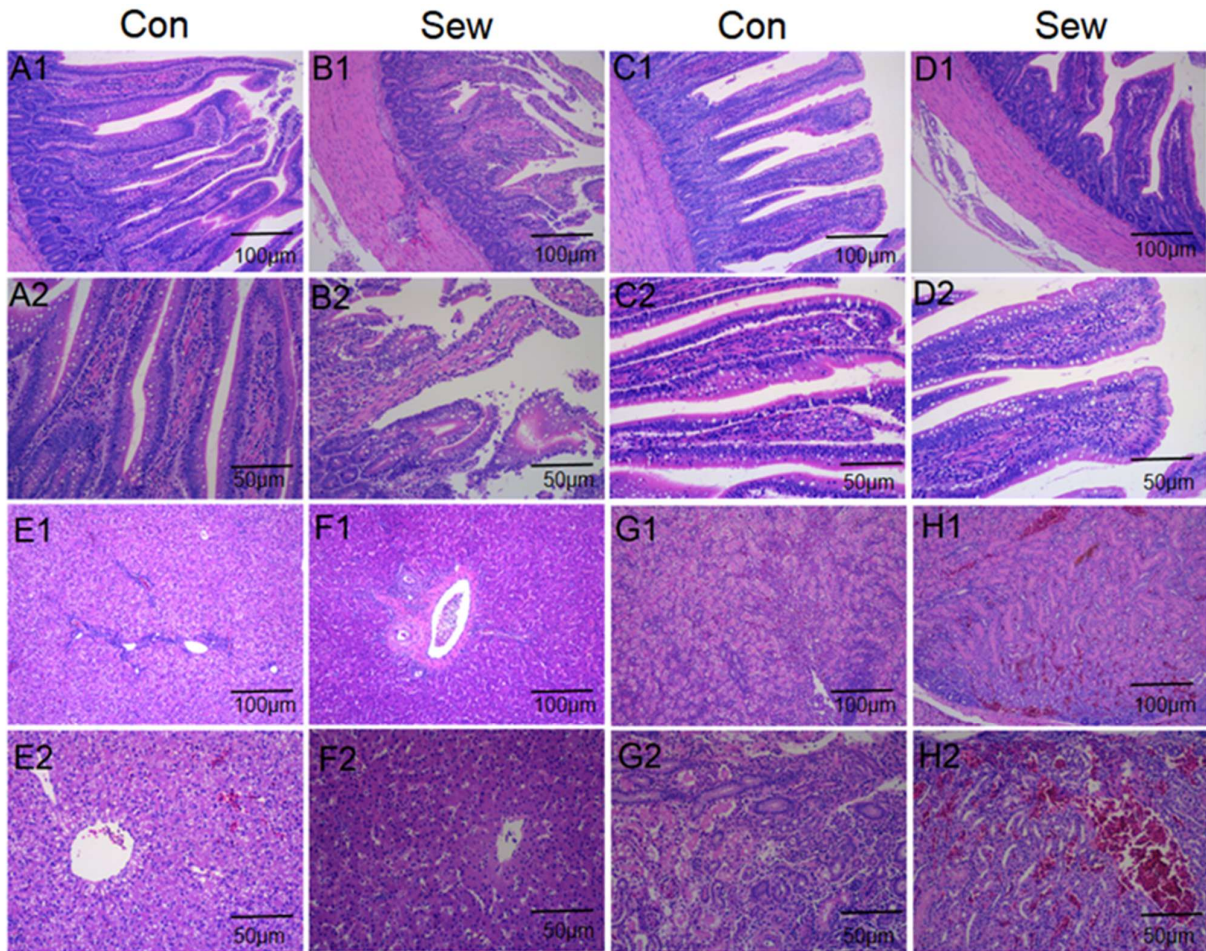


Fig. 2: Histological examination of the duodenum, ileum, liver, and kidney. (A1, A2) and (B1, B2) represent the duodenum in the Con and Sew groups, respectively. (C1, C2) and (D1, D2) represent the ileum in the Con and Sew, respectively. (E1, E2) and (F1, F2) represent the liver in the Con and Sew groups, respectively. (G1, G2) and (H1, H2) represent the kidney in the Con and Sew groups, respectively. The scale bar is 100 and 50µm, respectively.

Data acquisition and analysis: To characterize gut microbial alternations during sewage administration, this study amplicon-sequenced intestinal samples from ducks with both treatments, and a total of 147,3908 (Con = 746,007, Sew = 727,901) original sequences were obtained. After sequence optimization, we totally acquired 126,2131 (Con = 643,198, Sew = 618,933) valid sequences in the range of 78,795 to 119,421 sequences per sample (Table 1). Following taxonomic assignment, the effective sequences were clustered into 917 OTUs based on 97% sequence similarity, varying from 252 to 633 OTUs per sample. Furthermore, there were 760 core OTUs in the Con and Sew groups, which consisted of approximately 82.88% of the total OTUs identified (Fig. 3A). Multi-sample rarefaction and shannon abundance curves of both groups exhibited a saturated trend with the increased quantity of sequences sampled, suggesting that almost all the bacterial species were covered (Fig. 3B).

Microbial diversity index analysis in gut microbiota associated with sewage administration: Good's coverage estimates indicated that the sequenced sample contained nearly all bacterial species, suggesting sufficient coverage. There was no significant difference in the Chao1

(461.90 ± 143.41 versus 530.60 ± 142.99 , $P=0.43$), ACE (470.57 ± 152.8 versus 554.96 ± 139.05 , $p = 0.34$), Simpson (0.71 ± 0.19 versus 0.84 ± 0.12 , $p = 0.19$) and Shannon (3.20 ± 1.19 versus 4.48 ± 1.23 , $p = 0.10$) indices between the Con and Sew groups, suggesting that sewage administration did not impact the gut microbial abundance and diversity of ducks (Fig. 3C-F). Results of PCoA plots revealed that samples from both groups clustered together, indicating that sewage administration did not have a significant effect on the gut microbial structure (Fig. 3H, I).

Table 1: Basic bacterial information statistics of amplicon sequencing

Sample	Raw Reads	Clean Reads	Effective Reads	AvgLen(bp)	GC (%)	Effective (%)
Con1	125410	116714	106021	425	53.27	84.54
Con2	123685	114395	95826	427	52.8	77.48
Con3	123525	115240	103062	428	52.4	83.43
Con4	113231	106323	102034	424	52.72	90.11
Con5	127889	120439	116834	410	50.49	91.36
Con6	132267	123558	119421	414	50.93	90.29
Sew1	123278	115835	112280	417	51.35	91.08
Sew2	129566	120273	108746	418	54.7	83.93
Sew3	129342	119222	107602	424	53.09	83.19
Sew4	120935	113139	100709	427	54.28	83.28
Sew5	130683	120967	110801	423	54.28	84.79
Sew6	94097	88989	78795	419	52.97	83.74

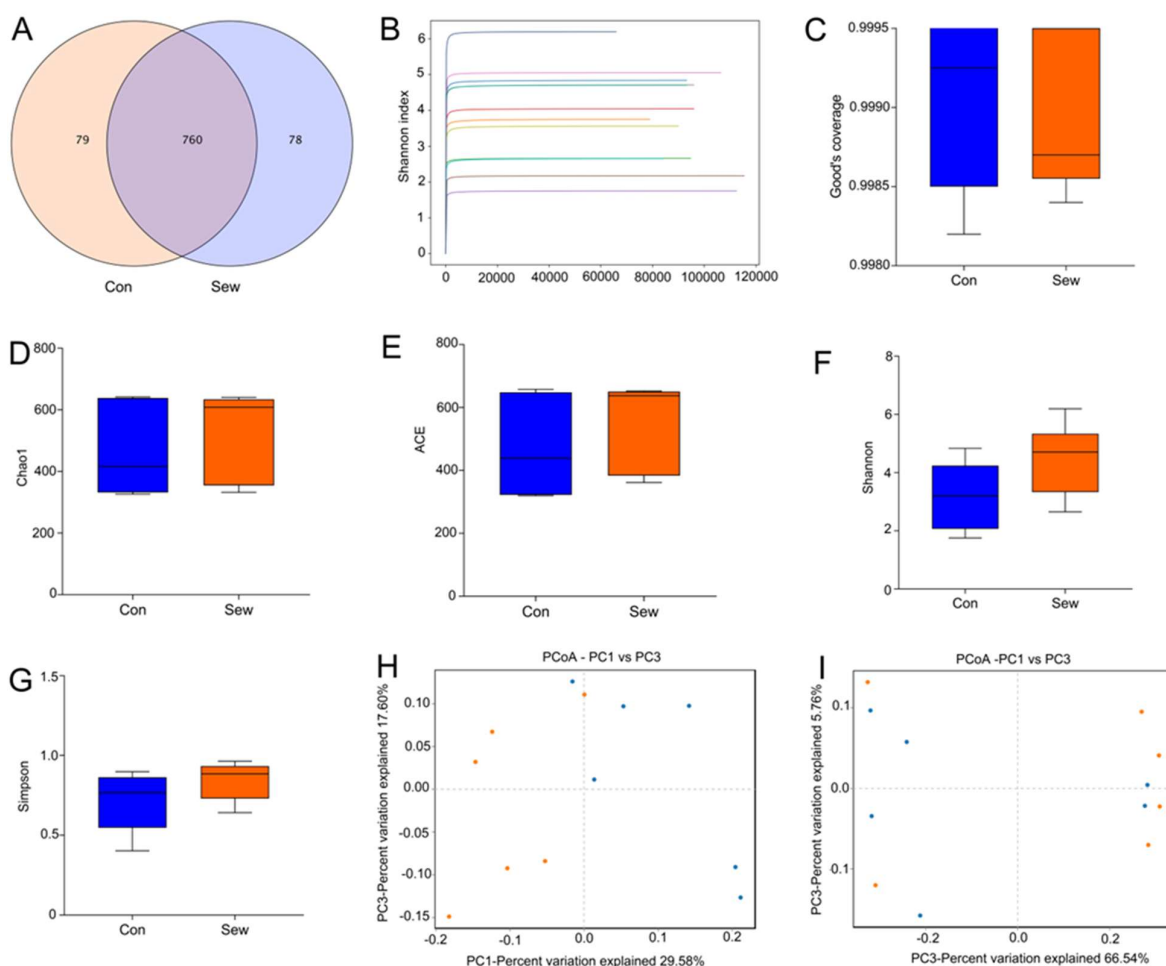


Fig. 3: Venn diagram and rarefaction curves. A: The numbers in the Venn diagram indicate the shared or unique OTUs of control and sewage exposure groups. B: Rank-abundance and rarefaction curves represent depth and evenness of amplicon sequencing. C-I: Gut microbial alpha-diversity differences between both groups were computed by the Chao1, ACE, Shannon, and Simpson indices, whereas beta-diversity was evaluated via PCoA scatterplot based on the weighted and unweighted UniFrac distance. Venn diagram displaying unique and common OTUs.

Sewage administration altered the gut microbial composition: There were 33 phyla and 414 genera detected in the gut microbiota of all ducks, varying from 11 to 27 phyla as well as 138 to 281 genera per sample. Specifically, the phyla *Firmicutes* (81.66% and 49.36%), *Proteobacteria* (10.03% and 18.964%), *Actinobacteria* (4.22% and 18.38%) and *Bacteroidetes* (2.79% and 12.26%) were the most dominant bacteria in the ducks regardless of treatment (Fig. 4A). Other phyla including *Euryarchaeota* (0.011% and 0.18%), *Epsilonbacteraeota* (0.081% and 0.073%), *Fusobacteria* (0.023% and 0.097%) and *Acidobacteria* (0.057% and 0.058%) in the Con and Sew groups were recognized in low abundances,

which together consisted of less than 0.5% of the total taxonomic groups identified. *Candidatus_Arthromitus* (28.04%) was the most dominant genus in the Con group, followed by *Enterococcus* (20.14%) and *Streptococcus* (11.19%). Furthermore, the dominant bacterial genus observed in the Sew group were *Escherichia-Shigella* (13.60%), *Staphylococcus* (11.32%) and *Rothia* (10.94%) in descending order (Fig. 4B). Furthermore, the heatmap based on genus-level cluster analysis revealed the bacterial abundance and distribution in both groups and indicated the significant alterations in gut microbial composition under the influence of sewage administration (Fig. 4C).

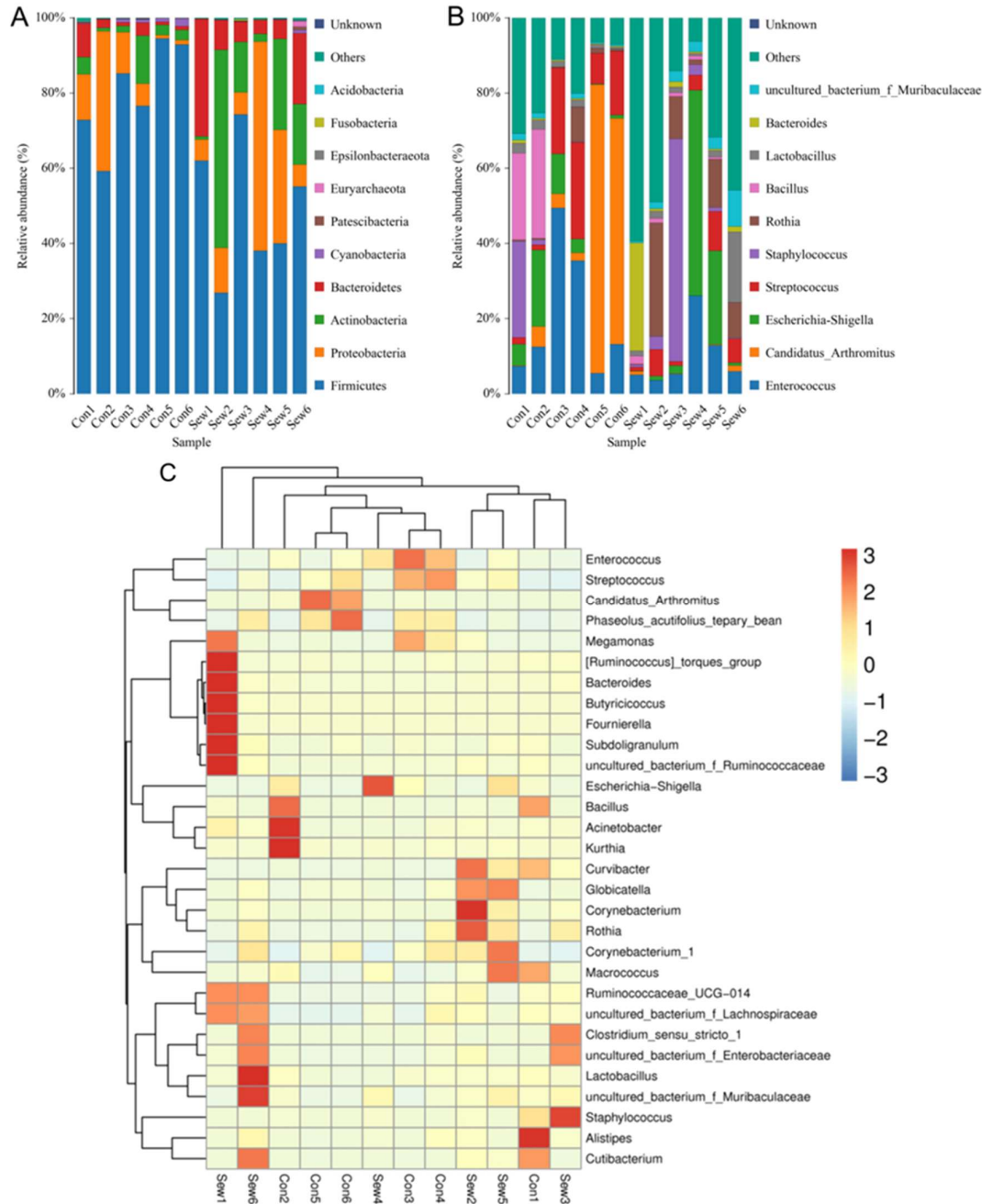


Fig. 4: Taxonomic profiles of the gut microbiota in the Con and Sew groups. The relative abundances of the dominant phylum (A) and genus (B) of gut microbiota are shown in the Con and Sew groups. C: The heatmap analysis presents the distribution and relative abundance of bacteria in the Con and Sew groups using hierarchical clustering. Each row and column in the heat map represents the bacterial genus and individual sample, respectively.

To further investigate the impact of sewage administration on gut microbial compositions in ducks, we used Metastats analysis to compare the different classification levels. At the phylum level, the gut microbial community in the Con group was characterized by a significant increase in the relative abundance of *Firmicutes*. Moreover, 24 genera were recognized as dramatically different between the Con and Sew groups. Of these differential taxa, *Muribaculum*, *Proteiniclasticum*, *uncultured_bacterium_c_Deltaproteobacteria*, *Arenimonas*, *Candidatus_Stoquefichus*, *Sulfurifustis*, *uncultured_bacterium_o_Actinomarinales*, *uncultured_bacterium_o_Candidatus_Kaiserbacteria*, *Solirubrobacter*, *uncultured_bacterium_o_Candidatus_Doudnabacteria*, *uncultured_bacterium_o_Microtrichales*, *Peptoniphilus*, *uncultured_bacterium_f_Lachnospiraceae*, *uncultured_bacterium_f_Enterobacteriaceae*, *Rothia*, *uncultured_bacterium_f_Muribaculaceae*, *Facklamia*, *uncultured_bacterium_f_Gemmatimonadaceae*, *Jeotgalicoccus* and *Ruminococcaceae_UCG-014* were observed to be more abundant in the Sew group than in the Con group, while the *Weissella*, *uncultured_bacterium_o_Chloroplast*, *Bilophila* and *Leuconostoc* showed the opposite trend (Fig. 5). Given the limitations of the above-mentioned discriminant analysis, LEfSe coupled with LDA were applied to recognize the specific bacterial species related to sewage administration. The Sew group was significantly enriched for *Bacteroidetes*, *Dependentiae*, *Corynebacterium*, *Prevotella_9*, *Sphingobium*, *Ruminiclostridium_9*, and *Sphingomonas*, whereas *Lactococcus* and *Candidatus_Arthromitus* were all significantly overrepresented in the Con group (Fig. 6A, B). Moreover, we also performed correlation network analysis to reveal the potential relationships among bacterial genera (Fig. 6C).

Muribaculaceae, *Facklamia*, *uncultured_bacterium_f_Gemmatimonadaceae*, *Jeotgalicoccus* and *Ruminococcaceae_UCG-014* were observed to be more abundant in the Sew group than in the Con group, while the *Weissella*, *uncultured_bacterium_o_Chloroplast*, *Bilophila* and *Leuconostoc* showed the opposite trend (Fig. 5). Given the limitations of the above-mentioned discriminant analysis, LEfSe coupled with LDA were applied to recognize the specific bacterial species related to sewage administration. The Sew group was significantly enriched for *Bacteroidetes*, *Dependentiae*, *Corynebacterium*, *Prevotella_9*, *Sphingobium*, *Ruminiclostridium_9*, and *Sphingomonas*, whereas *Lactococcus* and *Candidatus_Arthromitus* were all significantly overrepresented in the Con group (Fig. 6A, B). Moreover, we also performed correlation network analysis to reveal the potential relationships among bacterial genera (Fig. 6C).

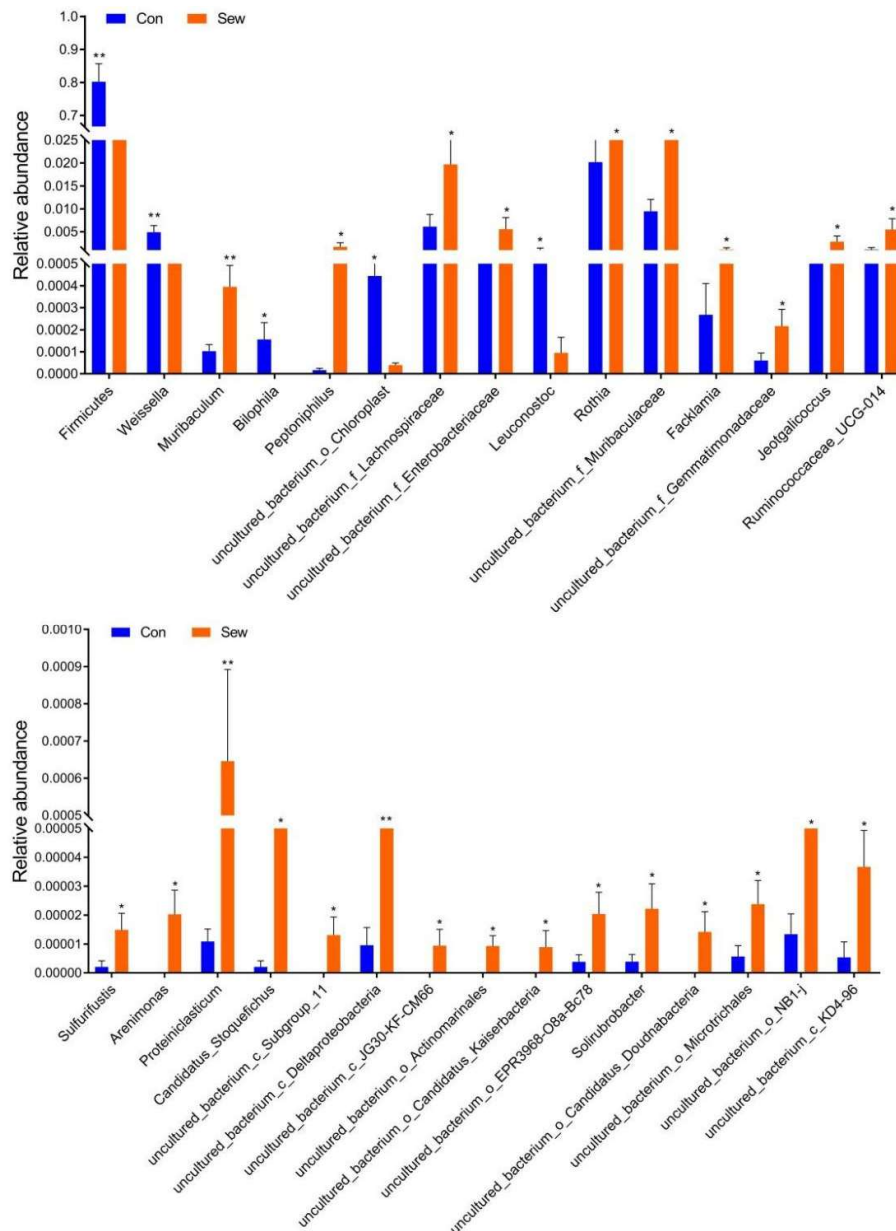


Fig. 5: The changes of the gut microbial compositions at the phylum and genus levels in ducks exposed to sewage.

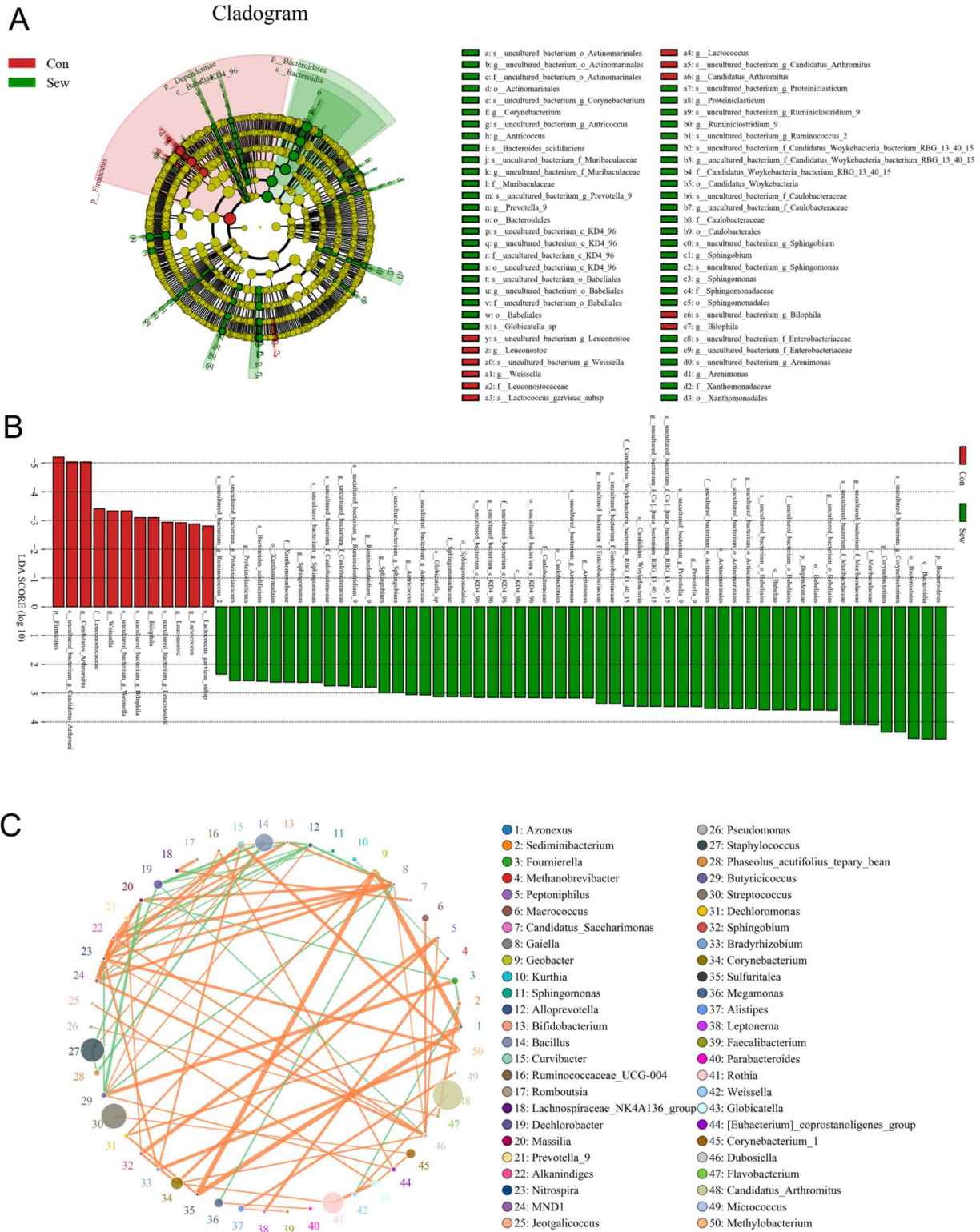


Fig. 6: LefSe analysis (A, B) was used to identify differential taxa in the gut microbiota between Con and Sew groups. C: Correlation analysis

Sewage exposure induced metabolic disorders in the intestine: The samples of sewage exposure group in PCA and OPLS-DA were distinctly separated from those of the control individuals, indicating the significant differences in intestinal metabolism between Con and Sew groups (Fig.

7A, B). Additionally, the differential metabolites were selected based on the criteria of VIP>1 and P<0.05, and their abundances were visualized through a clustering heatmap (Fig. 7C). Results indicated that 37 differential metabolites were totally identified between the Con and

Sew groups (Table 2). Among identified metabolites, the abundances of 5 metabolites were decreased dramatically, whereas 32 metabolites showed the opposite trend (Fig. 8A-C). MetaboAnalyst 4.0 was applied for metabolic pathway analysis to explore the metabolic mechanism associated with sewage exposure (Fig. 8D, E). Among enriched pathways, 8 pathways with significant difference were the glycerophospholipid metabolism, citrate cycle (TCA cycle), alanine, aspartate and glutamate metabolism, phosphatidylinositol signaling system, glyoxylate and dicarboxylate metabolism, glycerolipid metabolism, glycosylphosphatidylinositol (GPI)-anchor biosynthesis and pyruvate metabolism, which involved in 4 potential biomarkers including oxalacetic acid, PE(16:1(9Z)/20:1(11Z)), PC(18:1(9Z)/18:1(9Z)) and LPA(0:0/18:0).

gut microbiota and metabolites, we chose specific differential bacteria and metabolites to create a metabolite-microbe interaction heat map based on Spearman's correlation coefficient. Results revealed that glutathione was positively associated with *Bilophila* and *Leuconostoc* but negatively related to *Rothia*, *Prevotella_9*, *Corynebacterium*, *Peptoniphilus*, *Sphingobium*, *Arenimonas*, *Muribaculum*, *Candidatus_Stoquefichus*, *Sulfurifustis*, and *Solirubrobacter* (Fig. 8F). Famotidine was positively correlated with *Leuconostoc* but negatively related to *Rothia*, *Prevotella_9*, *Corynebacterium*, *Peptoniphilus*, *Sphingobium*, *Arenimonas*, *Muribaculum*, *Candidatus_Stoquefichus* and *Sulfurifustis*. Tolfenamic acid was negatively associated with *Proteiniclasticum*, *Rothia*, *Prevotella_9*, *Corynebacterium*, *Sphingobium*, *Sphingomonas*, *Muribaculum*, and *Candidatus_Stoquefichus*.

Correlation analysis for differential bacteria and metabolites: To further explore the connection between

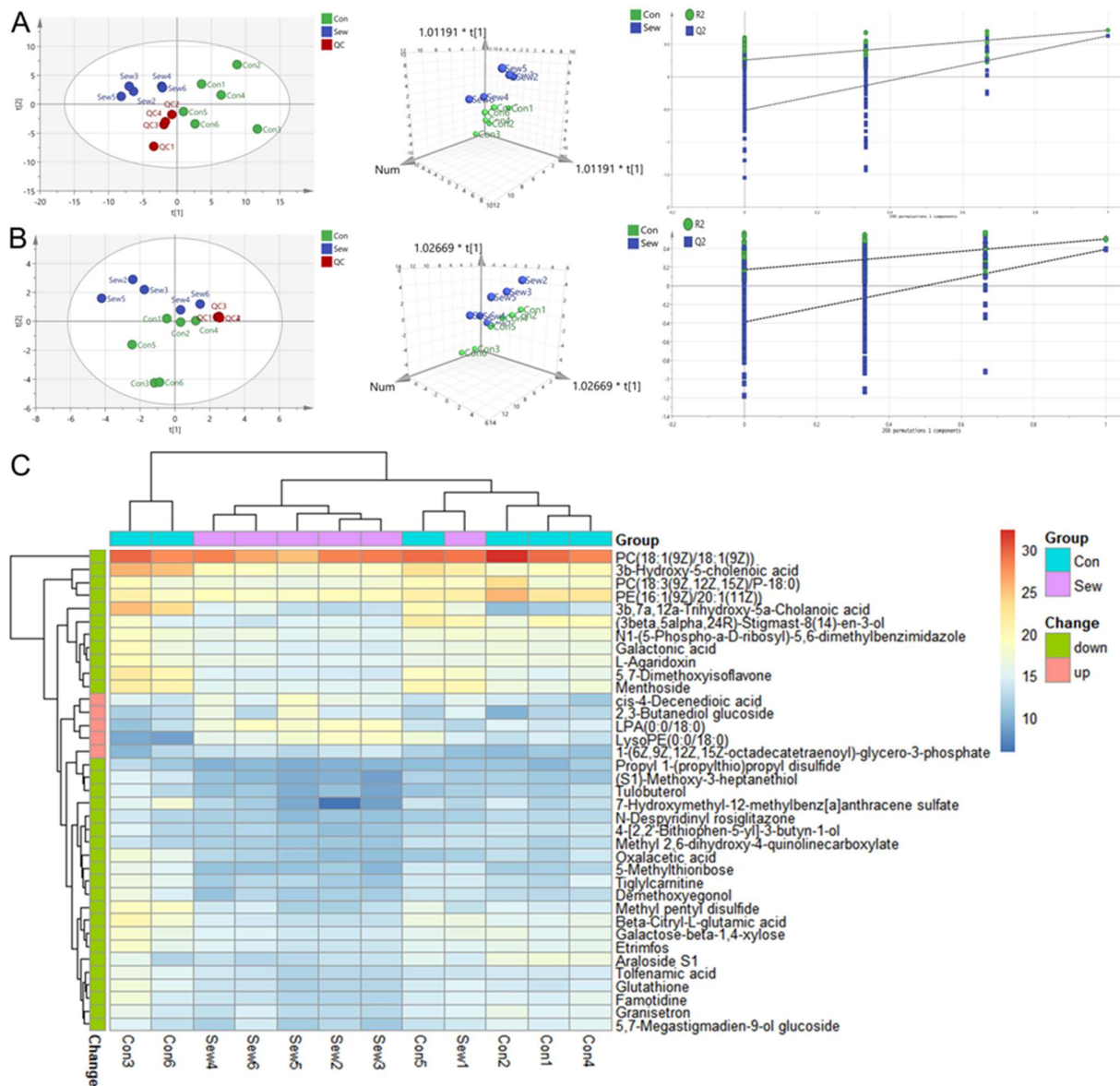


Fig. 7: Multivariate statistical analysis of intestinal metabolism exposed to sewage. (A) PCA, OPLS-DA, and Permutation test plots in positive-ion mode. (B) PCA, OPLS-DA, and Permutation test plots in negative-ion modes. (C) Heat map of differential metabolites.

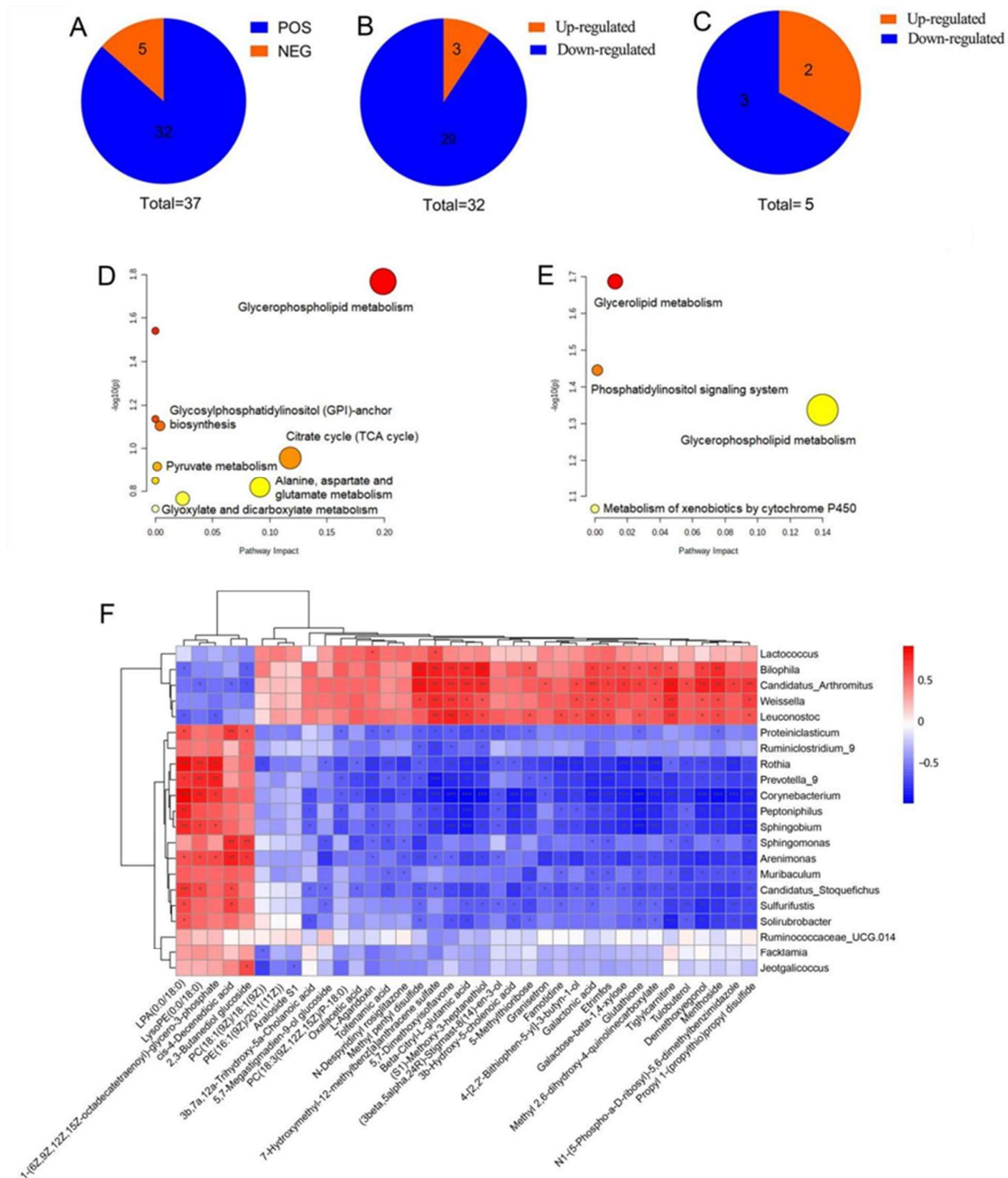


Fig. 8: Sewage exposure disturbed the intestinal metabolism of ducks. (A) The total quantity of differential metabolites. (B, C) Changes of metabolites in positive-ion mode (POS) and negative-ion mode (NEG). (D, E) Differential metabolic pathway analysis based on the POS and NEG. (F) Correlation analysis of gut microbiota and metabolites.

DISCUSSION

Recently, pathogen infection and gastrointestinal diseases caused by direct/indirect consumption of polluted water and poor health habits have become considerable public health problems in many countries. Thus, the potential threat of livestock farm wastewater contamination on environmental quality and animal health is generating growing concern. As waterfowl, duck is inevitably exposed to sewage contaminated by livestock farm wastewater, but studies regarding the influence of sewage exposure on

ducks were insufficient. Gut microbiota plays a crucial role in maintaining immunity, metabolism, and overall host health, all of which rely on normal intestinal morphology (Li *et al.*, 2021; Liao *et al.*, 2022). Given that the intestine is the primary route for harmful substances to enter the body and cause toxic effects, it is possible that both intestinal morphology and flora could be impacted. Here, we characterized the shifts of intestinal microbiota and metabolism of ducks exposed to livestock farm wastewater and revealed the distinct variabilities of microbial community and metabolism.

Table 2: Statistical analysis of differential metabolites between the Con and Sew groups

Metabolite	VIP	FC	P value	Trend
Glutathione	1.25	3.85	0.047	down
Oxalacetic acid	1.30	12.25	0.036	down
3β-Hydroxy-5-cholenoic acid	1.21	22.77	0.039	down
Galactonic acid	1.31	5.25	0.0094	down
PC(18:1(9Z)/18:1(9Z))	1.03	13.06	0.045	down
cis-4-Decenedioic acid	1.03	7.32	0.0021	up
5-Methylthioribose	1.16	5.50	0.049	down
Famotidine	1.30	4.17	0.010	down
Tiglylcarnitine	1.20	4.84	0.016	down
N1-(5-Phospho-α-D-ribose)-5,6-dimethylbenzimidazole	1.14	2.46	0.017	down
PC(18:3(9Z,12Z,15Z)/P-18:0)	1.12	20.83	0.040	down
PE(16:1(9Z)/20:1(11Z))	1.12	12.43	0.044	down
Galactose-β-D-1,4-xylose	1.20	3.11	0.017	down
Beta-Citryl-L-glutamic acid	1.47	11.85	0.017	down
Granisetron	1.14	2.72	0.010	down
L-Agaridoxin	1.12	3.82	0.018	down
5,7-Dimethoxyisoflavone	1.38	14.66	0.046	down
Demethoxyegonol	1.14	4.82	0.037	down
Etrimfos	1.18	3.76	0.018	down
(S1)-Methoxy-3-heptanethiol	1.29	6.81	0.0087	down
Propyl 1-(propylthio)propyl disulfide	1.00	2.63	0.038	down
4-[2,2'-Bithiophen-5-yl]-3-butyne-1-ol	1.19	2.96	0.016	down
Araloside S1	1.09	7.48	0.018	down
Menthoside	1.29	4.66	0.016	down
(3β,5α,24R)-Stigmast-8(14)-en-3-ol	1.15	3.63	0.030	down
Methyl 2,6-dihydroxy-4-quinolinecarboxylate	1.03	2.63	0.019	down
2,3-Butanediol glucoside	1.29	14.64	0.0025	up
5,7-Megastigmadien-9-ol glucoside	1.17	3.04	0.014	down
Tolfenamic acid	1.03	3.20	0.029	down
Tulobuterol	1.33	5.35	0.030	down
N-Despyridinyl rosiglitazone	1.10	2.76	0.010	down
1-(6Z,9Z,12Z,15Z-octadecatetraenoyl)-glycero-3-phosphate	1.21	3.66	0.020	up
3β,7α,12α-Trihydroxy-5α-Cholanoic acid	1.55	262.53	0.033	down
LysoPE(0:0/18:0)	1.45	5.67	0.023	up
7-Hydroxymethyl-12-methylbenz[a]anthracene sulfate	1.42	15.42	0.0092	down
LPA(0:0/18:0)	1.25	16.72	0.0088	up
Methyl pentyl disulfide	1.24	14.45	0.0028	down

This study found that *Firmicutes*, *Proteobacteria*, *Actinobacteria*, and *Bacteroidetes* were the predominant bacterial phyla in ducks, consistent with previous research (Liu *et al.*, 2021). These phyla were also abundant in the gut microbiota of broilers, geese, and pigeons, a common characteristic in poultry gut microbiota, suggesting their importance in intestinal health and functions (Yang *et al.*, 2022). Notably, although the types of dominant bacterial phyla did not change, the relative abundance of some bacteria, such as *Firmicutes*, altered significantly. Most *Firmicutes* members are recognized as beneficial bacteria in the intestines, crucial for maintaining intestinal homeostasis and improving the intestinal environment (Hou *et al.*, 2025; Zhu *et al.*, 2026). Moreover, *Firmicutes* have been linked to energy metabolism, including protein and carbohydrate digestion (Li *et al.*, 2021). In this study, sewage administration significantly decreased the abundance of *Firmicutes* in the intestine, which may impair intestinal homeostasis and affect the energy and nutritional demands of ducks during growth. Moreover, *Candidatus_Arthromitus* was the most dominant bacterial genus in the control ducks, which was inconsistent with the findings of ducks exposed to sewage. *Candidatus_Arthromitus* has been demonstrated to be a beneficial intestinal symbiotic bacterium, which is involved in the positive regulation of the immune system. The distinct changes of dominant bacterial phylum and genera in ducks exposed to sewage further indicated the gut microbial dysbiosis.

Importantly, this study also showed that exposure to sewage causes notable alterations in certain functional bacteria, potentially impacting intestinal homeostasis and

function. Notably, these quantitatively decreased bacteria (*Lactococcus*, *Weissella*, and *Leuconostoc*) in ducks exposed to sewage were considered as intestinal beneficial bacteria, indicating present intestinal environment is not conducive to the survival of these bacteria. *Lactococcus*, a key potentially beneficial bacteria, could inhibit bacterial proliferation and improve antioxidant capacity and intestinal immunity (Liu *et al.*, 2021). Recent investigations on *Lactococcus* have provided evidence that it could reduce the toxic effects of heavy metals and positively regulate gut microbiota (Kakade *et al.*, 2022). *Weissella*, an important intestinal beneficial bacterium, has several important physiological functions such as antioxidation and anti-inflammatory properties, all of which contribute to enhancing growth performance and disease resistance in poultry (Choi *et al.*, 2021). Moreover, *Weissella* has also been demonstrated to alleviate fat accumulation and protect the liver in high-fat diet-induced obese mice (Choi *et al.*, 2021). *Leuconostoc*, another beneficial intestinal bacterium, is known to produce extracellular polysaccharides with immunocompetence, anti-tumor, antioxidant, and antibacterial effects, thus promoting host health (Schifano *et al.*, 2021). Moreover, some bacteria, such as *Peptoniphilus*, *Rothia*, *Facklamia*, *Jeotgalicoccus*, *Muribaculum*, and *Corynebacterium* are commonly found as pathogenic bacteria in the intestines, with significantly increased presence in ducks exposed to sewage. *Proteinclasticum* is abundant in wastewater (Zeng *et al.*, 2021). *Sulfurifustis* is abundant in landfill-leachate contaminated aquifer (Abiriga *et al.*, 2021). *Peptoniphilus* can cause blood infection and endocarditis (Brown *et al.*,

2014). *Rothia* is closely related to multiple diseases such as endocarditis, peritonitis, and sepsis (Monju *et al.*, 2009). *Facklamia* may be relevant to bacteremia, meningitis, septicemia, and *Jeotgalicoccus*, a common drug-resistant bacterium, is negatively correlated with poultry productivity (Zhao *et al.*, 2021). Additionally, the addition of manure could increase the abundance of *Jeotgalicoccus* in the sludge (Ding *et al.*, 2022). Song *et al.* (2021) indicated that *Muribaculum* exhibited a high correlation with obesity and obesity-related metabolic disorders. Wang *et al.* (2021) reported that *Arenimonas* were dominant in the river contaminated with heavy metals. *Corynebacterium* was previously reported to cause bacteremia and lung abscesses (Zhang *et al.*, 2020). *Sphingomonas* displayed the characteristics of organic matter degradation, indicating its key roles in host health and environmental protection. We speculated that the higher abundance of *Sphingomonas* in the intestine may be the host's response to weaken the damage caused by harmful substances in the sewage.

In this study, we also conducted untargeted metabolomics to analyze changes in intestinal metabolites in ducks exposed to sewage. Metabolomic results indicated that 8 metabolic pathways and 37 metabolites (glutathione, famotidine, tolfenamic acid, etc.) were involved in sewage exposure-induced intestinal dysfunction. These altered metabolites and metabolic pathways affected by sewage exposure may have a significant impact on the host's negative regulation. Numerous investigations indicated that environmental contaminants could dramatically affect lipid metabolism pathways (Guo *et al.*, 2022). In this study, pyruvate metabolism and glycerophospholipid metabolism were the two vital pathways in the intestine affected by sewage exposure. Environmental contaminants exposure could alter the levels of lipid metabolites and induce mitochondrial dysfunction (Liao *et al.*, 2022). Consistent with previous investigations, this research showed a significant change in lipid metabolites such as PC, PE, LPA, and LysoPE of the intestine in ducks exposed to sewage. Phospholipid components such as PE and PC are important components of the bilayer organelle mitochondria and play vital roles in maintaining the structure and function of mitochondrial membrane proteins. The shifts in lipid metabolites may further cause a decrease in mitochondrial quantity and quality. The tricarboxylic acid cycle, which mainly exists in mitochondria, is the hub of the link between carbohydrate, lipid, and amino acid metabolism. As the core of energy metabolism in organisms, the metabolic intermediates of the tricarboxylic acid cycle are also crucial precursors for the biosynthesis of various compounds, which are essential for biological development and maintenance of cell homeostasis (Stratmann *et al.*, 2022). Abnormal pyruvate metabolism has been reported to disrupt mitochondrial function, causing muscle damage, acidosis, or weakened heart, lung, kidney, and liver functions (Zhao *et al.*, 2022). Moreover, pyruvate plays a crucial role in connecting the metabolic pathways of sugar, fat, and amino acids in the body through acetyl-CoA and the tricarboxylic acid cycle. These results indicated that sewage exposure affected pathways related to mitochondrial metabolism and thus caused intestinal dysfunction. Notably, sewage exposure also significantly decreased the contents of glutathione,

famotidine, and tolfenamic acid in the intestine. Glutathione (GSH) is a vital antioxidant commonly found in almost all cells and tissues, displaying multiple physiological properties such as maintaining immune system function, anti-tumor, delaying senescence, and detoxification. Furthermore, GSH contributes to the tricarboxylic acid cycle and sugar metabolism, while also enhancing carbohydrate, fat, and protein metabolism by activating multiple enzymes (Szeligowska *et al.*, 2022). Famotidine exhibits a significant inhibitory effect on gastric acid secretion, commonly used in acute gastric mucosal hemorrhage, gastric and duodenal ulcer (El-Dakrouy *et al.*, 2022). Tolfenamic acid has been reported to possess anti-inflammatory, analgesic, and antipyretic properties (Blanco-Paniagua *et al.*, 2021). Interestingly, the above-mentioned metabolites and bacteria that were significantly reduced in sewage exposure all exhibited positive regulatory effects on the host. Consequently, we speculated that gut microbial and metabolic disturbances may be one of the ways for sewage to exert its negative effects.

Conclusions: In conclusion, this study explored the effects of sewage exposure on gut microbiota and metabolism. Results showed that sewage exposure reduced the growth performance and induced intestinal, liver, and kidney injury in ducks. Moreover, the ducks exposed to sewage also exhibited intestinal microbiota and metabolic dysbiosis, which may be one of the causes for their reduced growth performance. This study raised public concern about the health threat caused by livestock farm wastewater discharge, which also provides a basis for regulating waste discharge from livestock farms to ensure environmental quality and waterfowl health.

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