Combined Effects of Simulated Microgravity, Low Pressure and Noise Environment on the Intestinal Flora of C57BL/6 Mice

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Research

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Abstract

Background: The composition and function of intestinal microbial communities are important for human health. However, these intestinal floras are sensitive to changes in the environment. Adverse changes to intestinal flora can affect the health of astronauts in complex space environments, resulting in difficulties of implementing space missions.

Results: Under the influence of different space environmental factors, the species composition at the phylum and genus level were significantly affected by the combined effects environment. Furthermore, screening was conducted to identify biomarkers that could be regarded as environmental markers. And there have also been some noticeable changes in the function of intestinal floras. Moreover, the antibiotic resistance genes (ARGs) were also found to be differently expressed under different environmental conditions.

Conclusion: The combined effects environment could significantly affect the species composition, function and the expression of ARGs of intestinal flora which may provide a theoretical basis for space medical supervision and healthcare. Meanwhile, these changes require more attention because they may induce diseases that may further affect performance during space missions.

Background

There are a large number of anaerobic and facultative aerobic bacteria presenting in the intestines of humans. Under normal conditions intestinal floras are of a relatively fixed composition, interdependence, mutual restriction and maintain a dynamic balance. More importantly, gut microbiota are important for human health as they can produce short-chain fatty acids (SCFAs), vitamins and amino acids, and may even interact with humans to train the immune system for protection from pathogens and contribute to the maturation of the intestinal epithelium among other role(1–4). More importantly, several studies have proven that the development and improvement of the human immune system cannot be achieved without intestinal floral diversity(5–7). Slow development and weakened immune systems have been found in rodents models with sterile intestines(8). When the immune system of the body is weakened, opportunistic infections caused by opportunistic pathogens, such as fungi or staphylococci can develop.

The outer space environment is a highly complex environment filled with more diversity than the terrestrial environment(9). Common variation factors include weightlessness, intense radiation and high vacuum. In addition, astronauts on missions spend most of their time inside the capsule and are exposed to an environment with loud noises. Existing studies have confirmed that the human body experiences certain physiological and pathological changes in space environments, which are mainly manifested as reduced bone density, decreased blood volume, abnormal digestive function and immune dysfunction(10–13). Moreover, the composition and activity of gut microbes may be unstable as they are influenced by endogenous and environmental factors(14), as well as the intestinal microecology of the organism, which may undergo adverse changes in space environments. Meanwhile, the pathogenicity

and resistance of intestinal flora may also be enhanced, resulting in disruption of the balance between organisms and flora(15). These changes in immune function and intestinal microecology may increase the risk of developing diseases. Previous spaceflight research has shown that astronauts were easily infected by bacteria, viruses or opportunistic pathogens(16, 17). In addition, if the astronaut has the history of using large doses of antibiotics due to chronic intestinal diseases, treatment for infections would also be extremely difficult(18). Ground simulated experiments mostly explore the impact of a single environmental factor on the organism, while space environments are full of intricate environmental changes, and functional changes of the astronaut may result in interactions between various environmental factors. Therefore, we designed this experiment to explore changes in the intestinal flora of mice affected by the combined effects environment, which included microgravity, low pressure and noise environments, so as to provide a theoretical base for medical supervision and medical insurance for astronauts.

The intestinal microorganism community constitutes a gene pool that is large and complex, and contains both phylogenetic marker genes, as well as various metabolism genes, which are collectively known as the metagenome(19). Researchers are able to analyze and forecast these genes to study the specific composition and function of the microbial community. For example, 16 s rRNA genes can indicate the microbial status. In this study, stool samples of mice in the three different groups were collected and a large quantity of biological data and an abundance of information on microorganisms were obtained using Next Generation Sequencing. Then, a bioinformatics analysis of these data was performed to further elucidate the composition and functional changes and the expression of resistance genes of the gut microbes in the intestine. The project work flow is shown in Fig. 1. Furthermore, we expect that the results of this study will provide a theoretical basis for the maintenance of normal intestinal microecology of astronauts and reduce the impact of mission execution on their physical health.

Results

1. Screening and identification of differentiated expressed genes

We obtained a large amount of raw data (Table S1) through the extraction and sequencing of DNA from mice stool samples. After further assembling and screening of raw data, scaftigs were obtained to be used for gene prediction (Table S2). The results of the prediction and comparison between the Open Reading Frames (ORFs) are shown in Table S3. A total of 937,098 effective genes were obtained through the above mentioned process. It was observed that there were significant differences in the number of effective genes among the three groups (Fig. 2A). Further analysis showed that the total number of common genes between the three groups was 468,006, while 44,704, 82,421 and 18,096 genes were differentially expressed between the three groups (Fig. 2B).

2. *Combined effects environment significantly affected the species composition of gut microbiota in mice*

2.1 Overall results of species annotation

We used metaphlan2 software to predict effective genes and analyze species-related information. The merged information obtained from all species was visualized to determine the taxonomic composition of the intestinal flora of the mice based on GraPhlAn (Fig. 3A). The results of the species annotation obtained using KRONA software based on the mean value of three groups is shown in Fig. 3D-3F. The results of the Principal Component Analysis (PCA) and Non-metric multidimensional scaling (NMDS) of the three groups showed that there were significant differences between the three groups (Fig. 3B and 3C).

2.2 Relative abundance and cluster analysis of species

The relative abundance histogram of the top 15 species in each sample were drawn using the corresponding species annotation results based on the relative abundance of different classification levels, (Fig. 4A and 4B). The species with the highest relative abundance ranking top 35 in each sample were used to construct the cluster tree map at phylum and genus levels (Fig. 4C and 4D). At the phylum level, the relative abundances of Firmicutes, Bacteroides and Verminobacteria showed similar fluctuation trends in each sample of the three groups. Compared with the NC group, Firmicutes in particular showed an upward trend in the TS and TS + SM groups, but the increase in the TS group was the most obvious. The relative abundance of Bacteroides showed a downward trend, while Verrucomicrobia almost disappeared from the TS and TS + SM groups. It must be noted that there was a significant increase in deferrobacteria in the TS group, but there was no significant difference between the TS + SM group and the NC group. At the genus level, the relative abundance of β-retroviruses and γ-retroviruses decreased in the TS group, but showed a significant increase in the TS + SM group, compared with the NC group. On the contrary, the relative abundances of Anaerotruncus, Oscillibacter and Lachnospiraceae increased in the TS group, but decreased in the TS + SM group. Moreover, Mucispirillum relative abundance only increased in the TS group, while Akkermansia almost disappeared from both the TS and TS + SM groups.

2.3 Screening of representative biomarkers

To explore the differences among species in the gut microbiota that were affected by weightlessness or compound environment, we carried out a Linear discriminant analysis Effect Size (LEfSe) analysis to obtain species specific biomarkers. The gut microbial compositions of three groups were compared to obtain the Linear discriminant analysis (LDA) score of each species (Fig. 5A). All meaningful biomarkers are shown in the cladogram of species differences at all levels for each different species (Fig. 5B). After exposure to the environments with weightlessness or compound factors, the abundances of 28 species decreased in NC group, while the abundances of 24 and 32 species decreased at all levels in the TS group

and TS + SM group, respectively. f-Bacteroidales, f-Deferribacteraceae and f-Coriobacteriaceae were found to be the biomarkers with the highest scores in the NC group, TS group and TS + SM group, respectively.

3. Combined effects environment significantly affected the function of gut microbiota of mice

To investigate the influence of spaceflight on gut microbiota function, we blasted the Unigenes to the Kyoto Encyclopedia of Genes and Genomes (KEGG) and evolutionary genealogy of genes: Non-supervised Orthologous Groups (eggNOG) databases. The results showed that the weightlessness and combined environments significantly affected the gut microbiota function. In the KEGG database (Fig. 6A), compared with the NC group, the proportion of metabolic pathways in the TS and TS + SM groups decreased, while the decrease was most obvious in the TS + SM group. The influence of Environmental Information Processing and Cellular Processes pathways of the TS + SM group increased significantly. The influence of the Organismal Systems signaling pathway decreased in the TS group, but plateaued in the TS + SM group. Based on the eggNOG database (Fig. 6B), the relative proportion of various functions in the TS + SM group decreased significantly while the same increased in the TS group, compared with the NC group. However, there were certain changes that deserve further attention. Specifically, the function involving replication, recombination and repair, and transcription increased in the TS group, but decreased significantly in the TS + SM group. All functional changes shown by the two databases were clustered and was shown in Fig. 6C and 6D.

4. Identification and classification of ARGs

We used the Antibiotic Resistance Genes Database (ARGD) to annotate antibiotic resistance genes. Based on the results of the comparison between the Unigenes and the data in ARGD database, we performed differential analyses of the ARGs and significant differences in all three groups (Fig. 7A). Then, the relative abundance histogram of the top 15 ranking antibotics were drawn. As shown in Fig. 7B, the abundance of multidrug_efflux_pump, erythromycin, macrolide increased in TS group, while tetracycline and its derivatives decreased. However, there were no significant changes of ARGs in the TS + SM group, compared with the NC group. The above changes were also observed in the cluster heat map shown in Fig. 7C.

Discussion

Through the history of human development, the vastness of space has always been an infinite attraction for humans. Although magnificent, space is rife with various environmental changes that can adversely physically affect those who enter it to explore. More specifically, during the execution of space missions, astronauts are not only affected by changes in the space environment which mainly include weightlessness, high radiation and vacuum, but are also restricted inside a narrow and closed cabin environment. Under these conditions, changes in the symbiotic intestinal flora of the intestines of

astronauts may occur and further affect their physical health. The health problem of astronauts is a factor that could hinder the success of a space mission.

The development of complex molecular biology technology has allowed us to conduct different types of research studies. In order to further explore the influence of complex space environments on intestinal flora, we used the ground-based simulated module and a TS model to explore the influence of a combined effects on changes in species composition, function and resistance genes of intestinal flora in mice. For the experiment, nine C57BL/6 mice with the same genetic background were randomly divided into the NC group, TS group, and TS + SM group to observe changes in intestinal flora caused by different environments, and fecal samples were collected for further analysis after the end of the 45-day experiment. Then, DNA extraction, quality inspection, bank building and other processes were performed on the qualified feces samples to obtain effective Unigenes for species annotation, functional analysis and resistance gene detection.

We first found that different environments could cause changes in the species composition of intestinal flora in mice, and that these changes were distributed at different levels. This is consistent with the results of the species analysis of the intestinal flora of short-flying astronauts in study conducted by Turroni(28). Especially at the phylum and genus levels, the effects of a simulated microgravity environment and combined effects environment can be observed on species composition. Specifically, Firmicutes and Bacteroidetes showed an increasing and decreasing trend, respectively, these changes were significant in a combined effects environment. Under normal circumstances, Firmicutes and Bacteroidetes are the main components of intestinal flora(29). A variety of Firmicutes bacteria can metabolize carbohydrates to produce butyrate, which is not only beneficial for the metabolism of energy substances, but can also protect the intestinal mucosa and improve immune function(30). Firmicutes is a class of gram-positive bacteria and one of the largest families in the domain bacteria. Most Firmicutes bacterial walls contain high levels of peptidoglycans (50%-80%) and are thick enough to form spores, resulting in them being highly resistant to dehydration or extreme environments. In addition, several studies have shown that a large number of Firmicutes bacteria in the gut are associated with obesity(31–33). Although obesity does not affect the performance of space missions, its associated metabolic changes require further attention due to its metabolic dysfunction. Several studies have demonstrated that many changes in the lipid metabolism of gastric cancer cells and epidermal stem cells have been observed under simulated microgravity(34, 35). Bacteroidetes, a core flora of the human intestinal tract, contains a powerful polysaccharide degrading system to digest dietary fibers consumed by the body and convert it into short-chain fatty acids(36). Relying on its powerful metabolic capacity, Bacteroidetes has the main high levels of stability for human health(37). However, this stability depends on the balance between nutrient absorption and consumption in the body(38). In addition, the main source of Vitamin K in humans is also synthesized by Bacteroides(39). Vitamin K deficieny affects various systems in the body, such as the coagulation system and the musculoskeletal system(40, 41). Due to these important functions, Bacteroidetes are widely regarded as beneficial bacteria that can decrease intestinal inflammation, immune dysfunction and metabolic disorders, and may even function in preventing the occurrence of cancer(42). Moreover, the metabolites of Bacteroides, such as propionate and acetate, can

also block the absorption of enteric endotoxin(43) and induce the apoptosis of colon cancer cells(44), playing a preventive and therapeutic role. Zitomersky et al. found that the specific mechanism by which the protective role of Bacteroides is exerted may be through recruitment of intraepithelial lymphocytes to produce IL-6(45). Overall, Bacteroides is a protective barrier of the host intestinal tract(46). However, in certain cases Bacteroidetes can also become opportunistic pathogens that can cause diseases(47). Although there was a decrease in the number of Bacteroidetes in our study, these changes need to be addressed due as they indicate changes in immune function in space extreme environment. In addition, we screened biomarkers that could be used at the species level for the effects caused by simulated weightlessness or complex environmental factors. The increase of Bacteroidetes in the NC group could be used as biomarkers, which suggested that the relative abundance of Bacteroidetes decreased under weightlessness environment and combined effects environment. This is consistent with the results obtained through species annotation, and also indicates that the changes in Bacteroidetes abundance in combined effects environment are very important for health.

The KEGG enrichment analysis showed that compared with the NC group, the proportion of metabolic pathways under the two different environments decreased, while the decrease was most obvious in the combined effects environment. The changes in these metabolic pathways were highly correlated with changes in intestinal floral species composition. To respond to environmental changes, the functional genes involved in environmental information processing of the gut microbiota were increased significantly in the combined effects environment, which may be related to the adaptation of the body and intestinal flora under changes in the environment. It is worth noting that changes in intestinal floral species composition and functional genes in mice were not consistent with that of weightlessness and combined effects environment, which needs to be confirmed through further studies.

The abuse of antibiotics leads to irreversible changes in the human body and microbial communities in the environment, which poses risks to human health and the ecological environment. Therefore, the study of resistance genes has attracted extensive attention from researchers. We expect to study the changes in resistance genes in intestinal flora to provide new insights for the application of antibiotics and the prevention of diseases. The weightlessness environment and combined effects environment both significantly affected the expression of resistance genes in the intestinal flora of the mice. Although the total number of resistance genes were decreased in the TS and TS + SM groups, the fluctuation in the expression of the resistance genes was more meaningful. For example, the multidrug efflux pump is capable of transporting structurally varied molecules, including antibiotics, out of the bacterial cell. This efflux lowers the intracellular antibiotic concentrations, allowing bacteria to survive at higher antibiotic concentrations(48).

The manned spaceflight environment is a complex environment that is subjected to multiple physical changes. In this study, we focused on the effects of combined effects environment on species composition and function of intestinal flora in mice, but more studies on the effect of complex factors on intestinal flora are needed to confirm the applicability of these results. In addition, the specific reasons for the changes in intestinal floral species composition and functions in mice under weightlessness or

complex factors need to be further confirmed. Based on previously reported results, it remains to be determined whether there is a correlation between the changes in immune function and the changes in intestinal flora microecology under microgravity environment, while mutual influences induced by the two groups, as well as the recovery of intestinal flora after exposure to a space environment are also worthy of attention.

Conclusion

We designed and conducted this study to explore changes in intestinal flora of mice under the influence of different space environmental factors to enrich our understanding and provide a theoretical basis for space medical supervision and healthcare. The results showed that different environments, especially a combined effects environment could significantly alter the species composition and function of intestinal flora in mice. In addition, the changes in ARGs under the influence of these environmental factors may be useful for the treatment and prevention diseases. These changes in intestinal flora caused by exposure to space environment should be taken seriously to avoid the adverse effects induced by participating in space missions. More importantly, further studies are warranted to analyze the mutualistic relationship between humans and gut microbiota under complex environmental conditions.

Methods

1. Preparation of experimental animals and collection of specimens

Nine C57BL/6 mice with the same genetic background that were 2 months old and male were selected and randomly divided into three groups: the normal control (NC) group (the normal environment), the tail-suspension (TS) group (the simulated microgravity environment) and the tail-suspension in simulated module (TS + SM) group (the combined effects environment). The TS model was constructed using the Globus method and the mice were suspended by their tails to restrict movement(20). The ground-based simulated module (SM) for animal research was used to build compound factors of space environment. It contains 200lux Light intensity, circadian rhythm of 12 hours light/darkness respectively; Air pressure: 0.9 atm pressure; and 85 dBA environment noise. The experiment period for all three groups was 45 days. During the first 7 days, all mice were allowed to settle under normal conditions (1 atmosphere and noiseless, 10 dbA) to adapt to the new environment. Beginning from the 8th day, mice were kept under the different environmental conditions based on their experimental grouping. An ambient temperature of 25 degrees was maintained. In addition, because the SM was shielded and was not affected by external light, the two other groups of mice were artificially exposed to the same lighting conditions. On the end of the 45th day, the mice were sacrificed and fecal samples were collected from the ileocecal region for further analysis. All animal experiments were approved by the Institutional Animal Care and Use Committee of the PLA Strategic Support Force Characteristic Medical Center.

2. Sample preparation and treatment

The fecal samples (50 mg) were weighed in 1 ml micro centrifuge tubes and placed in liquid nitrogen and were subsequently stored at -80°C until used. Total DNA was extracted from frozen fecal samples for metagenomic sequencing using a QIAamp Fast DNA stool Mini Kit. The whole extraction process was performed as instructed by the manufacturer. Agar-gel electrophoresis (AGE), Nanodrop, and Qubit 3.0 system (Thermo Fisher Scientific, Inc.) were used to determine the purity and integrity of the extracted DNA. DNA of a sufficient purity and integrity were tested for library construction and sequenced using an Illumina HiSeq high-throughput sequencing platform along with a KAPA Hyper Prep Kit. The raw data obtained from the sequencing were further filtered to obtain a higher quality of clean reads for subsequent informational analysis. SOAPdenovo Assembly software was used to assemble the Clean Data and Scaftigs were obtained(21). The scaftigs were further filtered for statistical analysis and subsequent genetic prediction (fragments cut-off: 500 bp).

3. Data analysis

The Open Reading Frames (ORFs) were predicted and mapped using CD-HIT and SoapAligner software and were filtered using the scaftigs(22, 23). Redundant genes were eliminated to obtain the gene catalogue (Unigenes). The abundance information of each gene in each sample was calculated and visualized using a violin diagram. A Venn diagram was used to present the differently expressed genes. Then, DIAMOND software(24) (Version 0.7.9; https://github.com/bbuchfink/diamond/) was used to blast and compare the Unigenes with sequences of the bacteria, fungi, archaea and viruses extracted from the NR database (Version: 20161115; https://www.ncbi.nlm.nih.gov/) of NCBI (e-value < 0.05). An LCA algorithm was used for species annotation to obtain abundance information of each sample at various classification levels. PCA and NMDS were used to analyze the correlation between samples. All annotated results were visualized using Krnoa and heat maps. LEfSe multivariate statistical analysis was used to screen the biomarkers of the representative groups. Then, the putative amino acid sequences were translated from the gene catalog and aligned against the proteins/domains in the KEGG and eggNOG databases using default setting(25, 26). Subsequently, the Antibiotic Resistance Genes Database (ARGD) was used to annotate the Unigenes in order to identify antibiotic resistance genes (ARGs)(27). The ARGs were classified and the specific antibiotics tolerated by them were also identified. Meanwhile, relative abundance analysis and abundance clustering analysis were performed based on the results of each sample.

Abbreviations

short-chain fatty acids (SCFAs)

antibiotic resistance genes (ARGs)

The Open Reading Frames (ORFs)

the gene catalogue (Unigenes)

Agar-gel electrophoresis (AGE)

comprehensive antibiotic resistance database (CARD)

Principal Component Analysis (PCA)

Non-metric multidimensional scaling (NMDS)

Linear discriminant analysis Effect Size (LEfSe)

Linear discriminant analysis (LDA)

normal control (NC)

tail-suspension (TS)

simulated module (SM)

Declarations

Availability of data and materials

The datasets generated and/or analysed during the current study are not publicly available due request of the project sponsor but are available from the corresponding author on reasonable request.

Ethics declarations

The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Experiments were performed under a project license (NO.: K2019-098-01) granted by institutional ethics board of PLA Strategic Support Force Characteristic Medical Center, in compliance with China national or institutional guidelines for the care and use of animals. This study does not involve gene editing, nor does it involve bacteria or virus infection experiments, but mainly physiological and behavioral experiments, so there is no potential harm from these aspects. In the process of handling animals, we treated animals well according to the regulations of animal ethics and properly disposed animal carcasses according to the regulations.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Authors' Contributions

The conception and design of the present research was performed by PS, JY, HY and YC. Material provision, data collection, and analysis were performed by PS, JY, BW, HM, YZ, and JG. The first draft of the manuscript was written by PS, JY, and BW. XC, JZ, HS, JY, HY and YC interpreted the data. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Figures

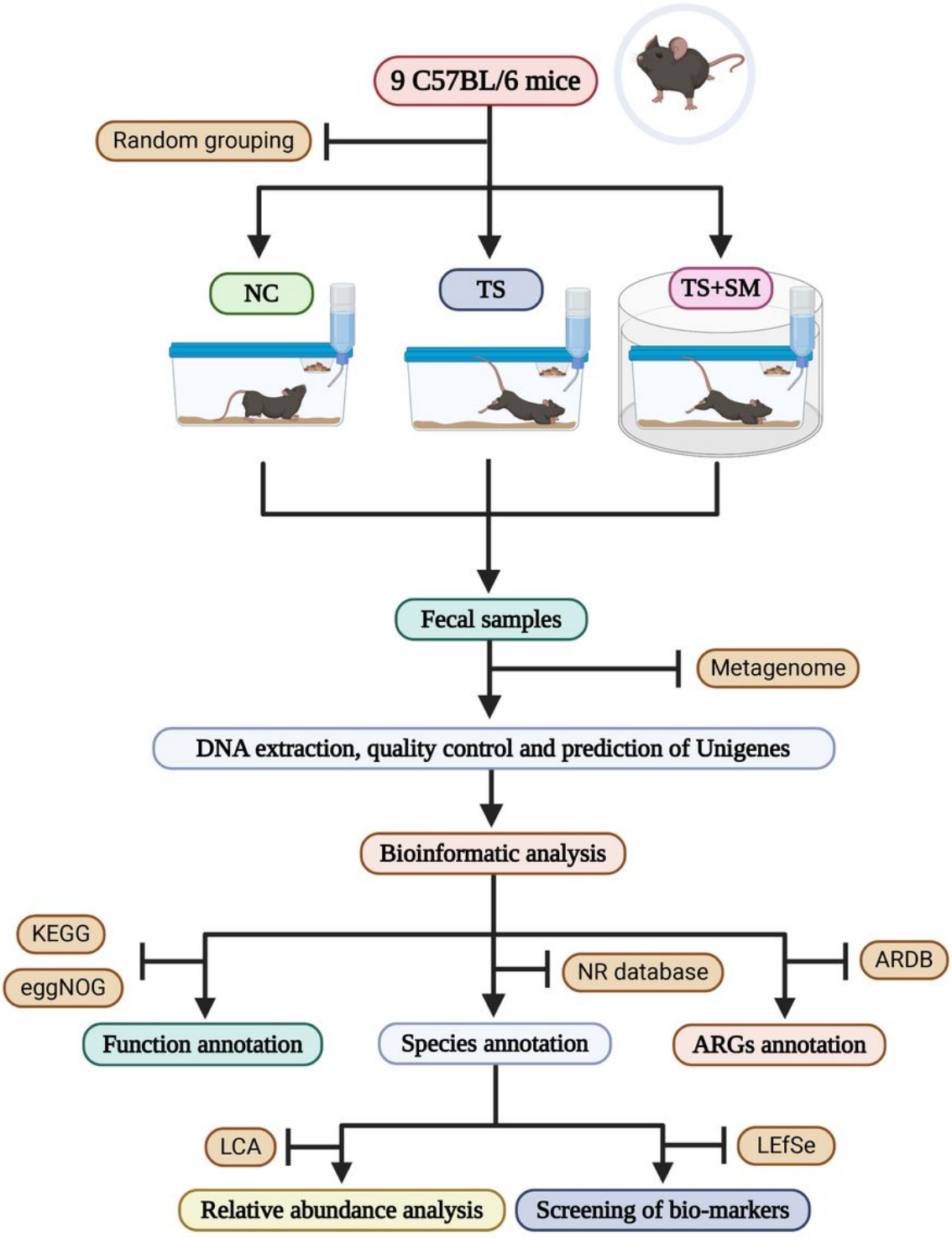

Figure 1

The work flow diagram of the study

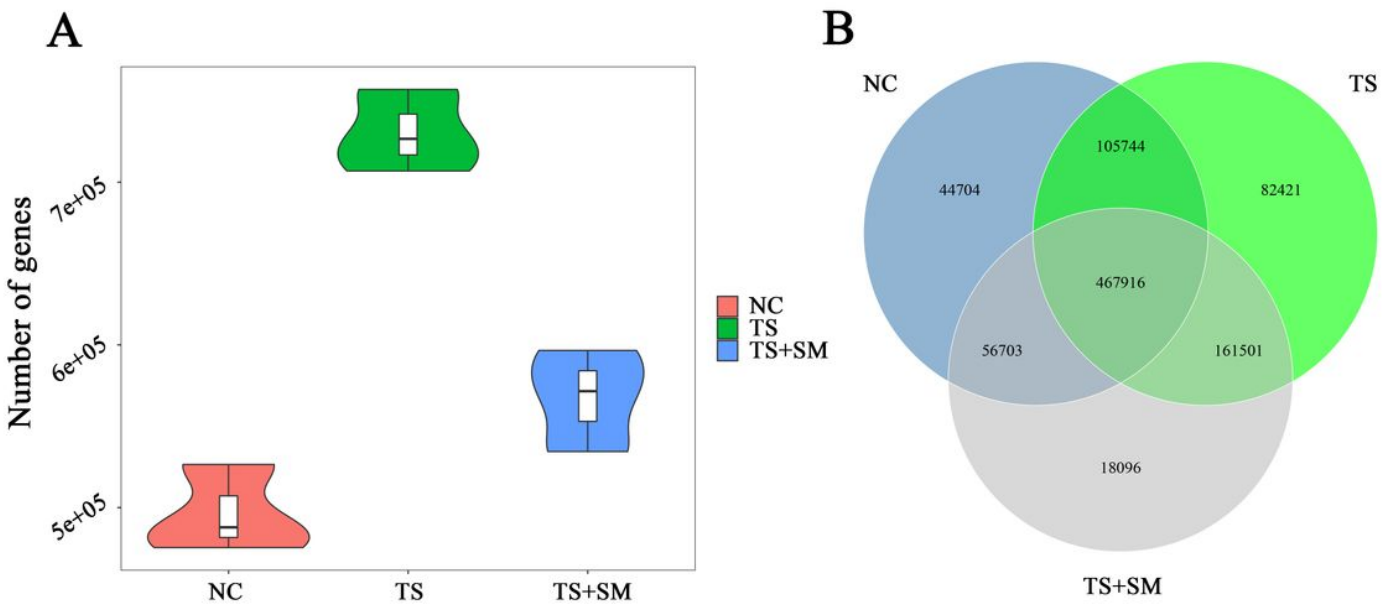

Figure 2

The results of the prediction of genes and their relationships. (A) The number of genes in the three groups are shown in the violin plot. (B) The intersection of the predicted genes among the three groups.

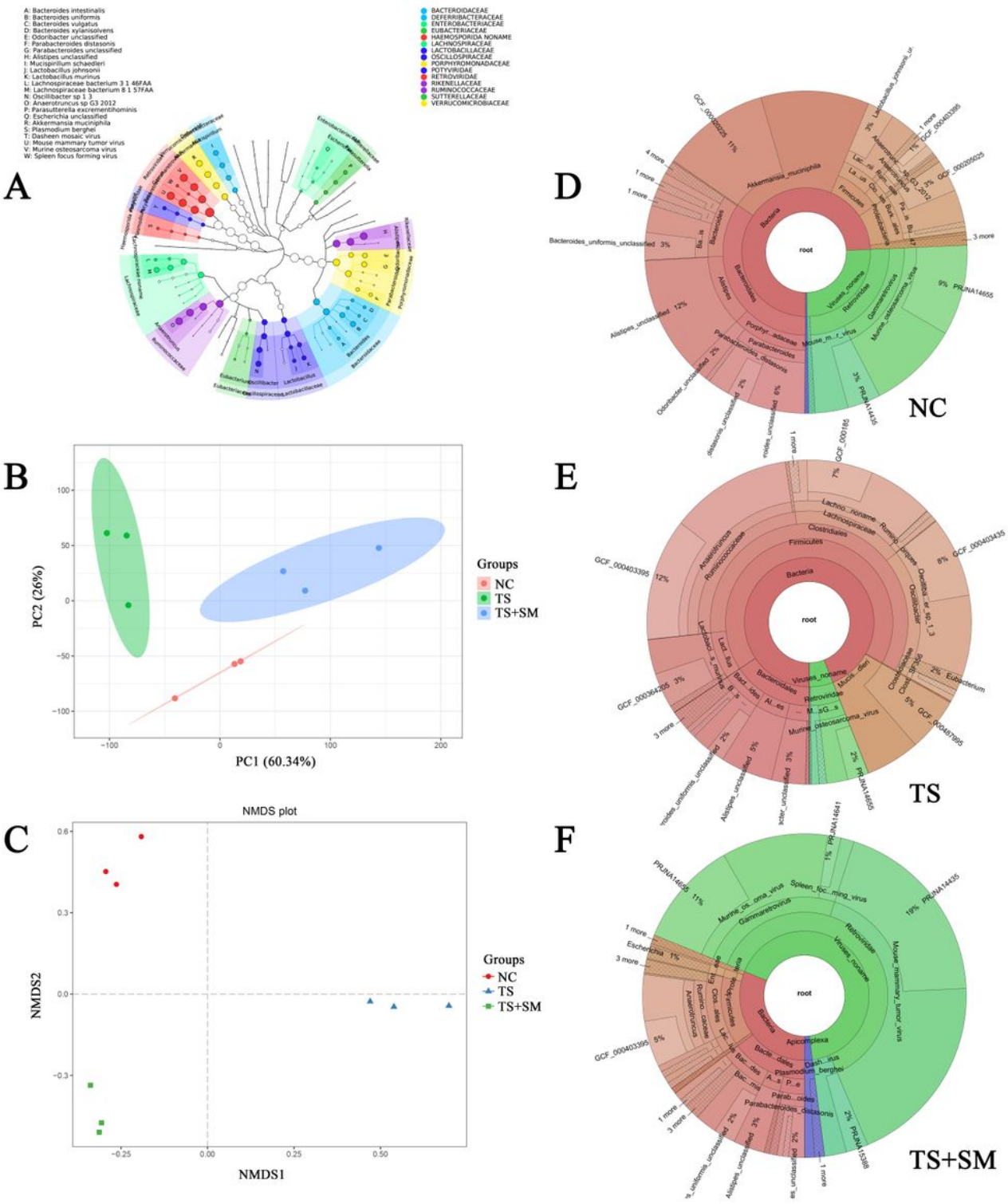

Figure 3

The overall results of species annotation and the correlation analysis of all samples. (A) Merged species information of all three groups based on GraPhlAn. (B, C) The PCA and NMDS analysis of all samples. (D-F) D, E and F represent the species annotation of the NC, TS and TS+SM groups, respectively.

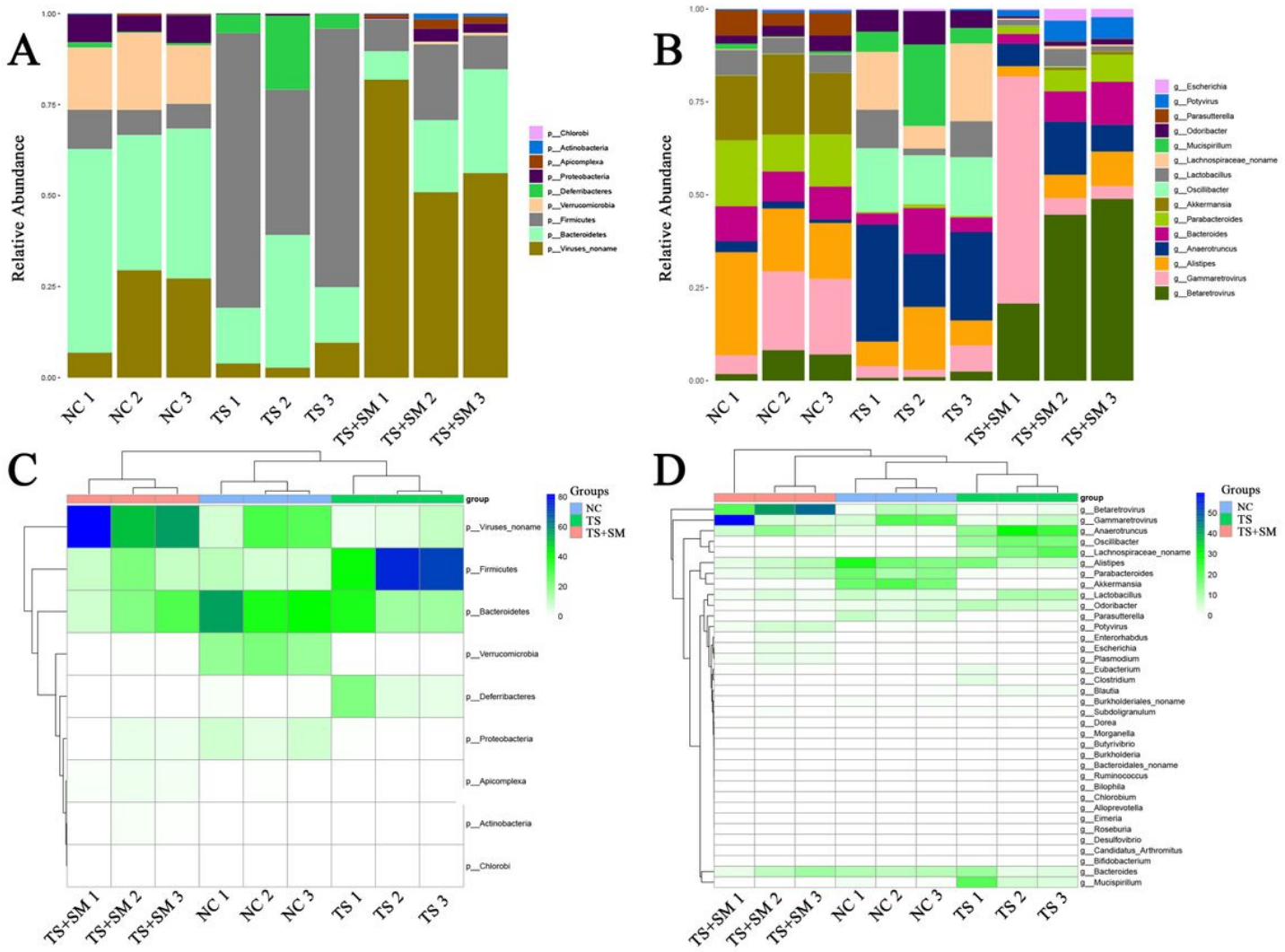

Figure 4

The relative abundances of species and heat maps of all samples. (A, B) The relative species abundance at phylum level and genus level. (C, D) The heat map of species abundance at phylum level and genus level.

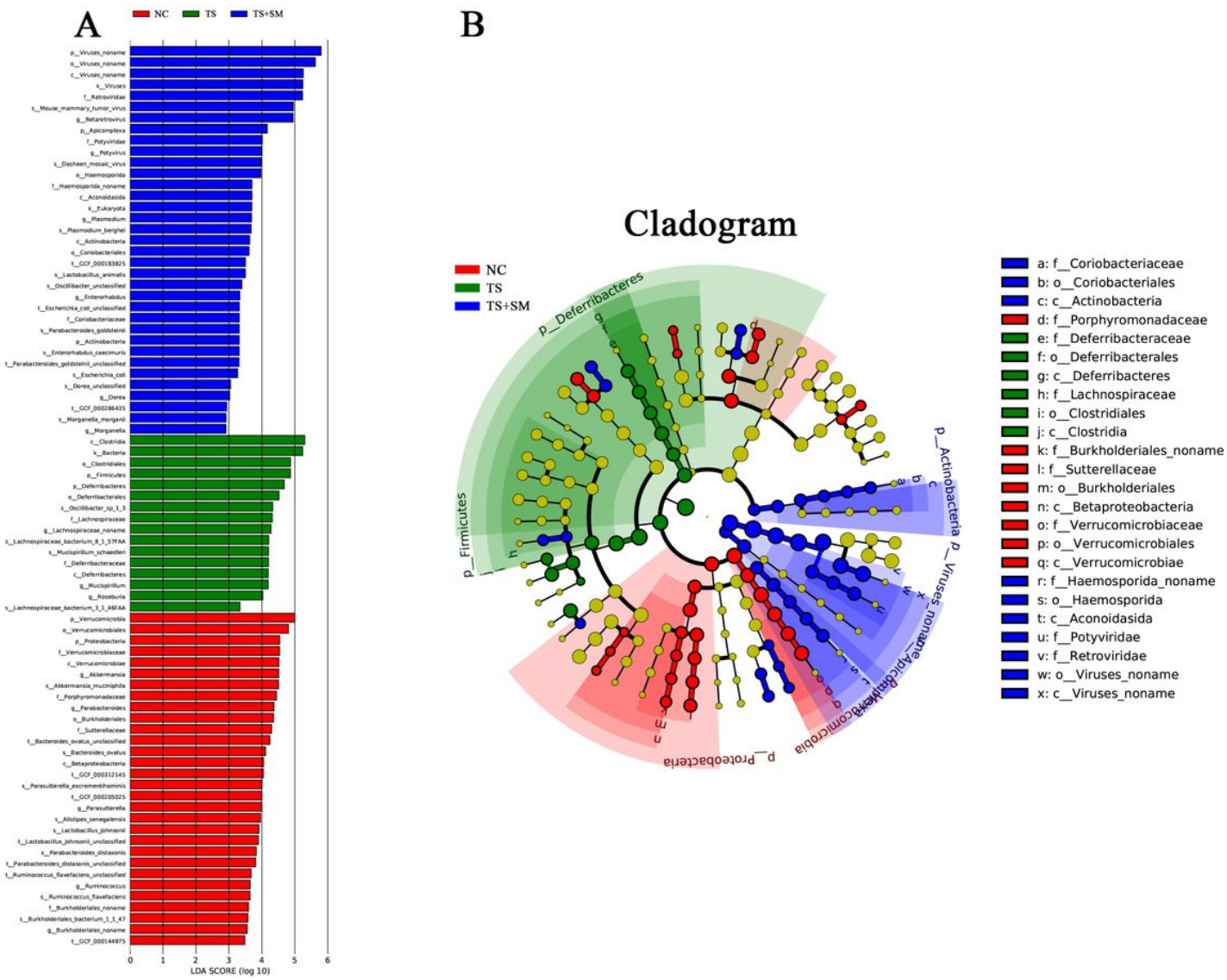

Figure 5

The results of the screening of biomarkers from all three groups. (A) The LEfSe score of the biomarkers in the three groups. (B) The cladogram of representative biomarkers and their evolutionary relationship.

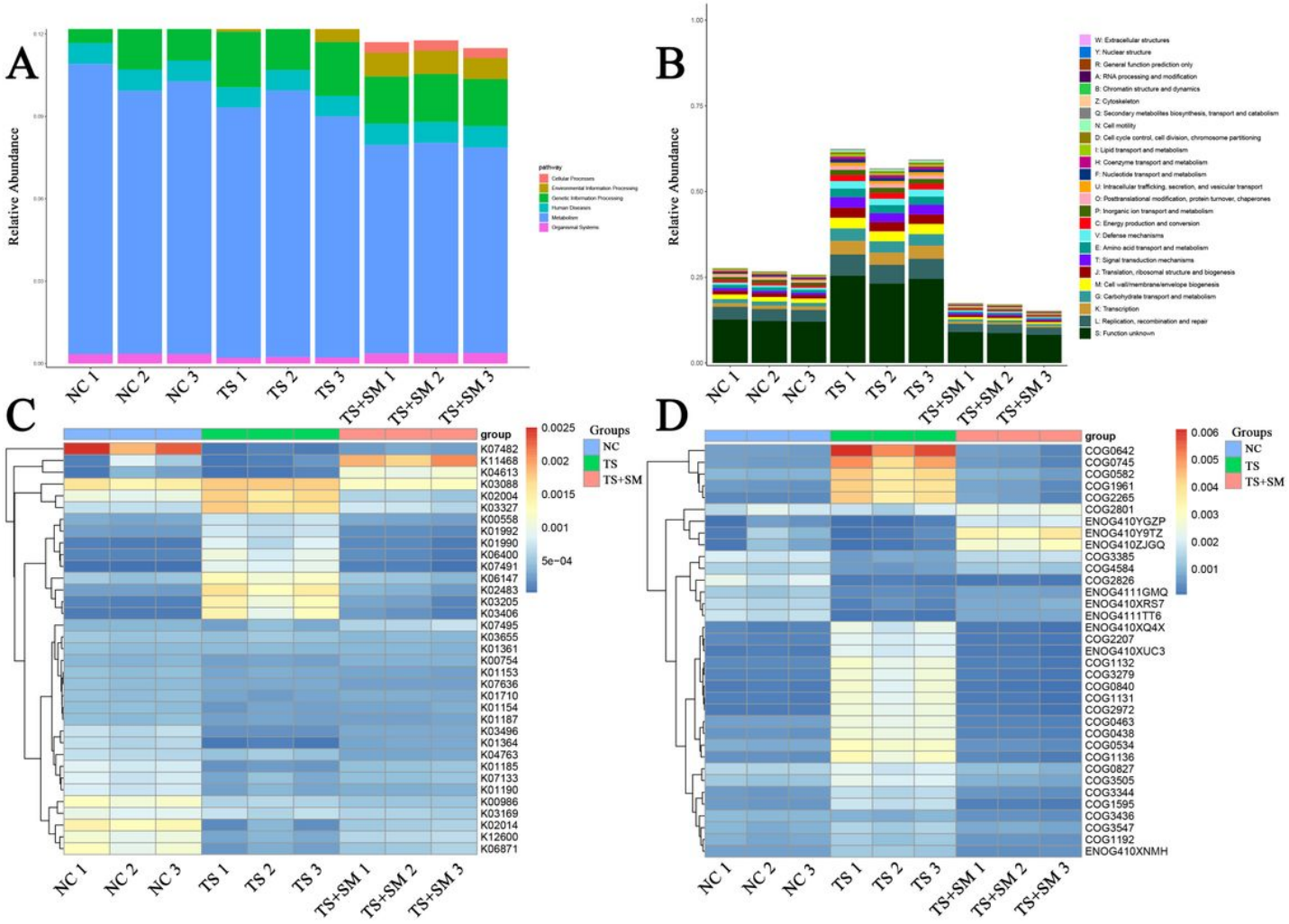

Figure 6

Functional enrichment analysis of all samples. (A, B) The results of the functional enrichment analysis of all samples using the KEGG and eggNOG databases. (C, D) The heat map showing the enriched functions of all samples obtained using the KEGG and eggNOG databases.

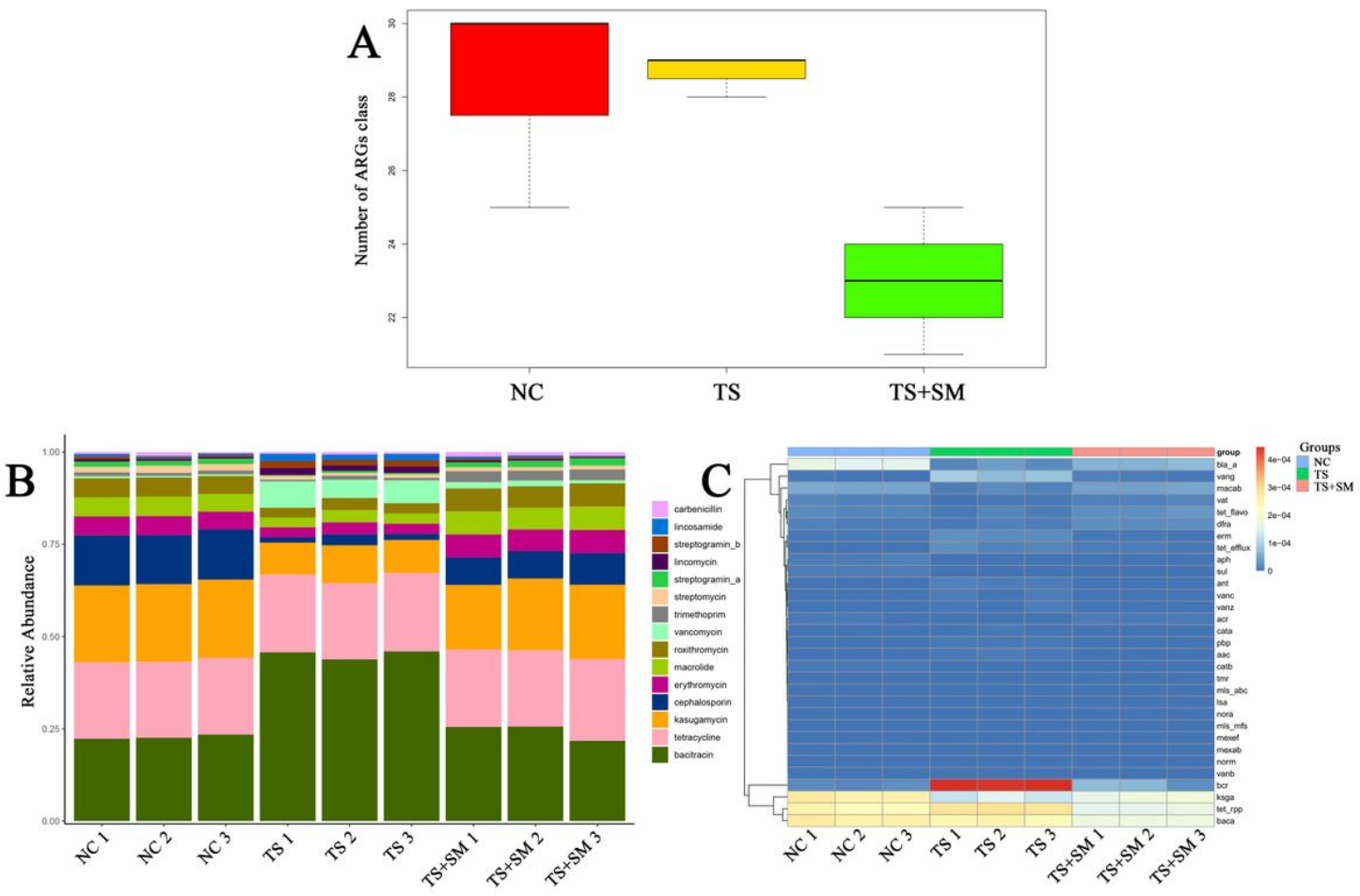

Figure 7

The predicted ARGs and their relative abundance in all samples. (A) The number of ARGs identified in the three groups. (B) The relative abundances of the top 15 ARGs in all samples. (C) The cluster heat map of the ARGs in all samples.

Supplementary Files

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- Tables.docx