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Research Report

Abnormalities of the composition of the gut microbiota and short-chain fatty acids in mice after splenectomy

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ABSTRACT

The brain–gut–microbiota axis is a complex multi-organ bidirectional signaling system between the brain and microbiota that participates in the host immune system. The spleen, as the largest immune organ in the body, has a key role in the brain–gut–microbiota axis. Here, we investigated whether splenectomy could affect depression-like phenotypes and the composition of the gut microbiota in adult mice. In behavioral tests, splenectomy did not cause depression-like behaviors in mice. Conversely, splenectomy led to significant alterations in the diversity of gut microbes compared with the findings in control (no surgery) and sham-operated mice. In an unweighted UniFrac distance analysis, the boxplots representing the splenectomy group were distant from those representing the other two groups. We found differences in abundance for several bacteria in the splenectomy group at the taxonomic level compared with the other two groups. Finally, splenectomy induced significant changes in lactic acid and n-butyric acid levels compared with those in the other groups. Interestingly, there were significant correlations between the counts of certain bacteria and lactic acid (or n-butyric acid) levels in all groups. These data suggest that splenectomy leads to an abnormal composition of the gut microbiota. It is likely that the spleen–gut–microbiota axis plays a crucial role in the composition of the gut microbiota by regulating immune homeostasis.

1. Introduction

The brain–gut–microbiota axis is a complex multi-organ bidirectional signaling system between the brain and microbiota with an important role in host homeostasis (Cryan et al., 2019; Cussotto et al., 2018; Dinan and Cryan, 2017; Fung et al., 2017; Long-Smith et al., 2020). Accumulating evidence suggests that the immune system plays a key role in the brain–gut–microbiota axis (Cerf-Bensussan and Gaboriau-Routhiau, 2010; Cryan et al., 2019; Fung, 2020; Round and Mazmanian, 2009). The composition of the gut microbiota in patients with psychiatric disorders such as depression (Chen et al., 2020; Jiang et al., 2015; Lin et al., 2017; Liu et al., 2020; Wong et al., 2016; Zheng et al., 2016) and schizophrenia (Ma et al., 2020; Xu et al., 2020b; Zheng et al., 2019; Zhu et al., 2020a, Zhu et al., 2020b) is altered compared with that in healthy control subjects. Collectively, it is likely that abnormalities of the brain–gut–microbiota axis influence the pathogenesis of these psychiatric disorders (Flux and Lowry, 2020). In addition, accumulating preclinical studies suggest that an abnormal gut microbiota composition could contribute to depression-like behaviors in rodents exposed to stress (Jianguo et al., 2019; Szyszkowicz et al., 2017; Wang et al., 2020a, 2020b; Yang et al., 2017b, 2019; Zhang et al., 2019a, 2020b).

The spleen is the largest secondary lymphoid organ in the body (Lewis et al., 2019). Accumulating evidence has identified a key role of the spleen in stress-related disorders (Bronte and Pittet, 2013; Lewis et al., 2019; Mebius and Kraal, 2005). For example, chronic stress (Bailey et al., 2007; McKim et al., 2018; Powell et al., 2009; Zhang et al., 2019b) or lipopolysaccharide (LPS)-induced stress (Zhang et al., 2020b) impairs splenic function in rodents. Collectively, it has been proposed that the brain–spleen axis plays a crucial role in stress-related psychiatric disorders (Hashimoto, 2020; Xu et al., 2020a; Yang et al., 2017a; Zhang et al., 2019b, 2020a, 2020b). Recently, Zhang et al. (2020c) demonstrated that splenic denervation in mice specifically compromises the formation of

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plasma cells during T cell-dependent immune activity, indicating key roles of brain–spleen communication in antibody production and brain–body interactions in the adaptive immune system (Cathomas and Russo, 2020; Whalley, 2020). Despite the well-known effects of stress on the spleen, the precise molecular mechanisms underlying stress-induced changes in splenic function remain unknown. Moreover, no study had demonstrated the role of spleen in the brain–gut–microbiota axis in rodents.

Splenectomy is a surgical procedure that partially or completely removes the spleen as a treatment for a wide variety of disorders, although subjects can experience a number of serious complications after surgery (Weledji, 2014). Given the crucial role of the spleen in the immune system, the present study investigated whether splenectomy affects the composition of the gut microbiota in mice. Furthermore, we sought to clarify previous findings (Haile et al., 2016) indicating that splenectomy causes depression-like phenotypes in mice.

2. Materials and methods

2.1. Animals

Male C57BL/6 mice (8 weeks old, weighing 20–25 g, n = 25) were purchased from Japan SLC Inc. (Hamamatsu, Shizuoka, Japan). Mice were housed (4 or 5 per cage) under a 12-h/12-h light/dark cycle (lights on 07:00 a.m.) under controlled conditions for temperature and humidity. Mice were granted access to food (CE-2; CLEA Japan, Inc., Tokyo, Japan) and water ad libitum. The experiment was approved by the Chiba University Institutional Animal Care and Use Committee (Permission number: 1–365). All efforts were made to minimize animal suffering.

2.2. Splenectomy

Splenectomy (or sham) surgery was performed under continuous isoflurane inhalation anesthesia. Briefly, the mice were anesthetized with 3% isoflurane through an inhalation anesthesia apparatus (KN-1071 NARCOBIT-E; Natsume Seisakusho, Tokyo, Japan). In the splenectomy group, each mouse was kept in a left lateral recumbent position, and an approximately 1-cm incision was made from the abdominal wall under the left costal margin. The skin was dissected, and subcutaneous, muscle, and fascia layers were removed individually until the spleen was exposed. The peripheral ligament of the spleen was separated, associated blood vessels and nerves were ligatured using 6-0 silk sutures, and the spleen removed by transecting the blood vessels distal to the ligature. Abdominal muscles and the skin incision were closed sequentially using 4-0 silk sutures. During sham surgery, the abdominal wall was similarly opened, and the wall was closed immediately after identifying the spleen.

2.3. Behavioral tests

The locomotion test (LMT), tail suspension test (TST), forced swimming test (FST), and 1% sucrose preference test (SPT) were performed as described previously (Chang et al., 2019; Wang et al., 2020a, 2020b). Behavioral tests were performed in a blind manner.

LMT: An automated animal movement analysis system (SCANET MV-40; MELQUEST Co., Ltd., Toyama, Japan) was used to measure the locomotor activity of mice. The cumulative ambulatory activity counts were recorded continuously over a period of 60 min after the mice were placed in the experimental cages (56 cm [length] × 56 cm [width] × 33 cm [height]). The cages were cleaned between the testing sessions.

TST: The TST was performed using a small piece of adhesive tape placed approximately 2 cm from the tip of the tail for each mouse. A single hole was punched in the tape, and mice were hung individually on hooks. The immobility time was recorded for 10 min. Mice were considered immobile only when they hung passively and they were completely motionless.

FST: The FST was performed using an automated forced-swim apparatus (SCANET MV-40). The mice were individually placed into a cylinder (23 cm [diameter] × 31 cm [height]) with a water depth of 15 cm (water temperature, 23 ± 1 °C). The immobility time was recorded and calculated by the analytical software of the apparatus throughout a 6 min observation time.

SPT: For the SPT, mice were exposed to both water and 1% sucrose solution for 48 h, followed by 4 h of water and food deprivation and a 1-h exposure to two identical bottles (water and 1% sucrose solution). The bottles containing water and sucrose were weighed before and after the exposure period. The sucrose preference was calculated as the percent sucrose solution consumption relative to the total liquid consumption.

2.4. Fecal sample DNA extraction

Fresh fecal samples of mice were collected before the behavioral tests. The fecal samples were placed into sterilized screw-cap microtubes immediately after defecation and stored at −80 °C until use. DNA from the mouse fecal samples of the three different groups (control, sham, and splenectomy) was extracted using a NucleoSpin DNA stool kit (MACHEREY-NAGEL, Germany) according to the manufacturer's protocol. The DNA concentration was determined using a BioPhotometer® Plus system (Eppendorf, Germany). Purity was determined by analyzing the ratio of absorbance at 260 nm and 280 nm (OD 260/OD 280) and also monitored using 1% agarose gels. The DNA samples were stored at −80 °C before further analysis.

2.5. 16 S rRNA analysis of fecal samples

The 16 S rRNA analyses of fecal samples were performed at Novogene Bioinformatics Technology Co. (Tianjin, China). PCR amplification was performed before sequencing, and a barcoded sequencing approach was used to study the bacterial composition of each fecal sample. The V3–V4 regions of the 16 S rRNA gene were amplified with the specific primers 341 F (CCTAYGGGRBGCASCAG) and 806 R (GGACTACNNGGGTATCTAAT). Sequencing was performed on the Illumina NovaSeq 6000 platform.

Sequences analysis for operational taxonomy units (OTU) production was performed using Uparse software (version 7.0.1001, http://drive5.com/uparse/). Sequence reads were further processed to remove low-quality and short reads according to the Quantitative Insights Into Microbial Ecology (QIIME, version 1.7.0, http://qiime.org/index.html) and UCHIME algorithms http://www.drive5.com/usearch/manual/uchime_algo.html). Then, the effective tags were obtained according to the aforementioned data filtration. For species annotation, the remaining reads (46,218 reads) were clustered into OTUs at 97% identity using Mothur software against the SILVA Database (SILVA132, http://www.arb-silva.de/) at each taxonomic rank of kingdom, phylum, class, order, family, genus, and species (threshold value = 0.8–1). The OTU abundance bioinformation was normalized using the sequence number corresponding to the sample sequences for alpha and beta diversity.

Alpha diversity, defined as the gut microbiota richness (Rhoads et al., 2018), was used to analyze the complexity of species diversity for a sample through six indices, including the observed OTU, Chao 1, Shannon, Simpson, ACE, and Fisher indices. These indices were analyzed using QIIME (Kuczynski et al., 2012) and displayed using R software (version 2.15.3).

Beta diversity was used to determine the differences of species complexity among samples. The beta diversity of unweighted UniFrac and weighted UniFrac distances were analyzed using QIIME software (version 1.7.0). Unweighted pair group method of arithmetic means (UPGMA) clustering was used as a hierarchical clustering method to

interpret the distance matrix using the average linkage with QIIME software (version 1.7.0). Metastats (Duan et al., 2020) and R software were used to analyze the effects of splenectomy on the beta diversity of the gut microbiota at the taxonomic level as demonstrated using a heatmap, and significance was indicated by $P < 0.05$ or $P < 0.01$. Differences in bacterial taxa between groups at the species or higher level (depending on the taxon annotation) were calculated via linear discriminant analysis (LDA) effect size (LEfSe) using LEfSe software (LDA score > 4.0, $P < 0.05$) (Segata et al., 2011).

2.6. Prediction of functional profiles of gut microbiota using PICRUSt

Based on the 16 S rRNA gene sequencing data and Kyoto Encyclopedia of Genes and Genome (KEGG) orthology, PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) analysis and Statistical Analysis of Metagenomic Profiles (STAMP) software package were applied for the functional prediction of gut microbiota (Langille et al., 2013; Parks et al., 2014).

2.7. Measurement of short-chain fatty acid (SCFA) levels in fecal samples

Determination of SCFAs (i.e., acetic acid, propionic acid, butyric acid, lactic acid, succinic acid) in fecal samples was performed at TechnoSuruga Laboratory, Co., Ltd. (Shizuoka, Japan), as reported previously (Wang et al., 2020a, 2020b; Zhang et al., 2019a). The concentrations of SCFAs were determined using gas chromatography with a flame ionization detector. The SCFA data were expressed as milligrams per gram of feces.

2.8. Statistical analysis

Data are expressed as the mean ± standard error of the mean (S.E.M.). The body weight data were analyzed using repeated-measures one-way analysis of variance (ANOVA), followed by Fisher's least significant difference (LSD) test. Data for behavioral tests, and SCFA levels were analyzed using one-way ANOVA, followed by Fisher's LSD test. Data for alpha-diversity of the gut microbiota were analyzed using the Kruskal–Wallis test, followed by the Dunn's test for *post-hoc* analysis. For beta-diversity of the gut microbiota, principal component analysis (PCA) of OUT level and the weighted UniFrac phylogenetic distance were performed using analysis of similarities (ANOSIM) by R package vegan (2.5.4) (Xia and Sun, 2017). The unweighted UniFrac phylogenetic distance was also calculated to estimate beta-diversity using ANOSIM by R package vegan (2.5.4). To compare gut microbiome structure among different groups for beta-diversity based on the unweighted UniFrac phylogenetic distance, we used a Wilcoxon-signed rank test. Metastats analysis was used to determine the differentially abundant genera between the two groups. Correlations between SCFAs and the relative bacterial abundance were analyzed using Spearman's correlation analysis. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of splenectomy on depression-like phenotypes

A previous study found that splenectomy caused depression-like phenotype in mice (Haile et al., 2016). Therefore, we performed behavioral tests for depression-like behaviors in mice after splenectomy (Fig. 1A). Body weight did not differ before and 7 days after splenectomy among the three groups (Fig. 1B). No differences were noted in any behavioral tests, including the LMT (Fig. 1C), TST (Fig. 1D), FST (Fig. 1E), and SPT (Fig. 1F), among the three groups.

3.2. Effects of splenectomy on the OTU clustering of the evolutionary tree and taxon composition profile of the gut microbiota

To understand the distribution of gut bacteria after splenectomy at different taxonomic levels, the evolutionary tree of the 100 most abundant genera was constructed to observe the representative sequences of bacteria at the genus level with color-coding at the phylum level for the three groups (Fig. 2A). We identified different distributions of bacteria at the genus level in the splenectomy group. For example, the splenectomy group exhibited a lower abundance of *Lactobacillus* (Fig. 2A). Simultaneously, the taxon composition profile of gut bacteria was also presented

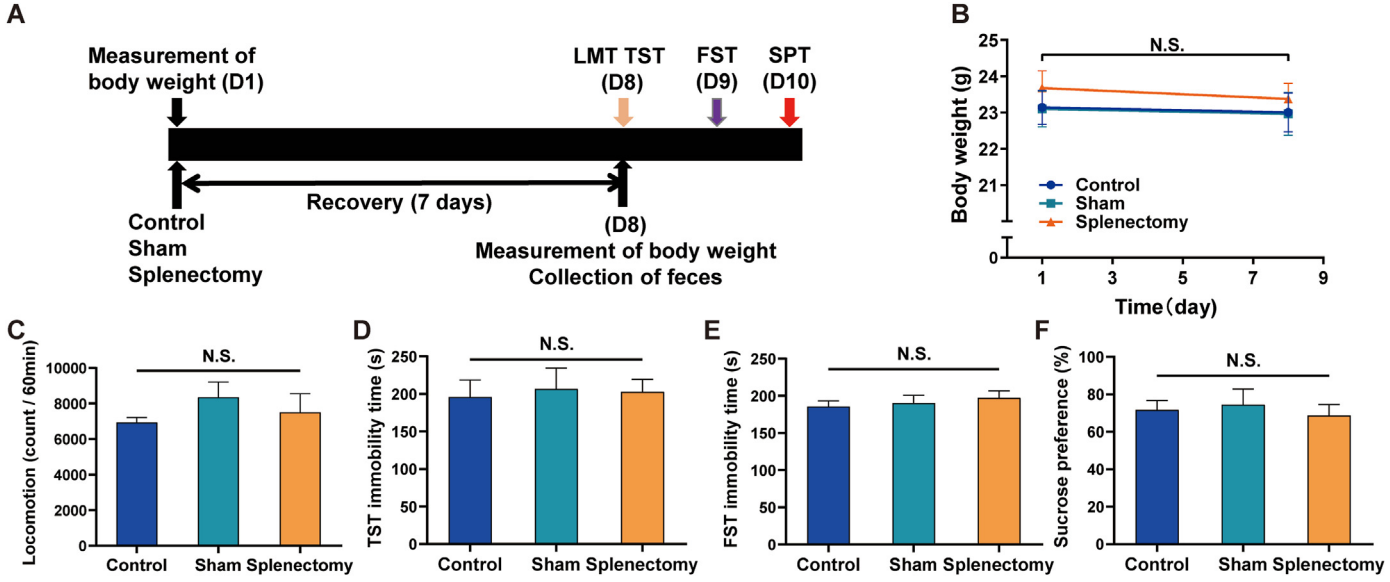

Fig. 1. Schedule of splenectomy, collection of feces and behavioral tests

(A): Splenectomy or sham surgery was performed on day 1. Collection of feces, locomotion test (LMT) and tail suspension test (TST) were performed on day 8. Forced swimming test (FST) and one % sucrose preference test (SPT) were performed on day 9 and day 10, respectively. (B): Body weight of three groups on day 1 and day 8 (repeated measure one-way ANOVA: $F_{2,22} = 0.321$, $P = 0.728$). (C): LMT (one-way ANOVA: $F_{2,22} = 0.7135$, $P = 0.5009$). (D): TST (one-way ANOVA: $F_{2,22} = 0.0573$, $P = 0.9444$). (E): FST (one-way ANOVA: $F_{2,22} = 0.3959$, $P = 0.6778$). (F): SPT (one-way ANOVA: $F_{2,22} = 0.2038$, $P = 0.8172$). The values represent the mean ± S.E.M. ($n = 8$ or 9). N.S.: not significant. LMT: locomotion test. TST: tail suspension test. FST: forced swimming test. SPT: 1% sucrose preference test.

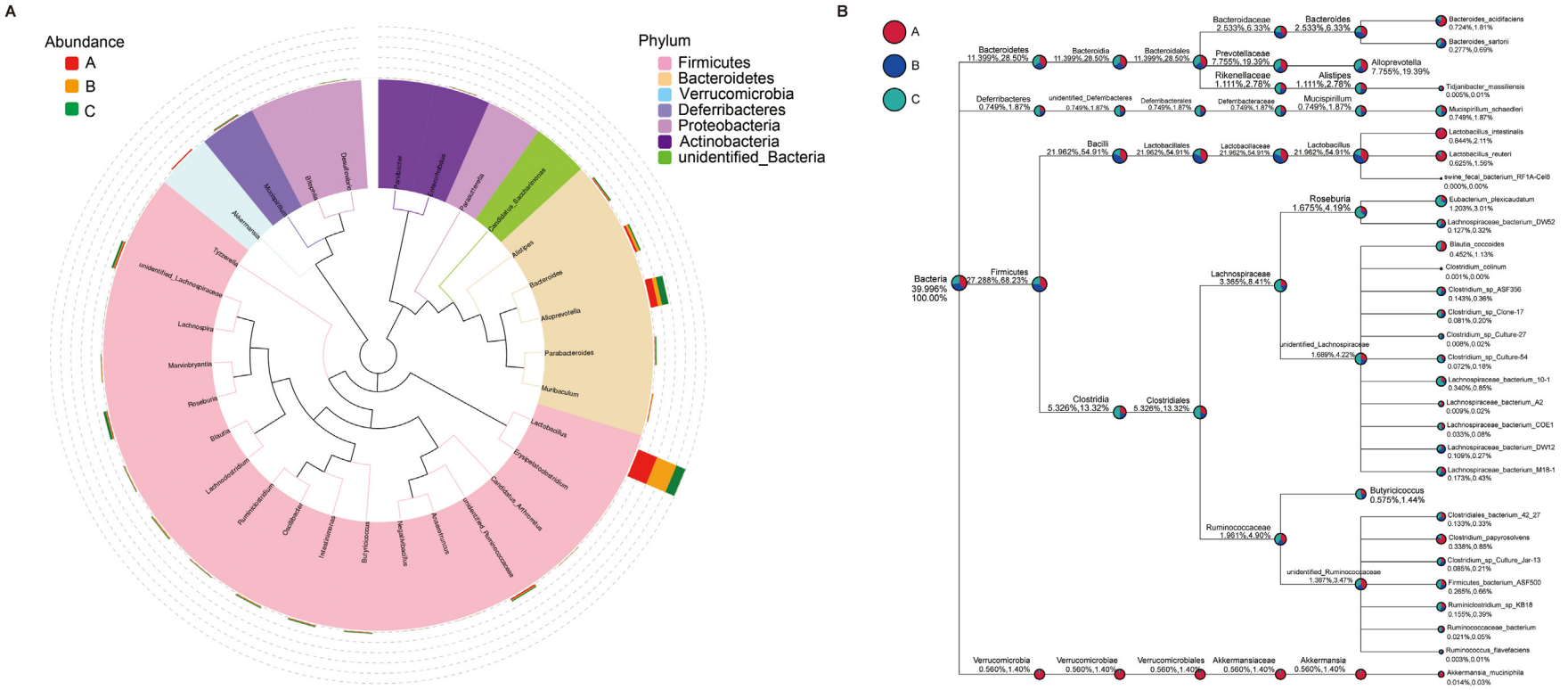

Fig. 2. Effects of splenectomy on the OTU clustering of evolutionary tree and taxon composition profile of gut microbiota

The evolutionary tree of top 100 abundant genera and taxonomy tree. (A): Circular phylogenetic tree at genus level. The inner band represents the genus taxonomy with color-coded by phylum level. Different colors of the branches represent different phylum. Relative abundance of each genus in each group was displayed outside the circle and different colors represent different groups. Red for control group, yellow for sham group, and green for splenectomy group. (B): Taxon composition profile of gut bacteria shown by taxonomy tree. Different colors represent different group of taxonomic ranks for the relative abundances in gut bacteria. Red for control group, blue for sham group, and green for splenectomy group. A, control (no surgery) group; B, sham group; C, splenectomy group. . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

using a taxonomy tree among the three groups (Fig. 2B). For example, higher abundance was observed for *Clostridia* at the class level, *Clostridiales* at the order level, and *Lachnospiraceae* at the family level in the splenectomy group (Fig. 2B).

3.3. Effects of splenectomy on the composition diversity of the gut microbiota

The composition of the gut microbiota among the three groups was analyzed using alpha- and beta-diversity. Kruskal–Wallis test revealed significant differences in the observed OUT, fisher and ACE indices among the three groups. *Post-hoc* analysis of Dunn's test showed that alpha-diversity was significantly higher in the splenectomy group than that in the control and sham groups (Fig. 3A-C). Regarding beta-diversity, PCA was applied to analyze the bacterial community composition of gut microbiota among three groups (Fig. 3D). PCA revealed significant separation in the community composition evaluated by ANOSIM (R = 0.3287, P = 0.001) (Fig. 3D) based on the OTU level. Furthermore, the UPGMA cluster analysis demonstrated that the splenectomy group was different from the control and sham groups based on the unweighted UniFrac distance (Fig. 3E and F). Importantly, the unweighted UniFrac distance among the three groups was also calculated by the ANOSIM (R = 0.2462, P = 0.001), and unweighted UniFrac distance illustrated that the boxplots representing the splenectomy group were distant from those representing in the control group (Wilcox, $P < 0.01$) and the sham group (Wilcox, $P < 0.01$) (Fig. 3G). In addition, the ordination of weighted UniFrac distance by PCoA revealed separation of splenectomy group from other two groups using the ANOSIM (R = 0.1486, P = 0.018) (Fig. 3H). Moreover, the heatmap of beta-diversity displayed significantly difference in splenectomy group compared with other two groups based on weighted UniFrac distance (Fig. 3I).

3.4. Effects of splenectomy on the beta diversity of the gut microbiota at the taxonomic level

At the phylum level, *Proteobacteria* and *Deferribacteres* had the highest abundance in the splenectomy group (Fig. 4A). The abundance of *Proteobacteria* in the splenectomy group was significantly higher ($P < 0.01$)

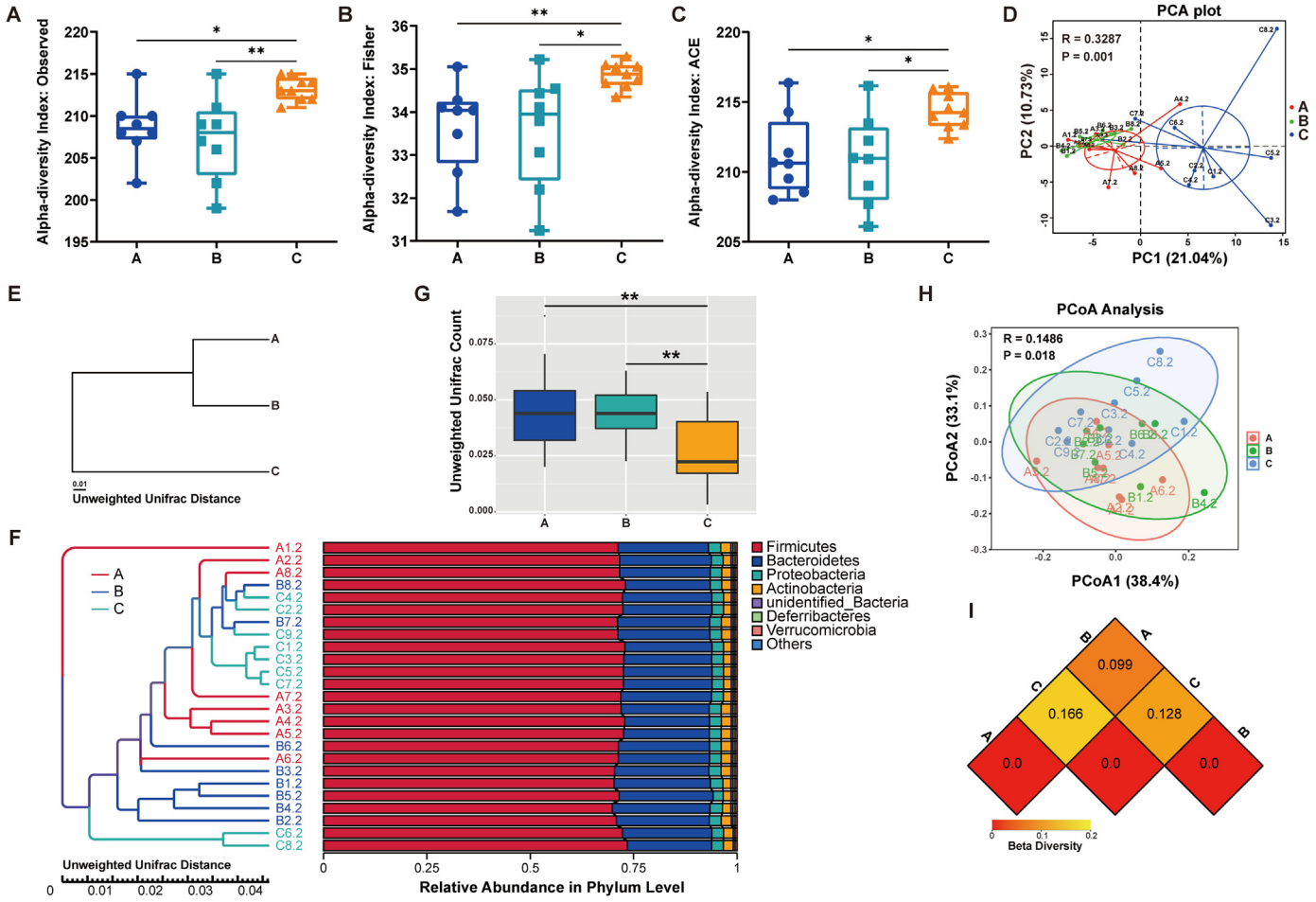

Fig. 3. Effects of splenectomy on the composition of gut microbiota in alpha-diversity and beta-diversity
(A): Alpha-diversity index of observed (Kruskal-Wallis test, $\chi2 = 10.085$, P = 0.006). (B): Alpha-diversity index of fisher (Kruskal-Wallis test, $\chi2 = 10.125$, P = 0.006). (C): Alpha-diversity index of ACE (Kruskal-Wallis test, $\chi2 = 6.801$, P = 0.033). (D): Principal component analysis (PCA) of beta-diversity based on the OTU level, where each point represents a single sample colored by group circle, indicated by the second principal component of 10.73% on the Y- axis and the first principal component of 21.04 on the X-axis (ANOSIM) (R = 0.3287, P = 0.001). (E) and (F): Unweighted Unifrac distance matrix was calculated by UPGMA cluster analysis. Splenectomy group was different with control and sham groups based on the Unweighted Unifrac distance. UPGMA hierarchical clustering results and the relative abundance of each sample at phylum level. (G): Boxplot of beta-diversity based on Unweighted Unifrac distance (ANOSIM, R = 0.2462, P = 0.001). Wilcox rank tests are performed for analysis of significance of difference between groups. (H): PCoA plot based upon weighted UniFrac distance (ANOSIM, R = 0.1486, P = 0.015). Each dot represents a single sample indicated by a principal component of 38.4% on the X-axis and another principal component of 33.1% on the Y- axis, contributing to discrepancy among the three groups. (I): Heatmap of beta-diversity based on weighted UniFrac distance. The values represent the mean ± S.E.M. (n = 8 or 9). *P < 0.05; **P < 0.01 ***P < 0.001. A, control (no surgery) group; B, sham group; C, splenectomy group.

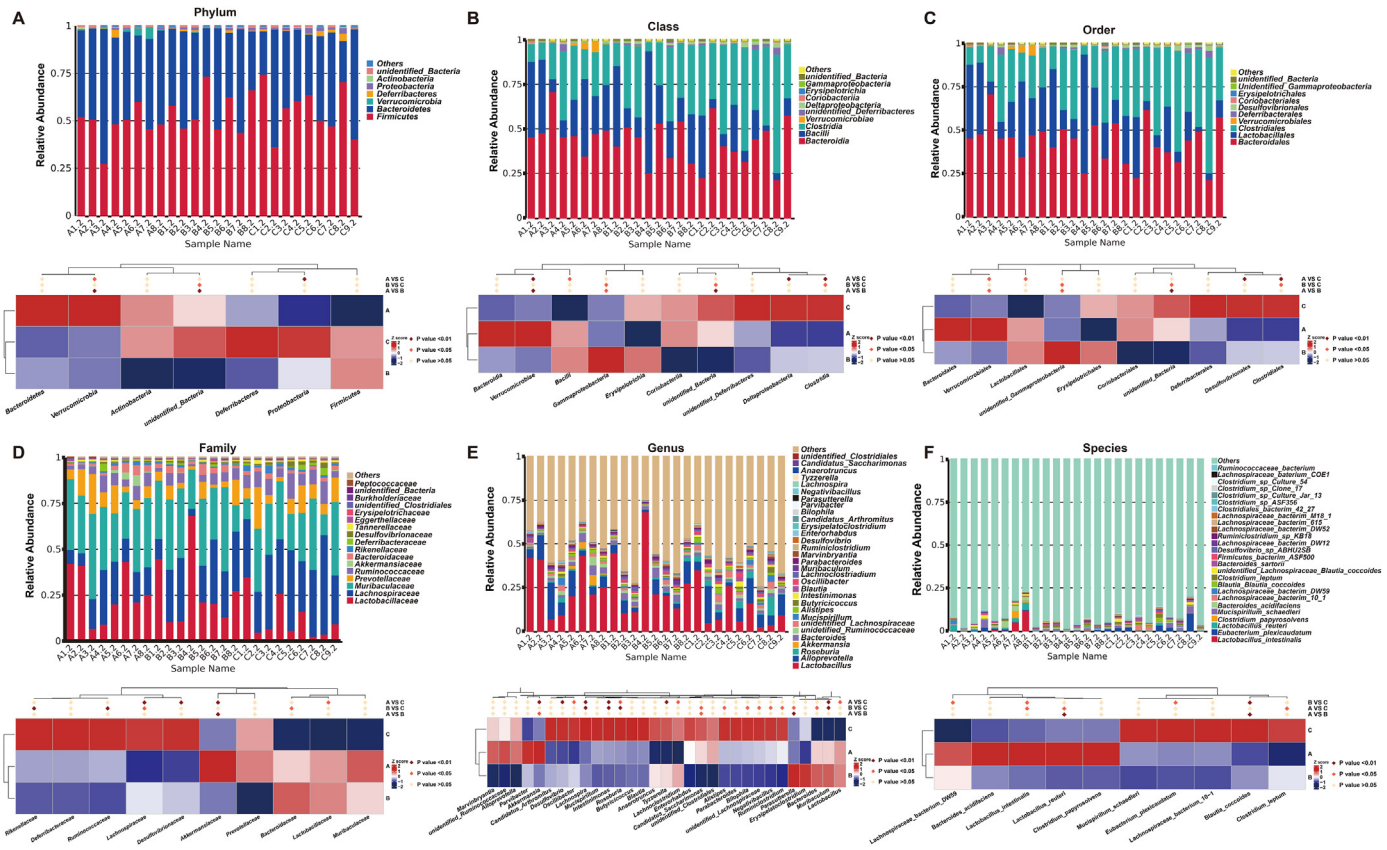

Fig. 4. Effects of splenectomy on the taxonomic level of gut microbiota in beta-diversity Relative abundance distribution and comparison of relative taxon abundances at different taxonomic level. The taxonomic level includes phylum (A), class (B), order (C), family (D), genus (E) and species (F), displayed by histogram on each fecal sample among the three groups. Heat map showed the relative abundance of the significantly changed bacteria at different taxonomic level based on MetaStat method. Relative abundance from high to low of bacteria was indicated by Z score with the color from red to blue. P value ($P < 0.01$, $P < 0.05$ and $P > 0.05$) was also shown in different color from red to yellow. A, control (no surgery) group; B, sham group; C, splenectomy group. . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

than that in the control group (Fig. 4A).

At the class level, *Deltaproteobacteria* and *Clostridia* had the highest abundance in the splenectomy group, whereas *Bacilli* had the lowest abundance (Fig. 4B). The abundance of *Deltaproteobacteria* was significantly higher ($P < 0.01$) in the splenectomy group than in the control group, whereas that of *Bacilli* was significantly lower in the splenectomy group than in the control group ($P < 0.05$) (Fig. 4B). In addition, the abundance of *Clostridia* was significantly higher in the splenectomy group than in the control ($P < 0.01$) and sham groups ($P < 0.05$, Fig. 4B).

At the order level, *Deferribacterales*, *Desulfovibrionales*, and *Clostridiales* had the highest abundance in the splenectomy group, whereas *Lactobacillales* had the lowest abundance (Fig. 4C). The abundance of *Desulfovibrionales* was significantly higher ($P < 0.01$) in the splenectomy group than in the control group, whereas that of *Lactobacillales* was significantly lower ($P < 0.05$, Fig. 4C). Moreover, the abundance of *Clostridiales* was significantly higher in the splenectomy group than in the control ($P < 0.01$) and sham groups ($P < 0.05$) (Fig. 4C).

At the family level, *Rikenellaceae*, *Deferribateraceae*, *Ruminococcaceae*, *Lachnospiraceae*, and *Desulfovibrionaceae* had the highest abundance in the splenectomy group, whereas *Bacteroidaceae*, *Lactobacillaceae*, and *Muribaculaceae* had the lowest abundance (Fig. 4D). The abundance of *Lactobacillaceae* was significantly higher ($P < 0.05$) in the splenectomy group than in the control group, whereas that of *Bacteroidaceae* was significantly lower in the splenectomy group ($P < 0.05$) (Fig. 4D). Meanwhile, the abundance of *Desulfovibrionaceae* was significantly higher ($P < 0.01$) in the splenectomy group than in the control group, and those of *Rikenellaceae* and *Ruminococcaceae* were significantly higher ($P < 0.01$) in the splenectomy group than in the sham group (Fig. 4D). More importantly, the abundance of *Lachnospiraceae* was significantly higher in the splenectomy group than in the control ($P < 0.01$) and sham groups ($P < 0.05$, Fig. 4D).

At the genus level, the abundance of *Desulfovibrio*, *Oscillibacter*, *Lachnospira*, *Mucispirillum*, *Intestinimonas*, *Roseburia*, *Alistipes*, *Bilophila*, unidentified *Lachnospiraceae*, *Negativibacillus*, and *Ruminiclostridium* was elevated in the splenectomy group, whereas that of *Bacteroides*, *Muribaculum*, and *Lactobacillus* was decreased (Fig. 4E). Obviously, the abundance of *Desulfovibrio* ($P < 0.01$) and *Oscillibacter* ($P < 0.05$) was higher in the splenectomy group than in the control group, and that of *Lactobacillus* ($P < 0.05$) was lower in the splenectomy group. Furthermore, the abundance of *Alistipes*, *Bilophila*, unidentified *Lachnospiraceae*, *Negativibacillus*, and *Ruminiclostridium* was significantly higher ($P < 0.05$) in the splenectomy group than in the sham group, whereas that of *Bacteroides* was significantly lower ($P < 0.05$, Fig. 4E). More importantly, the abundance of *Lachnospira* ($P < 0.01$ vs. control and sham), *Intestinimonas* ($P < 0.01$ vs. control and sham), and *Roseburia* ($P < 0.05$ vs. control and $P < 0.01$ vs. sham) was significantly higher in the splenectomy group than in the control and sham groups (Fig. 4E). Contrarily, the abundance of *Muribaculum* was significantly lower ($P < 0.01$) in the splenectomy group than in the control and sham groups (Fig. 4E).

The beta-diversity of the gut microbiota at the species level in the

three groups is presented in Fig. 4F. The abundance of *Clostridium leptum*, *Blautia coccoides*, *Lachnospiraceae bacterium* 10–1, *Eubacterium plexicaudatum*, and *Mucispirillum schaedleri* was increased in the splenectomy group, whereas that of *Clostridium papyrosolvens*, *Lactobacillus reuteri*, *Lactobacillus intestinalis*, *Bacteroides acidifaciens*, and *Lachnospiraceae bacterium* DW59 was decreased (Fig. 4F). Importantly, the abundance of *Lactobacillus reuteri* was significantly lower ($P < 0.05$) and that of *Clostridium leptum* was significantly higher ($P < 0.05$) in the splenectomy group than in the control group (Fig. 4F). The abundance of *Lachnospiraceae bacterium* DW59 was significantly lower ($P < 0.05$) in the splenectomy group than in the sham group, whereas that of *Blautia coccoides* ($P < 0.01$) and *Eubacterium plexicaudatum* ($P < 0.05$) was significantly higher in the splenectomy group (Fig. 4F). Moreover, the abundance of *Lactobacillus intestinalis* was significantly lower in the splenectomy group ($P < 0.05$) than in the control and sham groups (Fig. 4F).

3.5. Effects of splenectomy on the LEfSe algorithm of gut microbiota

The gut microbiota changes of the abundant taxa among the three groups were analyzed using the LEfSe algorithm, which permits the identification of microbial markers that are more important in one group than in another group. The color differences illustrated differences in the abundant taxa among the groups (Kwak et al., 2020). As presented in Fig. 5A and C, a similar distribution for potential microbial markers was found between the control and splenectomy groups and between the sham and splenectomy groups. Six mixed-level phylotypes, including the

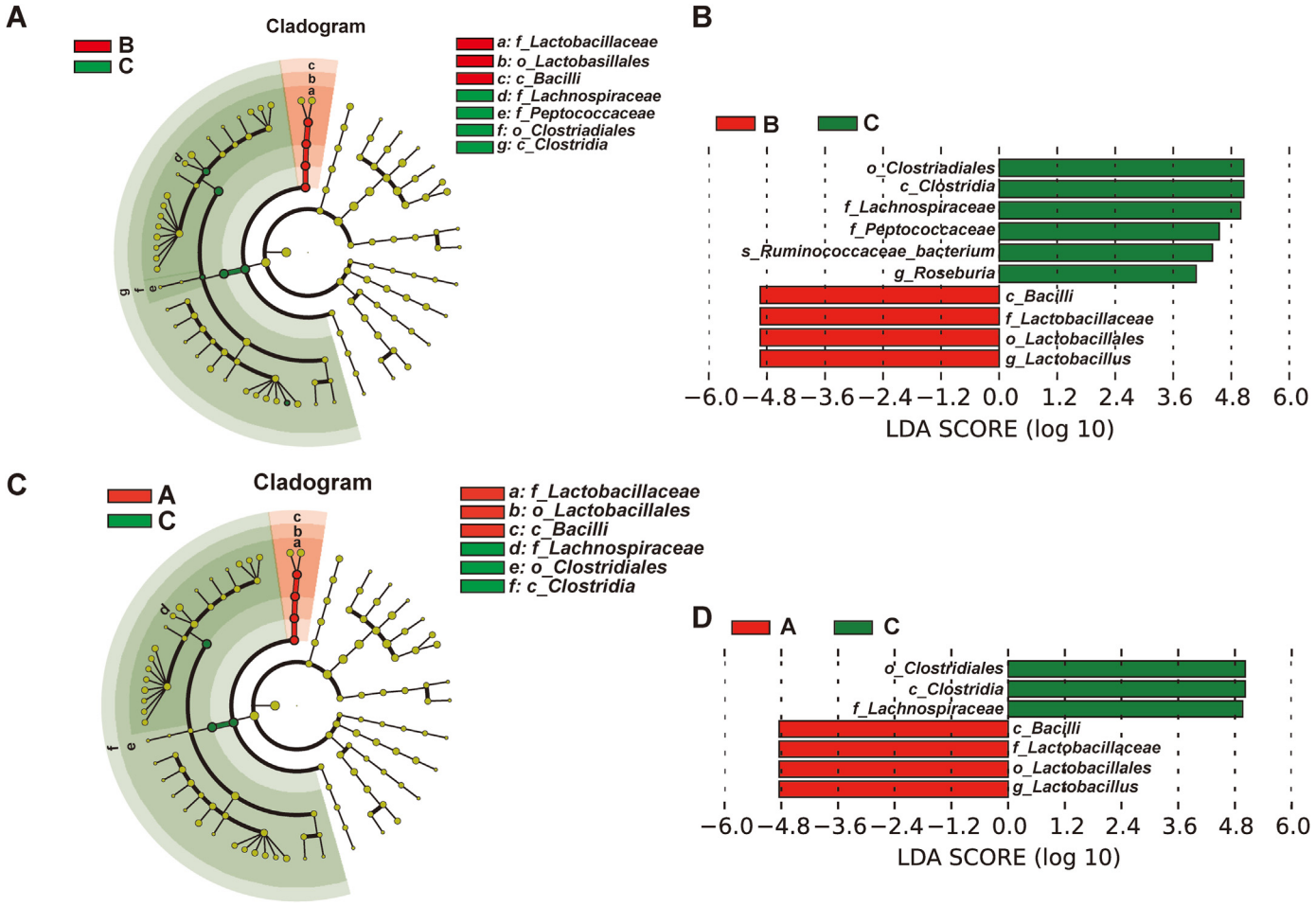

Fig. 5. Effects of splenectomy on LEfSe algorithm of gut microbiota

Linear discriminant analysis Effect Size (LEfSe) algorithm of gut microbiota changes in abundant taxa between the two groups. The colors showed that the group of abundant taxa was different with the other groups. (A): Cladogram (LDA score > 4.0, P < 0.05) showed the taxonomic distribution difference between the sham group and splenectomy group, indicating with different color region. Each successive circle represents a differentially abundant taxonomic clades at phylum, class, order, family, genus and species level from the inner to outer rings. (B): Histograms of the different abundant taxa based on the cutoff value of LDA score (log10) > 4.0 and P < 0.05 between the sham group and splenectomy group. The LDA scores of the sham group was negative, while those of the splenectomy group was positive. (C): Cladogram (LDA score > 4.0, P < 0.05) showed the taxonomic distribution difference between the control group and splenectomy group indicated with different color region. Each successive circle represents a differentially abundant taxonomic clades at phylum, class, order, family, genus and species level from the inner to outer rings. (D): Histograms of the different abundant taxa based on the cutoff value of LDA score (log10) > 4.0 and P < 0.05 between the control group and splenectomy group. The LDA scores of the control group was negative, while those of the splenectomy group was positive. A, control (no surgery) group; B, sham group; C, splenectomy group. . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

order *Clostridiales*, the class *Clostridia*, the families *Lachnospiraceae* and *Peptococcaceae*, the species *Ruminococcaceae bacterium*, and the genus *Roseburia*, were identified as potential microbial markers in the splenectomy group compared with the findings in the sham group (Fig. 5B, Table 1). Interestingly, three mixed-level phylotypes, including the order *Clostridiales*, class *Clostridia*, and family *Lachnospiraceae*, were also identified as potential microbial markers in the splenectomy group compared with the findings in the control group (Fig. 5D). As we expected, the same four mixed-level biomarkers, including the class *Bacilli*, family *Lactobacillaceae*, order *Lactobacillales*, and genus *Lactobacillus*, were found in both the control and sham groups compared with the results in the splenectomy group (Fig. 5B and D, Table 1).

3.6. Predictive functional metagenomes after splenectomy

The functional alterations of gut microbiota were predicted using the PICRUSt among the three groups. Predominant KEGG pathways involved in metabolism (Fig. 6A), including biosynthesis of other secondary metabolites, carbohydrate metabolism, lipid metabolism, metabolism of cofactors and vitamins, metabolism of terpenoids and polyketides, xenobiotics biodegradation and metabolism (Fig. 6B). Significant differences among the three groups were identified in 9 pathways on KEGG level 3, including beta−lactam resistance, penicillin and cephalosporin biosynthesis, amino sugar and nucleotide sugar metabolism, starch and sucrose metabolism, primary bile acid biosynthesis, secondary bile acid biosynthesis, ubiquinone and other terpenoid−quinone biosynthesis, biosynthesis of ansamycins and styrene degradation ($P < 0.05$) (Fig. 6C). In addition, other pathways on KEGG level 3 were also different among the three groups, such as ubiquitin system, renal cell carcinoma, plant−pathogen interaction, germination, sporulation ($P < 0.05$) (Fig. 6C), which related to genetic information processing (folding, sorting and degradation), human diseases (cancers), organismal systems (environmental adaptation) and cellular processes and signaling (Fig. 6A and B).

3.7. Measurement of SCFA content in fecal samples and correlations between the relative bacterial abundance and SCFA levels

SCFAs produced by microbiome play a role in brain-gut communication (Dalile et al., 2019; Silva et al., 2020; Wu et al., 2020). We measured the concentrations of SCFAs in fecal samples because gut microbes produce SCFAs (Table 2). The levels of lactic acid were significantly lower in the splenectomy group than those in the sham groups, whereas those of n-butyric acid were significantly higher in the splenectomy group than those in the other two groups (Table 2 and Fig. 7A and B).

To explore the possible relationships between the relative bacterial abundance and SCFA levels in fecal samples, we performed logistic regression and correlation analyses. There were significant negative correlations of the relative abundance of *Clostridia* ($r = -0.4558$, $P = 0.0252$) at the class level (Fig. 7C), *Clostridiales* ($r = -0.4558$, $P = 0.0252$) at the order level (Fig. 7D), *Lachnospiraceae* ($r = -0.4532$, $P = 0.0261$) at the family level (Fig. 7E), *Lachnospira* ($r = -0.6444$, $P = 0.0007$) and *Intestinimonas* ($r = -0.4110$, $P = 0.0460$) at the genus level (Fig. 7F and G) and *Blautia_Blautia_coccoides* ($r = -0.6155$, $P = 0.0014$) at the species level (Fig. 7H) with the lactic acid content in all three groups.

Moreover, there was a significant negative correlation between the relative abundance of *Muribaculum* ($r = -0.4005$, $P = 0.0472$) at the genus level and n-butyric acid levels in all three groups (Fig. 7I). There was a significant positive correlation between the relative abundance of *Blautia_Blautia_coccoides* ($r = 0.4925$, $P = 0.0124$) at the species level and n-butyric acid levels in all three groups (Fig. 7K).

Table 1
Linear discriminant analysis (LDA) score for gut microbiota.

Mix taxonomic level	Abundance	Group	LDA score	P value
A vs C				
c_*Clostridia*	5.636	C	5.019	0.002
o_*Clostridiales*	5.636	C	5.019	0.002
f_*Lachnospiraceae*	5.541	C	4.961	0.003
g_*Lactobacillus*	5.422	A	4.845	0.034
o_*Lactobacillales*	5.422	A	4.845	0.034
f_*Lactobacillaceae*	5.422	A	4.845	0.034
c_*Bacilli*	5.422	A	4.845	0.034
B vs C				
g_*Lactobacillus*	5.438	B	4.935	0.045
o_*Lactobacillales*	5.438	B	4.935	0.045
f_*Lactobacillaceae*	5.438	B	4.935	0.045
c_*Bacilli*	5.438	B	4.935	0.045
f_*Peptococcaceae*	2.627	C	4.548	0.036
g_*Roseburia*	4.511	C	4.073	0.032
s_*Ruminococcaceae_bacterium*	2.523	C	4.409	0.011
f_*Lachnospiraceae*	5.541	C	4.991	0.009
c_*Clostridia*	5.636	C	5.058	0.009
o_*Clostridiales*	5.636	C	5.058	0.009

p, phylum; c, class; o, order; f, family; g, genus; s, species.
The cutoff value of LDA score (log10) > 4.0 and $p < 0.05$ were considered as significant differences. A, control (no surgery) group; B, sham group; C, splenectomy group.

4. Discussion

The major findings of the current study were as follows. First, splenectomy did not induce depression-like phenotypes in mice, inconsistent with previous findings (Haile et al., 2016). Second, the OTU clustering of evolutional tress revealed low abundance of the phylum *Lactobacillus* and higher abundance of the class *Clostridia*, the order *Clostridiales*, and the family *Lachnospiraceae* in the splenectomy group compared with the results in the other two groups. Third, splenectomy induced significant changes in the alpha- and beta-diversity of the host gut microbiota in adult mice. UPGMA cluster analysis indicated differences between the splenectomy group and the other two groups. Fourth, splenectomy altered the abundance of several bacteria at different taxonomic levels. Fifth, the LEfSe algorithm identified *Clostridiales*, *Clostridia*, *Lachnospiraceae*, *Peptococcaceae*, *Ruminococcaceae bacterium*, and *Roseburia* as important phylotypes in the splenectomy group. Sixth, predictive functional metagenomes using PICRUSt showed that splenectomy caused alterations in metabolisms, including biosynthesis of other secondary metabolites, carbohydrate metabolism, lipid metabolism, metabolism of cofactors and vitamins, metabolism of terpenoids and polyketides, xenobiotics biodegradation and metabolism. Finally, lactic acid levels were significantly lower in the splenectomy group than in other two groups, whereas n-butyric acid levels were significantly higher in the splenectomy group. Interestingly, there were significant correlations between the fecal levels of lactic acid (or n-butyric acid) and the abundance of several components of the microbiome in all groups. Taken together, the current data indicated that splenectomy alters the composition of the gut microbiota and SCFAs in the host without causing depression-like phenotypes.

A previous study demonstrated that splenectomy induced decreased sucrose preference in the SPT 5 days after surgery and that splenectomy caused neuroinflammation (i.e., microglial activation, reactive astrocytosis) in the brain (Haile et al., 2016). Conversely, using three behavioral tests (i.e., TST, FST, SPT), we did not detect depression-like phenotypes in mice 7–9 days after splenectomy. In addition, there were no differences in the blood levels of pro-inflammatory cytokines such as interleukin-6 and tumor necrosis factor-alpha among the three groups (data not shown), indicating that splenectomy did not cause systemic inflammation. The reasons underlying the discrepancy concerning depression-like behaviors are currently unclear. One possible cause was the use of different mouse strains (Swiss Webster for the study by Haile et al. (2016) vs. C57/B6 in our study). The second possibility is the timing of the SPT (5 days for the Haile study (Haile et al., 2016) vs. 7 days for our

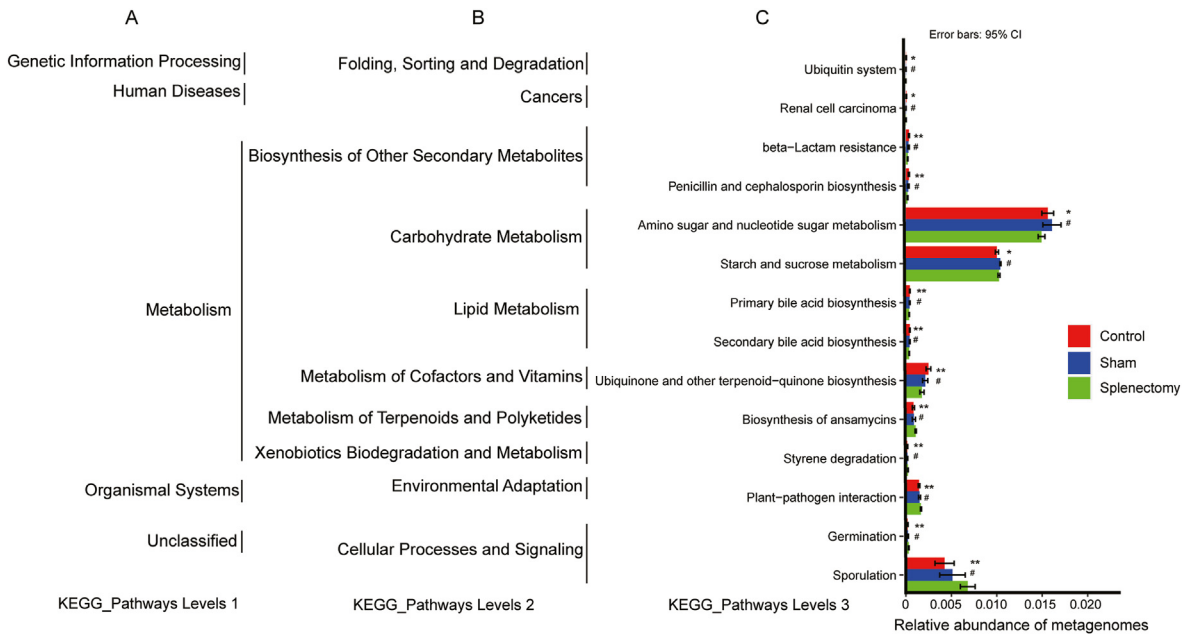

Fig. 6. Relative abundance of KEGG pathways of functional categories.
Functional predictions of the gut microbiota among the control, sham and splenectomy group generated from 16 S rRNA gene sequences using PICRUSt analysis. Significant differences of KEGG pathways at level 3 were determined by STAMP software based on the KEGG pathway database. *P < 0.05, **P < 0.01 and #P < 0.05 *vs* splenectomy group.

Table 2
Levels of SCFAs in the fecal samples.

SCFAs (mg/g)	A: Control	B: Sham	C: Splenectomy	One-way ANOVA
Succinic acid	0.364 ± 0.097	0.305 ± 0.039	0.193 ± 0.039	$F_{2,22} = 1.975$, P = 0.1626
Lactic acid	0.540 ± 0.093	0.651 ± 0.134*	0.254 ± 0.086	$F_{2,21} = 3.705$, P = 0.0419
Acetic acid	1.165 ± 0.082	1.525 ± 0.216	1.722 ± 0.216	$F_{2,22} = 2.319$, P = 0.1220
Propionic acid	0.369 ± 0.033	0.431 ± 0.044	0.454 ± 0.077	$F_{2,22} = 0.604$, P = 0.5556
n-butyric acid	0.349 ± 0.029**	0.523 ± 0.088*	0.854 ± 0.130	$F_{2,22} = 7.366$, P = 0.0036

The values are the mean ± SEM (n = 8 or 9). *P < 0.05, **P < 0.01 vs C group. One data of C group was under the limitation (0.05 mg/g).

study), as the recovery time after surgery may affect the results of behavioral tests such as the SPT. A third possibility was the changes in locomotor activity after surgery. Haile et al. (2016) reported decreased locomotor activity in mice after splenectomy. However, we did not find such changes. Thus, it is likely that reduced locomotor activity in the splenectomy group affects the results of SPT in mice. Therefore, further study on the effects of splenectomy on depression-like phenotypes is needed.

In this study, we found that splenectomy altered the abundance of several bacteria at different taxonomic levels. *Clostridium leptum* is one of the dominant groups of fecal bacteria in adult humans, constituting 16%–25% of the fecal microbiota (Lay et al., 2005). Prior research found that the abundance of *Clostridium leptum* and *Blautia coccoides* was negatively correlated with depression-like behaviors in mice (Tian et al., 2019), supporting the findings that splenectomized mice did not exhibit depression-like phenotypes in this study. *Eubacterium plexicaudatum* is a butyrate-producing bacterium (Breyner et al., 2019). The increase in n-butyric acid levels in the splenectomy group may be attributable to the higher abundance of *Eubacterium plexicaudatum*, although further study is needed. *Mucispirillum schaedleri*, a member of the phylum *Deferribacteres*, is a key antagonist of *Salmonella enterica* serovar Typhimurium colitis (Herp et al., 2019). Furthermore, a recent study indicated that neutralization using anti-mouse IL-1 alpha exerts anti-inflammatory effects in a mouse model of inflammatory bowel disease by modulating gut microbes such as *Mucispirillum schaedleri* (Menghini et al., 2019). Considering the anti-inflammatory actions of *Mucispirillum schaedleri*, it is likely that increases in the abundance of bacteria may reflect a compensatory response in the host after splenectomy. Nonetheless, further research is needed to investigate the mechanisms underlying increases in the counts of these bacteria after splenectomy.

Meanwhile, *Clostridium papyrosolvens* produce a wide variety of carbohydrate-active enzymes including extracellular multi-enzyme complexes termed cellulosomes with different specificities for enhanced cellulosic biomass degradation (Ren et al., 2019). The reduced abundance of *Lactobacillus reuteri* and *Lactobacillus intestinalis* in the splenectomy group may explain the reduced levels of lactic acid in splenectomized mice because lactobacilli ferment lactose into lactic acid (Fine et al., 2020). Furthermore, *Lactobacillus reuteri* is known to have several beneficial effects on anti-microbial activity, the host immune system, and microbial translocation (Fine et al., 2020; Mu et al., 2018). Furthermore, *Lactobacillus reuteri* is also known to produce the anti-microbial compound reuterin (Fine et al., 2020). Thus, it is possible that splenectomy affected the anti-microbial activity of reuterin in the host, although we did not measure reuterin levels in the same samples. Recently, we reported that *Lactobacillus intestinalis* and *Lactobacillus reuteri* may be responsible for the anhedonia-like phenotype in antibiotic-treated mice after fecal microbiome transplantation from mice with depression-like phenotypes (Wang et al., 2020a). Thus, the current data may support the findings that splenectomized mice did not exhibit depression-like phenotypes. *Bacteroides acidifaciens* was the most abundant *Bacteroidetes* species in mice. It has been reported that the levels of immunoglobulin A (IgA) in the gut were associated with the relative abundance of *Bacteroides fragilis* group species such as *Bacteroides acidifaciens* (Nakajima et al., 2020). Given the essential role of IgA in the defense of the intestinal mucosa against harmful pathogens, it appears that reduced counts of *Bacteroides fragilis* may be associated with inflammatory diseases induced by harmful pathogens. The functions of *Lachnospiraceae bacterium* DW59 are unknown. Although the precise

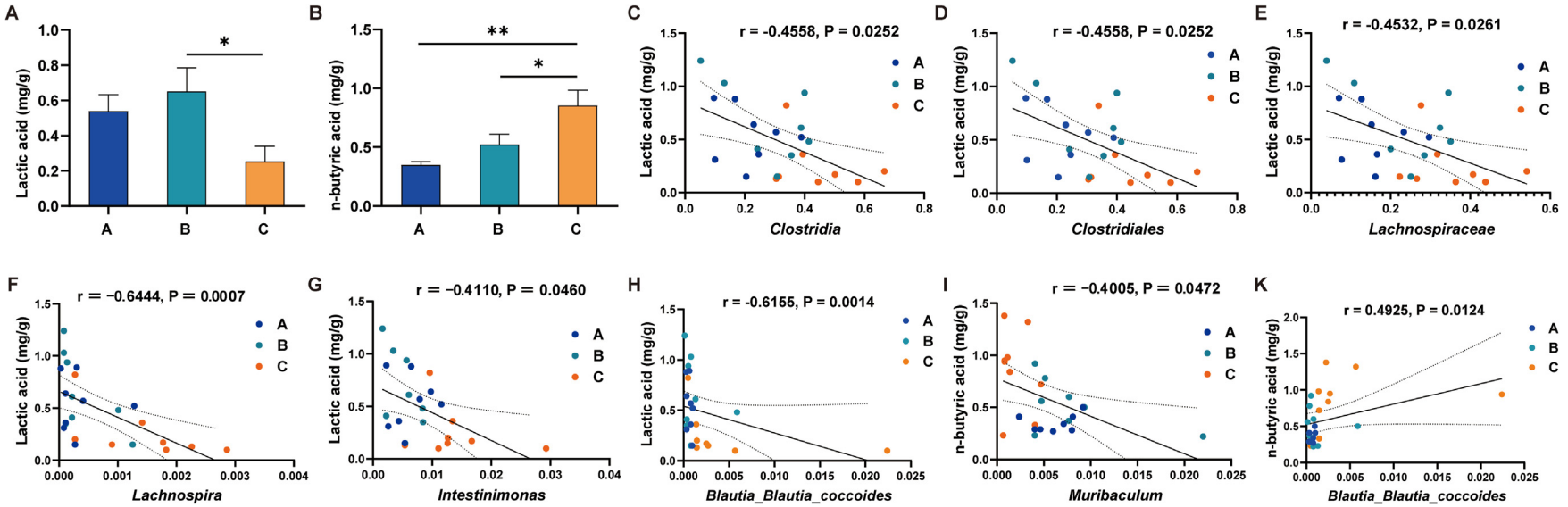

Fig. 7. Levels of SCFAs in the fecal samples and correlations between bacterial relative abundance and SCFAs

(A): Levels of lactic acid in fecal samples among the three groups (one-way ANOVA, $F_{2,21} = 3.705$, $P = 0.0419$). One data of C group was under the limitation (0.05 mg/g). (B): Levels of n-butyric acid in the fecal samples among the three groups (one-way ANOVA, $F_{2,22} = 7.366$, $P = 0.0036$). (C): There was a significant negative correlation between the relative abundance of the class *Clostridia* and lactic acid in the all groups. (D): There was a significant negative correlation between the relative abundance of the order *Clostridiales* and lactic acid in the all groups. (E): There was a significant negative correlation between the relative abundance of the family *Lachnospiraceae* and lactic acid in the all groups. (F): There was a significant negative correlation between the relative abundance of the genus *Lachnospira* and lactic acid in the all groups. (G): There was a significant negative correlation between the relative abundance of the genus *Intestinimonas* and lactic acid in the all groups. (H): There was a significant negative correlation between the relative abundance of the species *Blautia_Blautia_coccoides* and lactic acid in the all groups. (I): There was a significant negative correlation between the relative abundance of the genus *Muribaculum* and n-butyric acid the all three groups. (K): There was a significant positive correlation between the relative abundance of the species *Blautia_Blautia_coccoides* and n-butyric acid the all three groups. The values represent the mean ± S.E.M. (n = 8 or 9). *$P < 0.05$; **$P < 0.01$. A, control (no surgery) group; B, sham group; C, splenectomy group.

mechanisms underlying the abnormal composition of the gastrointestinal microbiota after splenectomy are currently unknown, it is likely that the abnormal composition of the gut microbiota and subsequent abnormalities in the levels of metabolites such as SCFAs may contribute to short- and long-term complications after splenectomy.

The spleen plays a major role in hematological and immunological functions (Lewis et al., 2019). Subjects lacking a spleen have impaired immune activity because this organ is responsible for producing antibodies and removing bacteria and aged, antibody-coated, and damaged blood cells (Weledji, 2014). Splenectomy is a surgical procedure that partially or completely removes the spleen to treat a wide variety of disorders such as splenic trauma, splenic abscess (e.g., tuberculous infection), neoplasm, and aneurysm of splenic arteries (Weledji, 2014). However, there are a number of serious complications in the early (i.e., atelectasis, pulmonary embolism, bleeding) and late periods (i.e., pulmonary tuberculous, overwhelming postoperative infection) (Cadili and de Gara, 2008; Weledji, 2014). A recent study using the database of the Taiwan National Health Insurance Program found that splenectomy is associated with pyogenic liver abscess (Lai et al., 2015) and acute pancreatitis (Lai et al., 2016). In addition, it is reported that splenectomy may ameliorate altered composition of gut microbiota in patients with liver cirrhosis (Liu et al., 2018) and that altered composition of gut microbiota in splenectomized patients were associated with plasma LPS levels (Zhu et al., 2018). It is suggested that the immunological role and structure of the spleen of humans may be quite different from those of rodents (Steiniger, 2015). Nonetheless, these studies suggest that splenectomy can affect the composition of gut microbiota in humans although it is unknown how splenectomy could affect levels of SCFAs. Collectively, splenectomy should be performed only after carefully assessing the short- and long-term risks and potential benefits to the patient. Given the key role of the spleen in the immune system and gut microbiota, the abnormal composition of the gut microbiota after splenectomy should be considered when assessing outcomes of splenectomized patients.

To understand the role of altered composition of gut microbiota after splenectomy, we examined the predictable function of gut microbiota using PICRUSt based on the 16rRNA gene sequence. According to the level 3 functional prediction map of KEGG pathway, the current data indicated that splenectomy may contribute to the altered metabolism induced by gut microbiota. Altered levels of SCFAs produced by gut microbes might be related with the functional prediction of the altered metabolism. It is shown that the production of SCFAs is regulated by the interaction among the environmental, host and microbiological factors (den Besten et al., 2013; Macfarlane and Macfarlane, 2003). Considering the relationship among the SCFAs, metabolism alteration and spleen, it is likely that SCFAs produced by gut microbiota can initiate and maintain the immune responses, which involved in the carbon metabolism (Bachem et al., 2019; Kim et al., 2016). Collectively, the KEGG pathways predicting the function of gut microbiota provide a functional view for the understanding the gut microbiota that makes a differential expression profile.

In conclusion, this study revealed that splenectomy induced abnormal changes in the gut microbiota in adult mice without causing depression-like phenotypes. The role of the brain–gut–microbiota and brain–spleen axes in splenectomized subjects should be considered.

5. Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declaration of competing interest

All authors report no biomedical financial interests or potential conflicts of interest.

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