BACTERIA AND BACTERIAL METABOLITES: MOLECULAR INTERPLAY WITH GUT IMMUNITY

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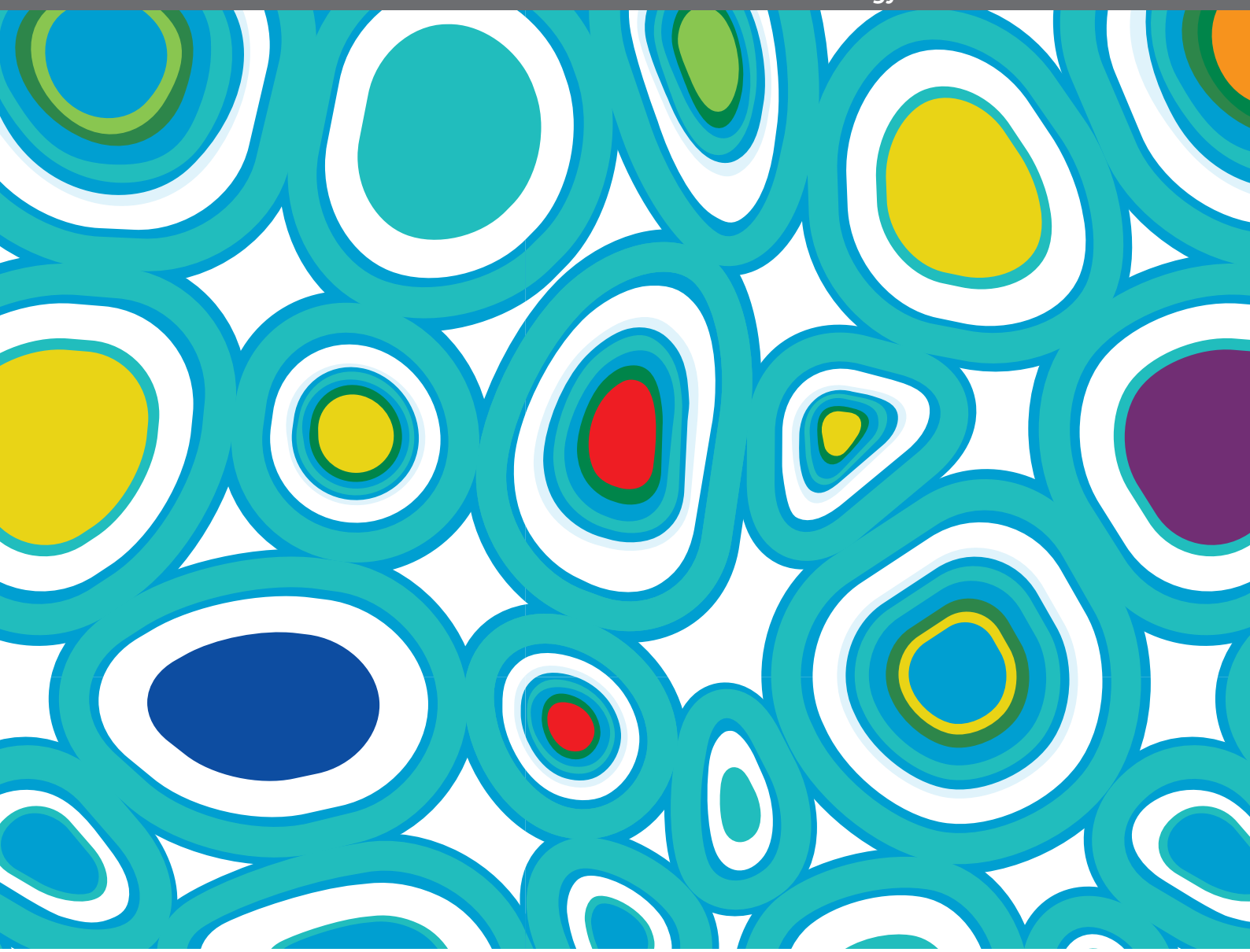

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BACTERIA AND BACTERIAL METABOLITES: MOLECULAR INTERPLAY WITH GUT IMMUNITY

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Heterogeneity of CD4$^+$CD25$^+$Foxp3$^+$Treg TCR β CDR3 Repertoire Based on the Differences of Symbiotic Microorganisms in the Gut of Mice

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Gut microbes play a crucial role in the occurrence and development of autoimmune diseases. The diversity of intestinal microorganisms affected by the living environment, regulate the immune function of peripheral immune organs and local tissues. In the study, the diversity of intestinal microorganisms of Germ-free (GF), Specific Pathogen-free (SPF), and Clean (CL) BALB/c mice were conducted by 16S rDNA sequencing. High-throughput sequencing technology was used to analysis the composition and characterization of TCR β chain CDR3 repertoires in Regulatory T cells (Treg) in intestine and spleen of GF, SPF, and CL mice, so as to investigate the effects of differential composition of intestinal microorganisms on the CD4$^+$CD25$^+$Foxp3$^+$Treg TCR β CDR3 repertoire of intestine and spleen. We observed that GF, SPF, and CL mice have different gut microorganism composition, and the abundance and quantity of microorganisms are positively correlated with the level of feeding environment. Interestingly, incomplete structure of spleen and small intestine in GF mice was found. Moreover, a significant difference in the usage of high frequency unique CDR3 amino acid sequences was detected in the intestinal Treg TCRβ CDR3 repertoire among GF, SPF and CL mice, and there were a greater heterogeneity in the usage frequency of *TRBV, TRBJ,* and *TRBV-TRBJ* combinations gene segments. However, the effect of different feeding environment on the mice Treg TCRβ CDR3 repertoire of spleen was weak, implying that the different composition of intestinal microbiota may primarily affect the diversity of the local Treg TCRβ CDR3 repertoire and does not alter the overall properties of the circulating immune system. These results provide basic data to further analyze the mechanism of gut microbes regulating the intestinal mucosal immune system.

Keywords: gut microbes, intestinal mucosal immune, Treg, TCR, CDR3 repertoire

INTRODUCTION

The colonization of gut microbes is not only very important for the metabolism of essential nutrients, but also plays an essential role in the development of the immune system (Rooks and Garrett, 2016). Bacteria and symbiotic microflora in the gut can dynamically regulate the occurrence and development of autoimmune diseases (Ivanov et al., 2009; Brown et al., 2011; Lee et al., 2011; Scher et al., 2013; Cantarel et al., 2015). Through natural selection and coevolution with the host, some microbial groups form an inseparable host microorganism symbiosis relationship with host in physiology and pathology (Sommer and Bäckhed, 2013). Symbiotic microflora affects the formation of immune system by participating in innate and adaptive immunity as well as the development and maintenance of various supervision mechanisms.

The diversity of symbiotic bacteria is different in mice raised in different environments, such as feed, drinking water and living environment, which has an important impact on the occurrence and development of their peripheral immune system. It was found that the development of peripheral immune organs of mice in aseptic feeding environment was significantly disordered compared with that of SPF mice (Telesford et al., 2015). However, transfer the intestinal microorganisms of SPF mice in GF mice, this defect was obviously improved (Macpherson and Harris, 2004; Mazmanian et al., 2005), indicated that the gut flora had an important regulatory effect on the development of peripheral immune system (Ivanov and Honda, 2012; Wu and Wu, 2012). Moreover, Ivanov et al. (2008) found that the differentiation of Th17 cells in the lamina propria of the small intestine required specific symbiotic microflora, which can regulate the balance between Foxp3 + Treg cells and Th17 cells in the lamina propria of the small intestine, suggesting that the composition of intestinal microflora is the key point of coordination (Agace and McCoy, 2017).

High throughput sequencing technology has the characteristics of accuracy, high efficiency and sensitivity, which has been widely used in the sequence analysis of T / B cell repertoire (Setliff et al., 2019; Roskin et al., 2020). At present, the knowledge is lack of influence on the CDR3 sequence of β chain of Treg cells in peripheral immune organs (spleen) and local tissues (intestine) caused by gut microbes. In this study, 16S rDNA sequencing and High-throughput sequencing were applied to detect the gut microbes composition and $CD4^+CD25^+Foxp3^+$Treg repertoire of spleen and intestine in GF, SPF, and CL mice. The aim of this study was to investigate the relationship between gut microbe composition and $CD4^+CD25^+Foxp3^+$Treg repertoire.

MATERIALS AND METHODS

Material and Sample Collection

A total of nine female BALB/c mice were purchased from the Department of experimental zoology, the basic Department of the China Third Military Medical University. Among them, there were three Germ-free (GF) mice, Specific Pathogen-free (SPF) mice and Clean (CL) mice, respectively, each weighing 21–24 g. GF, SPF, and CL mice were raised strictly according to the standard. All animals and experiments were conducted according to the guidance of animal care and use of laboratory animals (Ministry of Health, China) and approved by the experimental animal and use Ethics Committee of Zunyi Medical University. Feces of all mice were collected and sent to Beijing Genomics Institution (BGI) for sequencing of intestinal microorganisms. Spleens, intestine and mesenteric lymph nodes were collected at the time of sacrifice.

Morphological Analysis

The spleen, intestine and mesenteric lymph nodes were fixed in 4% paraformaldehyde overnight at 4°C, embedded in paraffin, sectioned into 8-μm thick slices, deparaffinized, and stained with hematoxylin and eosin (H&E). Tissue morphology was observed under a light microscope.

Sequencing of Intestinal Microorganisms

Fecal DNA was extracted as described previously (Nishijima et al., 2016). 16S rDNA obtained from the fecal extraction was analyzed by Illumina sequencing according to the 16S Metagenomic Sequencing Library Preparation (15044223 B) protocol. The experimental method of 16S rDNA sequencing was described by Watanabe et al. (2020). The DNA library was sequenced using MiSeq Reagent Kit V3 (Illumina Inc.) in the MiSeq platform according to the manufacturer's instructions. The 16S rDNA sequences were analyzed by the Quantitative Insights into Microbial Ecology (QIIME) pipeline version 1.9.1 (Caporaso et al., 2010). Through quality control, data filter out low-quality sequences, splice the sequences into tags through similarity relationship between sequences, gather tags into operational taxonomic units (OTU) based on whether they had 97% homology with the UCLUST algorithm, and then compare OTU with database (August 2013 version) to annotate species of OTU. Taxonomy from the phylum to the genus level was also performed by using the QIIME pipeline to analysis these sequences. GF mice are defined as having no detectable bacteria, fungi, protozoa and parasites in their bodies. However, one GF mouse has a certain number of gene sequencing results after PCR amplification, which may be caused by some dead microorganisms in the food after high pressure sterilization. The 16S rDNA sequences have been uploaded to NCBI repository[1].

$CD4^+CD25^+Foxp3^+$Treg Cells Isolation and RNA Extraction

Single cell suspensions were prepared by grinding the tissues with the plunger of a 5 mL disposable syringe. $CD4^+CD25^+Foxp3^+$Treg cells were obtained as described previously (Zhang et al., 2015). Briefly, $CD4^+CD25^+$Treg cells were enriched by depletion of non- $CD4^+$T cells, flow-through fraction of $CD4^+$T cells and positive selection of $CD4^+CD25^+$ cells according to the manufacture's instruction

[1]https://www.ncbi.nlm.nih.gov/bioproject/PRJNA646285/

of $CD4^+CD25^+$ Regulatory T Cell Isolation Kit (Miltenyi, Bergisch Gladbach, Germany). For the staining of Foxp3, cells were incubated with Cy-chrome-labeled anti-CD4 and FITC-labeled anti-CD25 monoclonal antibody. Then, according to the instructions provided by the company (eBioscience, San Diego, CA, United States), the cells were fixed and permeabilization with Intracellular Fixation Buffer after washing, stained with anti mouse Foxp3 monoclonal antibody. Finally, the purity of $CD4^+CD25^+Foxp3^+$Treg cells were detected by flow cytometry. Genomic DNA was extracted from $CD4^+CD25^+Foxp3^+$Treg cells using the QIAamp DNA Mini Kit (Qiagen, Milan, Italy) according to the manufacture's instruction.

TCR β Chain Library Preparation and High-Throughput Sequencing

The concentration and purity of genomic DNA of samples were conformed for TCR CDR3 sequencing[2]. And then genomic DNA were sent to Adaptive Biotechnologies Corp (Seattle, WA, United States) for sequencing. TCR β sequences were generated as our described previously (Ma et al., 2016). Briefly, a multiplex PCR amplification was performed consisting of 36 forward V segments (TRBV) and 14 reverse J segment primers (TRBJ) that targeted all possible somatic combinations of the rearranged TCR beta chain CDR3. Then, the TCR β CDR3 PCR library was loaded on an Illumina Flow Cell for sequencing on an Illumina Genome Analyzer (2 × 250 bp). Unfortunately, one of the samples failed to meet the requirements of database construction. The possible reason is that the DNA of SPF3-SP sample was degraded due to various factors, such as time and temperature during transportation, so it is impossible to conduct high-throughput sequencing through quality inspection.

Bioinformatics Analysis of TCR β-Chain Library

Raw sequences in the FASTA format were processed by Immuno-SEQ analyzer toolset and IMGT/High V-QUEST (version 1.3.1) to remove the No results and Un-known sequences as well as out of frame sequences. Using Immuno-SEQ analyzer toolset and IMGT/High V-QUEST, the characteristics of the TCR beta chain CDR3 repertoire sequences were defined, including CDR3 nucleotide, CDR3 amino acid; count (reads); frequency count (%); CDR3 length; V gene name; D gene name; J gene name; V deletion; n1 insertion; D 5′ deletion; D 3′ deletion; n2 insertion; J deletion; V index; n1 index; D index; n2 index; J index; sequence status (Has stop/in frame/out frame). TCR repertoire diversity was assessed by the Anti-Simpson index. The V-J and V-D-J rearrangement of the CDR3 repertoire, the proportion and frequency of unique CDR3 sequences, CDR3 repertoire clonality, CDR3 ANIMO ACID length, CDR3 amino acid usage, V deletion and J deletion, and dominant TRBV-TRBJ combination gene segments were also calculated in different mouse class and different tissue samples. Additionally, R package "ggplot2," "Venn Diagram," and GraphPad Prism (version 5) were used to plot the figures. Data analysis was performed by R studio (v3.3.3) and GraphPad Prism (version 5) software. $P < 0.05$ was considered statistically significant.

[2]http://www.immunoseq.com

RESULTS AND DISCUSSION

Sequencing of Gut Microorganisms

The gut microbial genome of BALB / c mice was obtained by 16S rDNA sequencing. All SPF and CL samples passed the quality inspection and established the database. For GF samples, only one passed the quality inspection. A detailed description of the total number of raw reads and filtered reads of each sample were displayed in Additional file 1. An average of 44,780 clean reads were obtained from each individual.

Germ-free mice are defined as no detectable bacteria, fungi, protozoa and parasites (GiLbert and NeufeLd, 2014) provides an irreplaceable sterile experimental model in the study of chronic gastrointestinal diseases (Grover and Kashyap, 2014), obesity (Gérard, 2017), spontaneous arthritis (Rehaume et al., 2014), and type I diabetes (Markle et al., 2013). In this experiment, a certain amount of gene sequences of gut microorganism of GF grade mice after PCR amplification may be caused by some dead microorganisms left in the feed after autoclave, the sequencing results met the definition of GF grade mice (Rodriguez-Palacios et al., 2018).

Species Diversity and Classification of Gut Microorganisms

The total and unique OTU number of each sample were showed in **Figure 1A** according to Edgar RC (Edgar, 2013). The largest number of OTUs was found in CL mice, followed by SPF mice. All mice shared 53 common OTU. In addition, the diversity of gut microorganism among different mice were analyzed by OTU rank curve (**Figure 1B**). The gut microbes of CL mice were the richest not only in quantity but also in species.

Compare with the database, OTUs were classified and the proportion of each sample species was analyzed in five classification levels, including phylum, class, order, family, genus and species (**Figure 1C**). The species classification of gut microorganisms in the GF mice was the lowest among the five levels, while the species classification of SPF and CL mice were significantly increased by compared with GF mice. Moreover, there was difference between the dominant species of SPF and CL mice in the five-species classification, and the diversity of gut microorganisms in CL mice was the most abundant.

Symbiotic microorganisms are necessary for the occurrence and development of some clinical diseases (McMurran et al., 2019; Zhu et al., 2020). Selecting the right mouse model will be beneficial to the study of specific diseases. The above results showed that the number and species of intestinal symbiotic microorganisms were different under different feeding conditions, and the dominant microflora was also different. The difference of species classification level of gut microorganisms in mice may participate in the development of immune system of

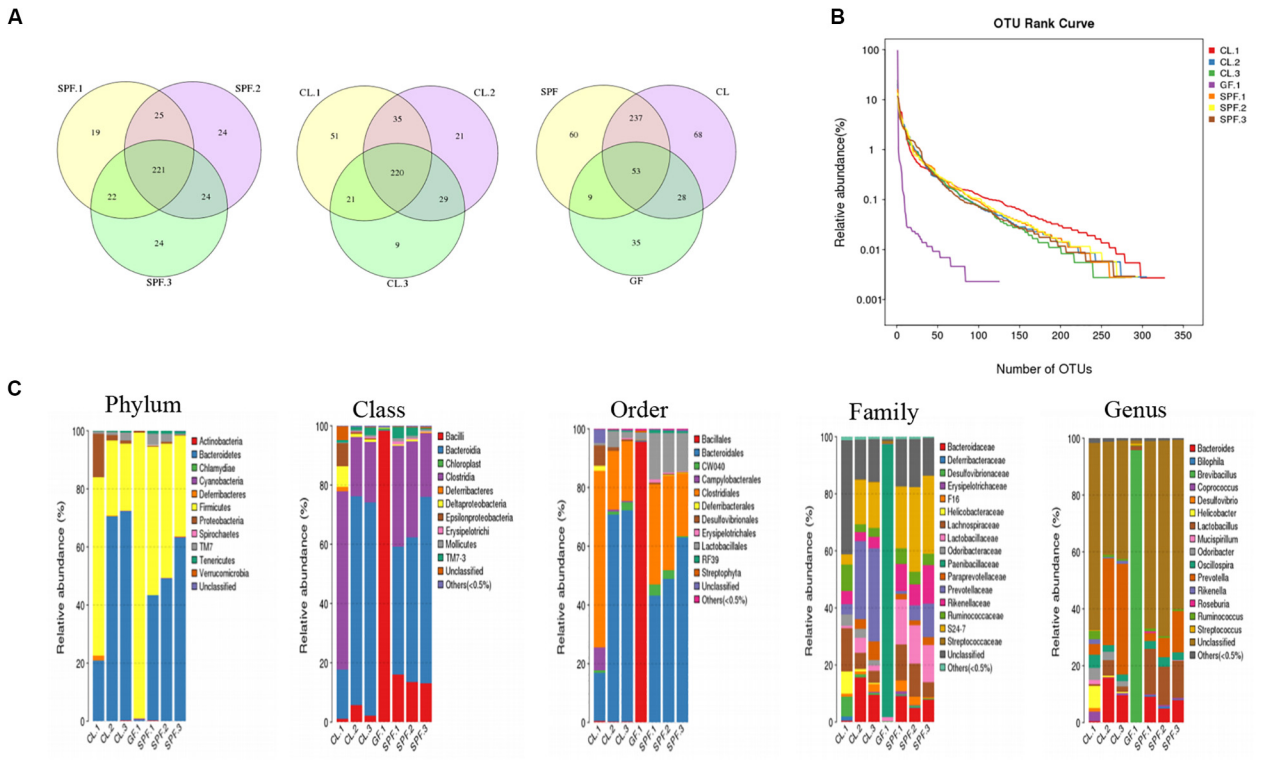

FIGURE 1 | Diversity and classification of intestinal microorganisms in three different feeding environments of mice. **(A)** Venn diagram analysis the total and unique OTU number of each sample; **(B)** OTU rank curve analysis of OTU species diversity among different mice; **(C)** Taxonomic analysis of fecal diversity in BALB / c mice. GF, Germ-free class mice; SPF, Specific Pathogen-free mice; CL, Clean Class mice.

mice in three different feeding environments in a symbiotic or pathogenic way (Caricilli et al., 2014).

Morphological Analysis of Peripheral Immune Organ

The Gut microorganism composition play an important role in the development of immune system (Martin et al., 2019). Among them, some microbial groups form an inseparable host microorganism symbiotic relationship with host physiology through natural selection and co evolution with the host (Sommer and Bäckhed, 2013). These symbiotic groups jointly affect the formation of the immune system by participating in the development and maintenance of innate and adaptive immunity and a variety of regulatory mechanisms.

In this study, the peripheral marginal zone of white pulp in GF mice was significantly smaller than that in CL mice by H&E staining (**Figure 2**). The peripheral marginal zone of white pulp contained a certain number of B and T cells (Macpherson and Harris, 2004). Therefore, we speculate that the difference of intestinal flora caused the incomplete structure of spleen, thus affecting the development of immune system in GF mice. In addition, by observing the sections of intestinal and mesenteric lymph nodes of mice of different feeding levels, we found that the angiogenesis in the center of intestinal villi of GF level mice was limited, the microvilli became longer, and the germinal center in the central area of mesenteric lymph nodes was also significantly reduced (**Figure 2**).

Sorting of $CD4^+CD25^+Foxp3^+$Treg Cells in Spleen and Intestine of Mice

Treg cells account for about 10% of the total number of peripheral T cells, which play a vital role in maintaining intestinal and peripheral tolerance and inhibiting autoimmune diseases. The expression of Foxp3 transcription factor is essential for the Treg cells development and maintenance (Hori et al., 2003; Fontenot et al., 2017). The number of $CD4^+CD25^+Foxp3^+$Treg cells in GF mice was reported significantly reduced (Hara et al., 2013). The decrease or even disappearance of intestinal flora in GF mice may have an important effect on Treg cells.

In this study, after sorting the Treg cells in the mouse intestine and spleen, the purity of the Treg cells both in spleen and intestine reached about 80% (Additional file 2), suggesting that the purity of $CD4^+CD25^+Foxp3^+$Treg cells can meet the requirements (Jenkins et al., 2015; Fuller et al., 2016).

Total Sequence Data Statistics of HTS in $CD4^+CD25^+Foxp3^+$Treg TCR β CDR3 Repertoire

$CD4^+CD25^+Foxp3^+$Treg TCR β CDR3 repertoire were successfully constructed. A detailed information of total

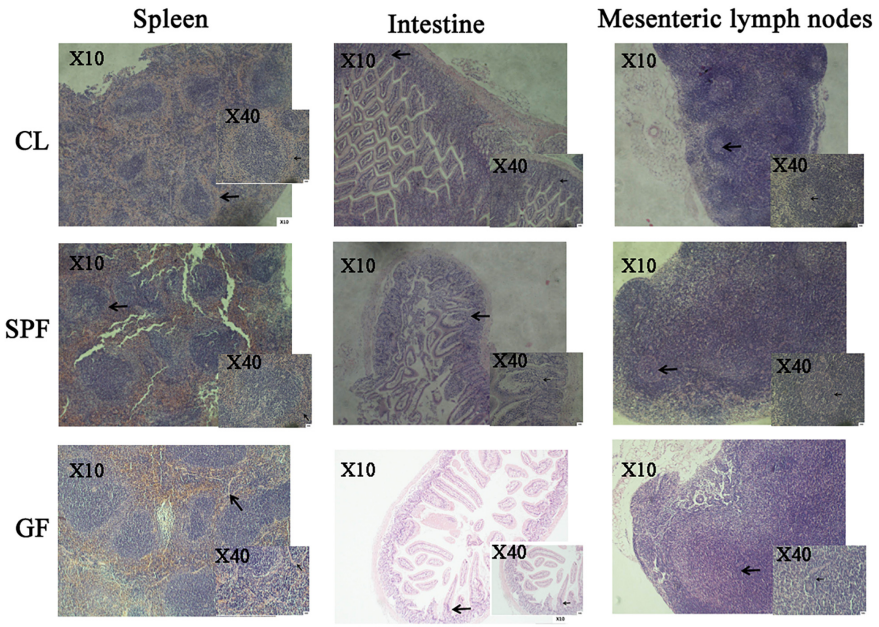

FIGURE 2 | The morphology of spleen, intestine, mesenteric lymph node in different classes of BALB/c mice by H&E staining. The arrow in the spleen picture indicates the peripheral edge of the white pulp in the spleen. The arrow in the intestine picture indicates the central villi of the small intestine. The arrow in the mesenteric lymph node image indicates the lymph node. GF, Germ-free class mice; SPF, Specific Pathogen-free mice; CL, Clean Class mice.

sequences, productive sequences, and unique sequences of each sample was displayed in **Table 1**. Unique sequence (Torella et al., 2014) is one of the key factors to analyze the specificity of the repertoire. The average value of unique sequences in intestine was 113, which is much lower than that in spleen.

TABLE 1 | Total sequences statistics of TCR beta chain CDR3 repertoire.

Sample	Total	Out and Stop of frame	In frame	Productive
CL1-In	192	58	134	126
CL1-SP	16926	4904	12022	12008
CL2-In	114	50	64	59
CL2-SP	27266	7875	19391	19351
CL3-In	44	26	19	15
CL3-SP	27126	7757	19369	19340
SPF1-In	113	53	60	58
SPF1-SP	28491	8692	19799	19772
SPF2-In	60	30	30	27
SPF2-SP	58428	16718	41710	41643
SPF3-In	997	420	577	569
GF1-In	223	122	101	99
GF1-SP	27088	8635	18453	18416
GF2-In	106	58	49	40
GF2-SP	4155	1538	2617	2604
GF3-In	195	94	101	101
GF3-SP	27879	8747	19132	19089

GF, Germ-free class mice; SPF, Specific Pathogen-free mice; CL, Clean Class mice; SP, spleen; In, intestine.

Clonal Expansion of Treg TCR β Chain CDR3 Repertoire

It is reported that the diversity of T cell receptor (TCR) can reach 2×10^6 (Nishio et al., 2015) in the peripheral of mice after massive replication. The diversity of TCRs is predominantly confined to the CDR. The CDR3 domain is in direct contact with peptide antigens and is highly diverse, allowing the recognition of various antigens (Attaf et al., 2015). The Anti Simpson Index (1 / DS) was used to evaluate the diversity of Treg TCR β chain CDR3 in BALB / c mice. The results showed that the diversity of Treg TCR β chain CDR3 repertoire in spleen was significantly higher than that in intestine of all samples (**Figure 3A**). However, there was no statistical significance through the statistical analysis of 1 / DS average distribution in different mice class of intestine and spleen, suggesting that differences in intestinal microorganisms has no obvious effect on the diversity of Treg TCR β CDR3 of intestine and spleen.

The expansive level of Treg TCR β chain CDR3 repertoire can directly reflect the immune response of T cells and can also be used for the immune reconstruction after antiviral treatment or bone marrow transplantation, as well as the detection of autoimmune diseases, including biomarkers for the treatment of type I diabetes (Kern et al., 2014; Shevyrev and Tereshchenko, 2019). The expansive level of each clonotype in intestine (**Figure 3B**) were conducted, GF mice was mainly in medium frequency (1–2%), while the CL and SPF mice were mainly in high frequency (>2%). For spleen, the clone and proliferation level of Treg TCR β CDR3 repertoire in all mice were mainly in low frequency (<0.02%), and with no statistical significance in the cloning frequency (**Figure 3C**). It is suggested that the

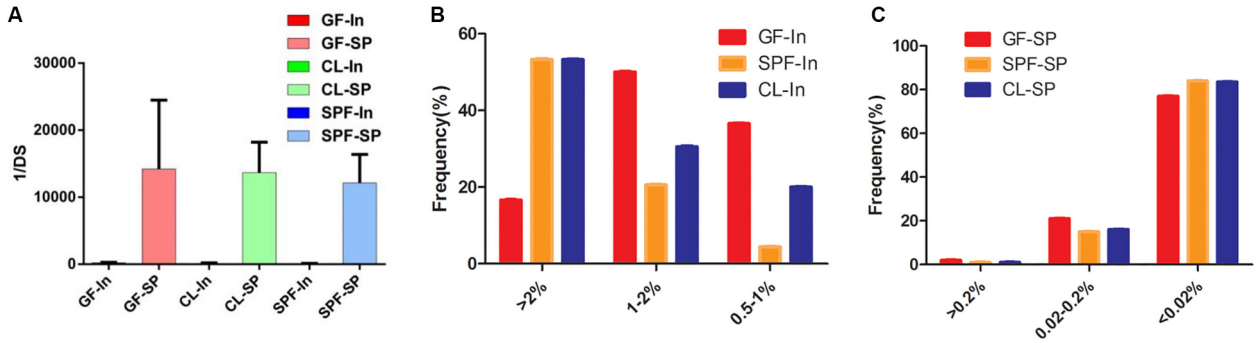

FIGURE 3 | Expansive of total Treg TCR beta CDR3 clones in spleen and intestine. **(A)** The inverse Simpson's diversity index average distribution; **(B,C)** Expansive degree in spleen and intestine. GF, Germ-free class mice; SPF, Specific Pathogen-free mice; CL, Clean Class mice. SP, spleen; In, intestine.

difference of feeding environment has no specific effect on the diversity and clonality of Treg TCR β CDR3 repertoire in the spleen of BALB / c mice.

All samples shared "YEQY" and "NTLY" amino acid motif among five sequences amplified at high frequency (Additional file 3), and the highest frequency was found in CL mice, which may be related to the stimulation of common antigen epitopes in the environment.

Usage Frequency of *V, J* and *V-J* Combination Gene Segments in TCR β Chain CDR3 Repertoires

The patterns of *TRBV* and *TRBJ* gene usage were determined dominantly by MHC alleles. The same high frequency usage *TRBV* and *TRBJ* genes were detected both in spleen and intestine among all samples, such as *TRBV19-01* and *TRBJ02-07*. Moreover, the intestine of GF mice has their own unique high-frequency usage genes, such as *TRBV13-02* and *TRBJ02-01* (**Figure 4**). Compared with GF mice, *TRBV05-01* and *TRBV01-04* were up-regulated in intestine of CL and SPF mice. However, high frequency usage *TRBV* and *TRBJ* genes do not differ in spleen among GF, SPF, and CL mice.

To further study the characteristics of *TRBV* and *TRBJ* gene usage, the expression level of the 100 most high frequently usage *TRBV-TRBJ* combinations were compared among GF, SPF, and CL mice. There are common dominant pairing genes in spleen and intestine of all mice and different group of mice also have their own unique advantage matching genes in the intestine and spleen (Additional file 4). *TRBV19-01-TRBJ02-07* and *TRBV05-01-TRBJ02-07* were dominant combinations identified in intestine and spleen, respectively in all mice.

Between-individual similarities in usage of V, J, and V-J combinations gene segments may stem from chromatin conformation, physical proximity of germline segments, and/or recombinatorial bias (Pickman et al., 2013). Selective bias of V and J gene usage may lead to a loss of diversity in the intestinal TCR repertoires and result in occurrence of some diseases, such as inflammatory bowel disease (Saravanarajan et al., 2020). Although the species of symbiotic bacteria have changed in different environmental levels, there is still a common specific antigen. Therefore, the same high frequency of *TRBV* and *TRBJ* gene family of Treg TCR β CDR3 repertoire in the gut and spleen of mice were found, and some specific high frequency of usage may be related to the change of specific symbiotic bacteria after the change of environmental level.

Amino Acid Analysis of TCR Beta Chain CDR3 Repertoire

Different types of T cells have different length distribution of CDR3 and can combine with different MHC complexes. It has been confirmed that the CDR3 length distribution of $CD4^+$ T cells is significant different in patients with IgG4-related disease (Wang et al., 2019). The distribution of amino acid length in all samples was similar, and the distribution of amino acids length was concentrated between 8 and 16 amino acids (**Figure 5A**). All mice formed bell-shaped distribution, which peaks at 12 amino acids, advocating that the change of intestinal flora does not affect the distribution of CDR3 amino acids sequence of Treg TCR β chain in spleen and intestine.

Moreover, hydroxy hydrophilic amino acids were taken at high frequency in the CDR3 region of Treg TCR β chain in spleen and intestine represented by serine (**Figure 5B**). However, no significant statistical significance was found among the samples after statistical analysis. These results suggested that the difference of environment has no specific effect on the high frequency use of amino acids in the CDR3 region of Treg TCR β chain in the gut and spleen of BALB / c mice.

The same amino acid overlapping sequence did not appear in the intestine among 3 groups but was found in the spleen (**Figure 5C**). The same amino acid overlapping sequence in the spleen may be the natural Treg (Cebula et al., 2013) derived from mouse thymus, which is not related to the specific antigen stimulation in the environment.

The cleavage and insertion of CDR3 region of Treg TCR β chain have been reported to be an important mechanism for the formation of CDR3 region diversity (Toor et al., 2016). However, our comparative analysis of the cleavage and insertion of CDR3 region of Treg TCR β chain in spleen and intestine of all experimental samples showed that nucleotide insertion and deletion occur at the junction of V, D, and J gene segments

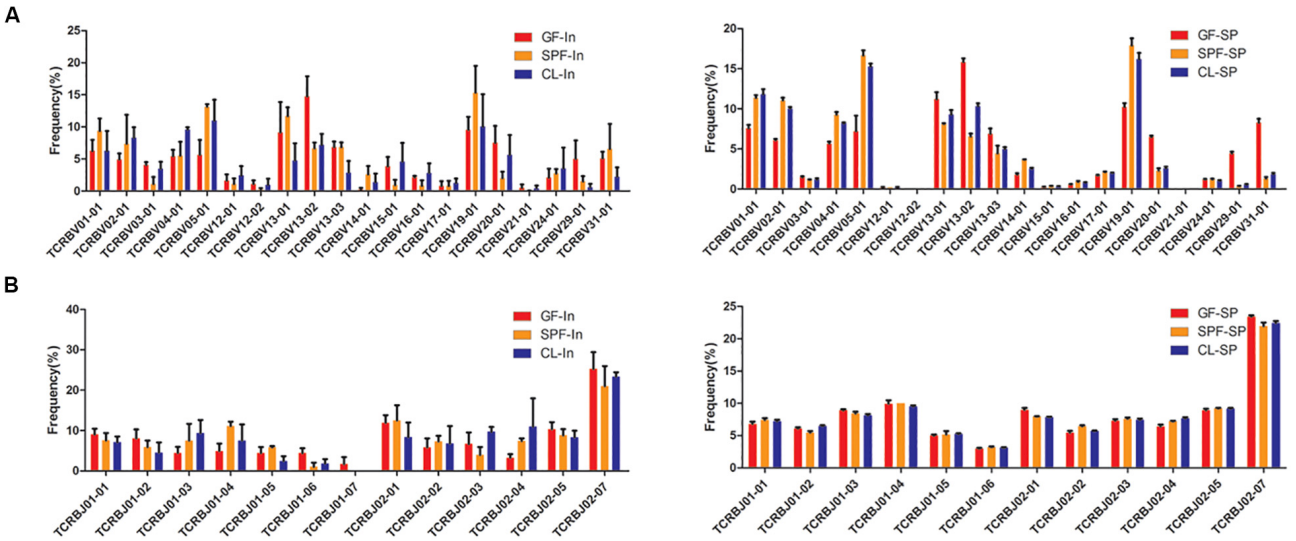

FIGURE 4 | Distinct usage percentages of the total *TRBV* and *TRBJ* genes in mice spleen and intestine. **(A)** usage percentages of the TRBV; **(B)** usage percentages of the TRBJ. GF, Germ-free class mice; SPF, Specific Pathogen-free mice; CL, Clean Class mice. SP, spleen; In, intestine.

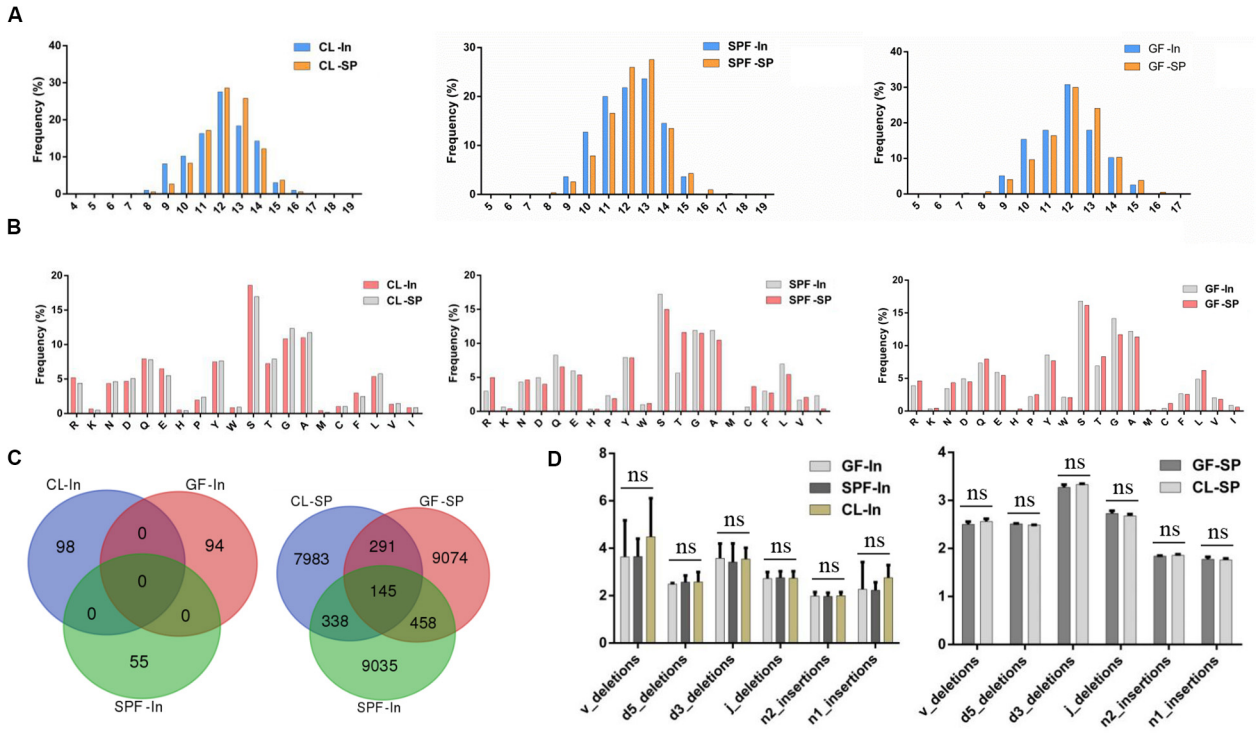

FIGURE 5 | Amino acid analysis of TCR beta chain CDR3 repertoire. **(A)** Length distribution of TCR beta chain CDR3 repertoire in spleen and intestine; **(B)** Top 20 AA usage distribution of Treg TCR beta chain CDR3 repertoire in spleen and intestine; **(C)** overlap of Treg TCR beta chain CDR3 regions amino acid sequences in spleen and intestine. **(D)** Statistical analysis of the distribution of TCR-β CDR3 region nucleotides deletion and insertion in spleen and intestine. GF, Germ-free class mice; SPF, Specific Pathogen-free mice; CL, Clean Class mice. SP, spleen; In, intestine.

did not significant difference among GF, SPF, and CL mice (**Figure 5D**), which may be caused by sample size. Therefore, future investigations will be needed to verify the associations in the larger population.

CONCLUSION

In this study, we evaluated the correlation between the differences of intestinal microflora and the heterogeneity of

$CD4^+CD25^+Foxp3^+$Treg TCR β CDR3 repertoire by analyzing the differences of intestinal microflora in different feeding environments and sequencing $CD4^+CD25^+Foxp3^+$Treg cell in the intestine and spleen. GF, SPF, and CL mice have different intestinal microbial composition, which may be related to the development and composition of different peripheral immune tissues and organs. In addition, the significant difference of Treg TCR β CDR3 repertoire among GF, SPF, and CL were found only in intestine, indicating that different intestinal microbial composition may mainly affect the diversity of intestinal Treg TCR β CDR3 repertoire. However, the mechanism of intestinal microbes regulating intestinal immune system is still unknown, and further confirmatory investigations will be needed to conduct the effects of intestinal microorganisms and metabolites on intestinal immune cells.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by all animals and experiments were conducted according to the guidance of animal care and use of laboratory animals (Ministry of Health, China) and approved by the Experimental Animal and Use Ethics Committee of Zunyi Medical University.

AUTHOR CONTRIBUTIONS

XY designed the research. JL and HX did the experiment and wrote the manuscript. QM, XH, LM, BS, and SS analyzed parts of the data. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.576445/full#supplementary-material

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REVIEW
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A Novel Insight at Atherogenesis: The Role of Microbiome

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There is an important task of current medicine to identify mechanisms and new markers of subclinical atherosclerosis in order to develop early targets for the diagnosis and treatment of this disease, since it causes such widespread diseases as myocardial infarction, stroke, sudden death, and other common reasons of disability and mortality in developed countries. In recent years, studies of the human microbiome in different fields of medicine have become increasingly popular; there is evidence from numerous studies of the significant contribution of microbiome in different steps of atherogenesis. This review attempted to determine the current status of the databases PubMed and Scopus (until May, 2020) to highlight current ideas on the potential role of microbiome and its metabolites in atherosclerosis development, its mechanisms of action in lipids metabolism, endothelial dysfunction, inflammatory pathways, and mitochondrial dysfunction. Results of clinical studies elucidating the relationship of microbiome with subclinical atherosclerosis and cardiovascular disease considered in this article demonstrate strong association of microbiome composition and its metabolites with atherosclerosis and cardiovascular disease. Data on microbiome impact in atherogenesis open a wide perspective to develop new diagnostic and therapeutic approaches, but further comprehensive studies are necessary.

Keywords: microbiome, atherosclerosis, inflammation, TMAO, mitochondria, lipoproteins

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INTRODUCTION

Today atherosclerosis remains one of the most important problems of current medicine, since it causes such widespread diseases as myocardial infarction, stroke, sudden death, and other common causes of disability and mortality in developed countries. The development of atherosclerosis has a long asymptomatic phase; the first clinical manifestations appear with a significant vascular lesion. Despite the fact that modern science understands the basic mechanisms of atherogenesis, individual factors, which determining the variability of the atherosclerosis progression, are still not well studied. In this regard, it is an important task to identify mechanisms and new markers of subclinical atherosclerosis in order to develop early targets for the diagnosis and treatment of this disease. Therefore, the need to search for additional factors of the pathogenesis of this disease remains relevant (Orekhov and Ivanova, 2017).

In recent years, studies of the human microbiome in different fields of medicine have become increasingly popular (Huttenhower et al., 2012). The relationship of the gut microbiome is shown

with autoimmune, inflammatory (Belkaid and Hand, 2014; Opazo et al., 2018), neurodegenerative (Endres and Schäfer, 2018), infectious diseases (Lazar et al., 2018), and cancer (Villéger et al., 2019). Studies of microbiome to understand its relationship with cardiovascular diseases, in particular, mechanisms of atherogenesis has great potential (Kazemian et al., 2020).

The gut microbiome is the first largest and most complex community of microorganisms in the human body, which consists of bacteria, viruses, archaea, fungi, protozoa, and bacteriophages. It is an ecological community of microorganisms inhabiting the gastrointestinal tract along its entire length consists of a trillion bacteria and is encoded by more than 100 times more genes than the human genome (Mills et al., 2019). The gut microbiome is an important component of homeostasis involved in physiology and metabolism in human body (Sommer and Bäckhed, 2013). It participates in the digestion processes, providing fermentation of undigested substances, the synthesis of certain vitamins, energy metabolism, and regulation of the intestinal barrier function (Ahmad et al., 2019; Derovs et al., 2019). Recent studies have shown the key role of the microbiome in the functioning of the human immune system (Belkaid and Harrison, 2017). The composition of the intestinal microbiota depends on a number of factors, such as nutrition, lifestyle, gender, age, and the use of antibiotics (Jandhyala et al., 2015; Kundu et al., 2017).

This review reflects current ideas about the role of the microbiome in the processes of atherogenesis. Using the electronic databases PubMed and Scopus, a search was conducted for the keywords "microbiome," "atherosclerosis," "metabolites of the microbiome," "TMAO" until May 2020. References' lists of the identified reviews and original research articles were hand-selected for papers those may have been missed during primary search. Only articles published in English were selected. We found about 500 articles and focused on the studies on the influence of the microbiome on the pathogenesis of atherosclerosis.

THE EFFECT OF MICROBIOME ON BASIC PATHWAYS OF ATHEROGENESIS

Numerous experimental and clinical studies investigate the role of microbiome and its metabolites in different steps of atherosclerosis development. Further in this article we will consider the main effects of the microbiome on lipid metabolism, inflammation, endothelial dysfunction, mitochondrial dysfunction during atherogenesis. **Figure 1** represents a brief scheme of microbiome effects in basic mechanisms of atherogenesis.

Microbiome and Lipid Metabolism

Lipid metabolism disturbance is the initial stage and one of the main reasons of atherosclerosis development, which is manifested in lipid accumulation in vessel wall (Pieczynska et al., 2020). The gut microbiota may disturb lipid metabolism both in mice and humans. Microbiome composition may further correlate with atherosclerotic lesion progression (Schoeler and Caesar, 2019). Intestinal microorganisms can modulate human metabolism at the level of small molecules, including the conversion of food components into hormone-like signals and biologically active metabolites. About 10% of small molecules circulating in the bloodstream have the origin of intestinal microbiome (Herrema et al., 2020). One of such biologically active metabolites of the intestinal microbiota, which may be involved in the development of atherosclerosis, is trimethylamine N-oxide (TMAO).

The increasing level of TMAO in plasma is correlated with the lipid deposition in blood vessels and endothelial dysfunction (Hardin et al., 2019). TMAO also may impair cholesterol reverse transport, which is promoted by bile acid biosynthesis (Sun et al., 2016). Bile acids can be ligands for nuclear farnesoid X receptor (FXR), which mediates the expression of genes regulating bile acid biosynthesis and excretion (Lefebvre et al., 2009). Members of cytochrome P450 superfamily, cholesterol 7 alpha-hydroxylase (CYP7A1) and cholesterol 27 alpha-hydroxylase (CYP27A1), are important bile acid synthetic enzymes responsible for maintaining cholesterol metabolism homeostasis (Sayin et al., 2013; Lau et al., 2017). Defective metabolism of bile acids and cholesterol is associated with atherosclerosis. Recently, the link of TMAO-induced atherosclerosis with bile acid metabolism was studied in apoE−/− mice on TMAO-containing diet (Ding et al., 2018). Specifically, it was elucidated that TMAO can repress hepatic bile acid synthesis pathway by activation of small heterodimer partner (SHP) and FXR, which can down-regulate of Cyp7a1 and Cyp27a1 gene expression (Koeth et al., 2013; Ding et al., 2018). Moreover, it was demonstrated that the levels of triglyceride, total cholesterol, low-density lipoprotein cholesterol (LDL-c), as well as, total plaque areas in the aortas in TMAO administration mice were significantly increased (Ding et al., 2018).

Human studies revealed the association of altered microbiota, represented mostly by some taxa from phylum Actinobacteria, with increased triglyceride level, decreased high-density lipoprotein cholesterol (HDL-c), and slightly changed LDL-c level. HDL mediates efflux of cholesterol from cells and then transports it to liver to be excreted from an organism (Fisher et al., 2012). It was elucidated in two studies on obese and healthy European individuals, that elevated fasting triglycerides and diminished HDL-c are associated with the low abundance of the gut microbiota (Cotillard et al., 2013; Nielsen et al., 2013). Furthermore, as a result of the systemic analysis of blood lipid levels, body mass index, and gut microbiota, conducted in 893 participants from the LifeLines-DEEP cohort, it was suggested that the gut microbiota may influence only certain aspects of lipid metabolism and distinct classes of lipoproteins, such as HDL and very low-density lipoproteins (VLDL) (Fu et al., 2015). Enrichment in Bacteroides, Eubacterium, and Roseburia microbes, which are common microbiota in healthy people, can correlate with decrement of body mass index and plasma triglycerides and increment of plasma HDL (Nakaya and Ikewaki, 2018). In addition, the full suppression of gut microbiota may enhance macrophage-to-feces reverse cholesterol transport (Canyelles et al., 2018). However, there are a lot of blind spots in the TMA/FMO3/TMAO/bile acids axes in human.

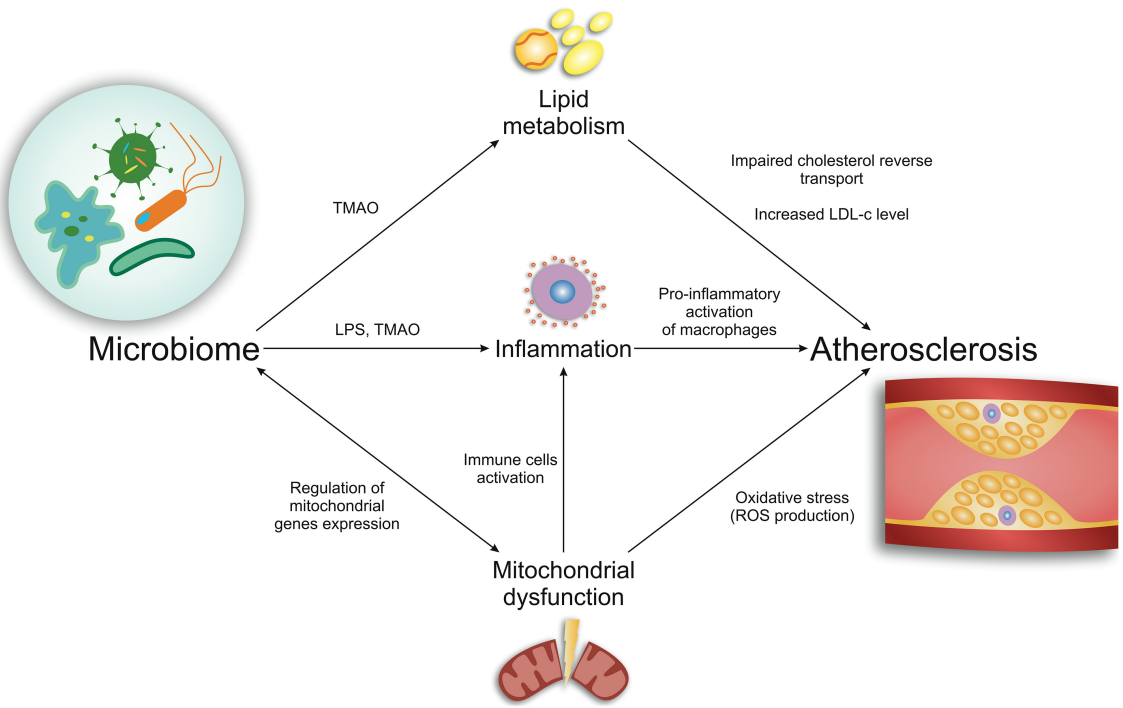

FIGURE 1 | Brief scheme of microbiome effects in basic mechanisms of atherogenesis.

Thus, further studies that help to clarify the exact role of gut microbiome and TMAO in cholesterol and bile acid metabolism in human, are necessary.

Microbiome and Inflammation

Atherosclerosis is a chronic inflammatory disease, which is characterized by the accumulation of monocytes and lymphocytes within the arterial wall, the release of chemokines, interleukins, and activation of pro-inflammatory pathways within endothelial cells (Libby, 2012; Tuttolomondo et al., 2012; Nikiforov et al., 2019). Possible association of gut microbiome composition, diet, atherosclerosis, and inflammation is under thorough investigation (van den Munckhof et al., 2018). For example, saturated fat rich diet can dramatically change the gut microbiome composition, elevating the intestinal absorption of gut-derived microbial products that lead to an increased concentration of plasma lipopolysaccharide (LPS), which is called metabolic endotoxemia (Sonnenburg and Bäckhed, 2016). The activation of inflammatory signaling pathways in the intestine is one of the factors determining the influence of the gut microbiome on the development of atherosclerosis. LPS, the main component of the outer membrane of gram-negative bacteria, can penetrate the bloodstream as a result of the intestinal mucosa barrier function disturbance (Ahmad et al., 2019). Consequently, metabolic endotoxemia provokes macrophage activation via TLR4/MyD88/TRIF signaling (Caesar et al., 2015). TLR4/MyD88/TRIF is activated by the lipid A component of LPS and this causes inflammatory signaling pathways (Herrema et al., 2020; Caesar et al., 2015). Thus, microbiome alteration based on a diet type may influence on pro-inflammatory activation, which is correlated with atherosclerosis since pro-inflammatory macrophages are one of the key cells in atherogenesis. The role of microbiome composition in pro-inflammatory status was further demonstrated in mice on high-fat diet, which is positively correlated with metabolic endotoxemia and endothelial dysfunction (Toral et al., 2014). It was shown, that *Lactobacillus coryniformis* administration as a probiotic promoted the increase in the intestinal mucus glycoprotein mucin-3, which restores gut barrier function and subsequently decreases plasma LPS level. Moreover, *L. coryniformis* administration lowered IL-6 and IL-1β expression in aorta of these mice. In addition, it was elucidated in germ free mice that the absence of microbiota has beneficial effect on arterial lesion and helps to decrease inflammatory cytokines in macrophages (Kasahara et al., 2017).

Microbiome and Mitochondrial Dysfunction

Genetic predisposition to atherosclerosis development is widely studied nowadays. Mitochondrial dysfunction is one of the possible genetic factors that may lead to the progression of atherosclerosis (Sazonova et al., 2017; Orekhov et al., 2020). In previous studies the association of mitochondrial heteroplasmy and atherosclerosis was demonstrated. Several variants of mitochondrial heteroplasmy were found in atherosclerotic lesions of human aorta (Sazonova et al., 2015; Sobenin et al., 2019) as well as the correlation of these mitochondrial mutations in white blood cells with carotid atherosclerosis was demonstrated in some clinical trials (Kirichenko et al., 2018,

2020a). Inflammatory changes leading to the progression of atherosclerosis, the manifestation of CVD and the development of their complications, develop with age due to many factors, including oxidative stress caused by mitochondrial dysfunction as well as inflammatory processes caused by changes in the composition of the microbiota described above (Ostan et al., 2016; Ferrucci and Fabbri, 2018). At the same time, association of mitochondrial dysfunction and alterations of gut microbiota are studied in several trials (Ma et al., 2014; Hirose et al., 2017). It was demonstrated in subjects with Crohn's disease that downregulation of mitochondrial proteins, which indicates the central role of mitochondria dysfunction in the pathogenesis of inflammatory bowel disease, is associated with a depletion of butyrate producers, suggesting a signaling role for butyrate in host mitochondrial genes expression (Mottawea et al., 2016). It is assumed that gut microbiota signaling to mitochondria leads to the activation of immune cells and inflammation, and changes the function of the epithelial barrier (Jackson and Theiss, 2019). It was proposed that production of toxins by dysbiotic gut microbiome activates neuronal innate immunity and inflammatory processes that lead to the development of neurodegenerative processes in patients with Parkinson's disease (Cardoso and Empadinhas, 2018). As for the possible relationship of the microbiome and mitochondria in the pathogenesis of atherosclerosis, the mechanisms of their interaction are not well studied at present. However, we can assume the combined influence of these factors on the development of atherosclerosis, since mitochondria are bacterial in origin, and therefore microbiome products can affect their functions, stimulating processes that play an important role in atherogenesis (Clark and Mach, 2017; Bajpai et al., 2018). In particular, gut microbiome influences the production of reactive oxygen species (ROS) in mitochondria and regulates their inflammatory activity (Saint-Georges-Chaumet and Edeas, 2016; Clark and Mach, 2017). Overproduction of ROS stimulates inflammatory reactions which affect the development of atherosclerosis (Forrester et al., 2018). On the other hand, in mice, engineered for overproduction of ROS in mitochondria and in aged mice, the composition of the intestinal microbiota changed compared to the initial one, Shannon diversity significantly decreased, that demonstrates the reverse effect of mitochondrial dysfunction on microbiome. At the same study, in mice treated with N-acetylcysteine or engineered to produce more mitochondrial catalase, ROS production decreased significantly that led to significant increase in Shannon diversity (Yardeni et al., 2019). So, the microbiome-mitochondria interaction may be an important factor in mechanisms of atherogenesis, but further studies are required.

THE ROLE OF MICROBIOME METABOLITES IN THE DEVELOPMENT OF ATHEROSCLEROSIS

TMAO Metabolism

The prevailing phyla of bacteria compounded the gut microbiome are the Actinobacteria, Bacteroides, Firmicutes, Proteobacteria, and Verrucomicrobiota. The balance between Bacteroides and Firmicutes may determine the health of gastrointestinal tract (Jama et al., 2019). Shift to Firmicutes domination may lead to dysbiotic environments, which is beneficial to TMAO accumulation. TMAO is produced in liver by flavin-containing monooxygenase 3 (FMO3) from the precursor trimethylamine (TMA), which is generated in gut from metabolic intermediates incorporating the TMA-moiety, such as betaine, γ-butyrobetaine, choline, phosphatidylcholine, and L-carnitine (Chen K. et al., 2017). TMA lyases are gut microbe enzymes, which convert choline to TMA. An inhibition of these lyases may reduce atherosclerosis in mice (Canyelles et al., 2018).

Circulating plasma TMAO levels are determined by several factors, including the consumption of its metabolic precursors, medication, and liver flavin monooxygenase activity (Janeiro et al., 2018). The level of TMAO largely depends on the composition of the intestinal microbiome. The predominant bacterial types are Bacteroides and Firmicutes, and Actinobacteria, Proteobacteria, and Verrucomicrobia are less common. The splitting processes of the main source of TMAO, choline, are regulated by the intestinal microbiota, in particular, the phylum Firmicutes and Proteobacteria, and the genera *Anaerococcus hydrogenalis*, *Clostridium asparagiforme*, *Clostridium hathewayi*, *Clostridium sporogenes*, *Escherichia fergusonii*, *Proteus penneri*, *Providencia rettgeri*, and *Edwardsiella tarda*. The main microbial species responsible for the degradation of another TMAO source of L-carnitine are the phylum Proteobacteria and Bacteroides and the family Prevotellaceae (Zhu et al., 2020).

Recent clinical studies confirm the close relationship of atherosclerosis with the level of TMAO. Increased plasma TMAO levels in patients are associated with an increased risk of adverse cardiovascular events such as stroke, heart attack, and death (Tang et al., 2013). It was found in the study on patients with acute coronary syndrome (ACS) that elevated TMAO value is a high predictor of long-term mortality risk (Senthong et al., 2016b).

TMAO and Foam Cells

The early stage of atherosclerosis is the accumulation of foam cells in the intima of the arteries. Most foam cells come from macrophages that can regulate lipoprotein metabolism (Poznyak et al., 2020). The association of TMAO with stimulation of atherosclerosis progression was first demonstrated in animal models where TMAO led to the formation of foam cells in atherosclerotic lesions by increasing expression of the CD36 and SR-A1 scavenger receptors (Wang et al., 2011). It is known that the main reason for the formation of foam cells is the excessive influx of modified LDL and the accumulation of cholesterol esters in macrophages. Macrophages express various CD36 and SR-A1 scavenger receptors, as well as the lectin-like receptor oxLDL-1 (LOX-1), with affinity to oxidized low-density lipoproteins (oxLDL). This in turn induces the formation of foam cells (Chistiakov et al., 2017). It has been shown that macrophage foam cell formation was increased in apoE−/− mice on TMAO-, choline-, or L-carnitine-fed diets (Wang et al., 2011). Up-regulation of macrophage scavenger receptors CD36 and SR-A by TMAO is one the plausible mechanisms

underlying this process (Koeth et al., 2013). TMAO is probably involved in the process of atherogenesis, contributing to the migration of macrophages, the accumulation of ox-LDL in them, and their transformation into foam cells (Mohammadi et al., 2016; Janeiro et al., 2018). In addition, studies revealed that TMAO positively regulates the expression of VCAM-1 in endothelial cells, leading to an increase in monocyte adhesion, which is an early sign of foamy cell formation (Ma et al., 2017).

Effect of TMAO on Inflammation and Endothelial Dysfunction

Trimethylamine N-oxide is believed to enhance atherosclerosis by influencing inflammatory processes in vascular wall by activating the inflammatory pathway (Ahmad et al., 2019). The processes of inflammation are involved in all stages of the development of atherosclerosis, from the initial lesions to the terminal stage of thrombotic complications. Most often, the processes begin with inflammatory changes in the vascular endothelium, characterized by the expression of VCAM-1 and monocyte adhesion (Ma et al., 2017). Elevating plasma level of TMAO is associated with the high risk of atherosclerosis and cardiovascular disease due to chronic inflammation and the recruitment of leukocytes to endothelium (Al-Obaide et al., 2017). TMAO promotes macrophage migration and an increase in the expression of inflammatory cytokines such as TNF-α and IL-6 and a decrease of the expression of the anti-inflammatory cytokine IL-10 (Zhu et al., 2020). In response to inflammatory activation, macrophages can penetrate the vascular endothelium and accumulate in the intima, thereby causing the formation of plaques (Chistiakov et al., 2017). It was shown that an increase in TMAO levels led to the activation of a mitogen-activated protein kinase (MAPK), a signaling cascade of nuclear factor-κB (NF-κB), and also to over-expression of pro-inflammatory genes, inflammatory cytokines, adhesion molecules, and chemokines, so TMAO elevation stimulates the inflammatory response within endothelial and smooth muscle cells (Seldin et al., 2016). In addition, TMAO can trigger vascular inflammation by the NLRP3 mechanism – inflammasome is an IL-1β family cytokine-activating protein complex, consisting of the pattern recognition receptor NLRP3, adaptor protein apoptotic speck-like protein, and inactive pro-caspase-1, and involved in the regulation of inflammatory response (Chen M. L. et al., 2017). It was also shown, that TMAO can activate inflammation via NOD-like receptor family and may lead to NLRP3 inflammasome activation through the SIRT3-SOD2-mtROS signaling pathway in human umbilical vein endothelial cell and aortas from apoE−/− mice. It was demonstrated at the same study that TMAO may induce the expression of cyclooxygenase-2 (COX-2), IL-6, E-selectin, and intercellular adhesion molecule-1 (ICAM-1) in primary human aortic endothelium cells. It can also promote production of COX-2, IL-6, TNF-α, and ICAM-1 in vascular smooth muscle cells (Matsumoto et al., 2020).

In addition to the activation of NLRP3 – inflammasome, TMAO can affect the processes leading to endothelial dysfunction, which is also an important factor in the development of atherosclerosis. In experiments on cell cultures, it has been shown that TMAO can induce oxidative stress and activate NLRP3 – inflammasome, results in the release of inflammatory cytokines, and reduce the level of endothelial nitric oxide synthase (eNOS) and the production of nitric oxide (NO) (Sun et al., 2016). Intracellular inflammation and oxidative stress reactions lead to the formation of ROS and a decrease in NO. In addition, this leads to inhibition of the function of circulating endothelial progenitor cells (EPCs), which also has a negative effect on maintaining the function of the vascular wall. All these pathophysiological processes ultimately lead to endothelial dysfunction and the development of atherosclerosis (Chou et al., 2019).

TMAO in the Processes of Thrombosis

The effect of TMAO on platelet activation processes and following thrombus formation, which play an important role in atherosclerotic events, has been shown in several studies (Kasahara and Rey, 2019). A dose-dependent effect was revealed between the increased content of TMAO in blood plasma and platelet hyperreactivity. Most likely, this is due to the influence of TMAO on the release of intracellular Ca^{2+} stores from platelets and their activation as a result of stimulation of ADP, thrombin, collagen, arachidonic acid (Zhu et al., 2016).

Other Microbiome Metabolites

Besides TMAO, which makes a significant contribution to the development of atherosclerosis, there are other biologically active metabolites of the gut microbiota. One of them is short chain fatty acids (SCFA), such as acetate, butyrate and propionate, resulting from the fermentation of undigested dietary fiber. They play an important regulatory role, act as substrates in cholesterol and lipid metabolism (Chen et al., 2020; Zeng and Tan, 2020). SCFA have anti-inflammatory effects by inhibiting the migration and proliferation of cells of the immune system and the production of cytokines, which can weaken the progression of atherosclerosis (Ohira et al., 2017).

Other metabolites of the gut microbiome are aromatic amino acids. The metabolism of phenylalanine, tyrosine, tryptophan, and histidine leads to the formation of compounds that can also affect the development of cardiovascular diseases. For example, tryptophan metabolism results in indole ethanol (IE), indolepropionic acid (IPA) and indole acrylic acid (IA), which are absorbed through the intestines and have anti-inflammatory activity, which can be a beneficial for CVD development (Kasahara and Rey, 2019). Other uremic toxins, indoxyl sulfate and p-cresyl sulfate, are metabolites derived from tyrosine, phenylalanine, and tryptophan by gut microbiota (Nallu et al., 2017; Pereira-Fantini et al., 2017). Indoxyl sulfate is produced in liver from the *Escherichia coli* metabolite indole. Indoxyl sulfate can activate IL-6 expression in both endothelium and smooth muscle cells via organic anion transporters/aryl hydrocarbon receptor/NF-κB pathway that has been elucidated in mice studies (Matsumoto et al., 2020). It has also been studied that indoxyl sulfate can increase ICAM-1, TNFα, and monocyte chemoattractant protein-1 (MCP-1) expression (Tumur et al.,

2010). Elevated plasma p-cresyl sulfate is a risk factor of cardiovascular disease in patients with chronic kidney disease, since it can up-regulate MCP-1, ICAM-1, TNF-α, and vascular cell adhesion molecule-1 expression in endothelium cells. It was also demonstrated that increased p-cresyl sulfate level may promote atherosclerotic lesion in apoE−/− mice with chronic kidney disease (Jing et al., 2016). Furthermore, it was shown in human study, that oat and barley beta-glucans, prebiotic fibers with proven cholesterol-lowering activity, significantly reduced LDL and total cholesterol, and serum p-cresyl sulfate levels, which may have positive effect on endothelial function (Cosola et al., 2017).

Circadian Rhythms of Microbiome and Atherogenesis

It is believed that circadian rhythms affect the development of atherosclerosis since immune cell activity depends on the circadian clock (Winter and Soehnlein, 2018). Conventional cardiovascular risk factors such as blood pressure, pulse rate, endothelial function are also subjected to diurnal variation (Kawano et al., 2002; Paschos and FitzGerald, 2010). The composition of gut microbiota, which is associated with atherogenesis, is also characterized by diurnal variations. However, it is still not entirely clear how the circadian clock and gut microbiota, in particular bacterial metabolites such as TMAO, are associated with the progression of atherosclerosis. Clock (Circadian Locomotor Output Cycles Kaput) and Bmal1 [Brain and Muscle ARNT (aryl hydrocarbon receptor nuclear translocator) -Like 1], two major circadian clock genes, play important regulatory role in atherogenesis, expression of Bmal1 and Clock decreased in patients with atherosclerosis (Winter and Soehnlein, 2018; Man et al., 2020). TMAO induces increased expression of lncRNA-NEAT1, Clock and Bmal1 and inhibits the MAPK pathways (Wu et al., 2019a). Some researchers suggest that pro-atherosclerotic changes in the composition of the microbiota are due to the influence of toxic products, in particular, the environmental pollutant acrolein. Acrolein exposure increased MMP9, decreased Clock and Bmal1, and activated MAPK-pathways in human umbilical vein endothelial cells in plasma of apolipoprotein-E deficient mice fed a high fat diet with acrolein. At the same, in this model feeding with acrolein changed the composition of the gut microbiota – increase of Firmicutes and decrease of Bacteroidetes and these changes correlated with atherosclerotic plaque, MMP9 and Bmal1 levels (Wu et al., 2019b). Some analogy in the change in daily fluctuations in the composition of the intestinal microbiota was found in the study on a mouse model of diabetes. Rhythmic oscillations of gut microbiota were observed in diabetic mice as well as analysis of circulating metabolites showed changes in the daily concentration of metabolites of the histidine pathway and the metabolism of betaine, methionine, cysteine, followed by an increase in TMAO production (Beli et al., 2019). Moreover, in the study of atherosclerosis, associated with infection of *P*orphyromonas *gingivalis* that causes periodontitis, circadian clock disruption enhanced atherosclerosis progression in Bmal1−/− ApoE−/− mice (Xie et al., 2020). Despite of a number of studies on the influence of circadian rhythms on atherogenesis and the composition of gut microbiome and its metabolites, the mechanism of the influence of diurnal variations in the microbiome on the development of atherosclerosis is still an actual topic for research in the near future.

THE ASSOCIATION OF HUMAN MICROBIOME WITH ATHEROSCLEROSIS AND CVD

It is known that one of the surrogate markers and an indicator of early atherosclerosis, widely used in epidemiological and interventional studies, is the carotid intima-media thickness (cIMT) measured in common carotid arteries by high-resolution ultrasonography (Baldassarre et al., 2010; Kirichenko et al., 2020b). On the other hand, there is increasing evidence showing the association of TMAO with

TABLE 1 | Clinical evidences on association of TMAO with atherosclerosis and CVD.

Study design	Results	References
TMAO and cIMT		
CARDIA study: 817 patients aged 33–45 years	No significant correlation of TMAO level with cIMT	Meyer et al., 2016
TULIP study: 220 participants with BMI > 27 kg/m^2 or previous diagnosis of impaired glucose tolerance; 9-months lifestyle improvement (diet and sports)	TMAO level correlated with cIMT at baseline ($p < 0.0001$) and was associated with cIMT increase ($p = 0.03$) as well as age ($p < 0.0001$) and visceral fat mass ($p = 0.0001$)	Randrianarisoa et al., 2016
175 CVD-free HIV-infected patients	No significant correlation TMAO and cIMT; Trend toward higher TMAO level in patients with cIMT > 0.9 mm ($p = 0.087$)	Montrucchio et al., 2020
TMAO in patients with CAD		
353 patients with evidence of significant coronary artery disease defined by diameter stenosis ≥50% in vessels ≥1.5 mm	TMAO level correlated with coronary atherosclerosis measured by angiography using SYNTAX score ($p < 0.0001$)	Senthong et al., 2016b
4007 participants mean aged 63 years old at least single-vessel CAD, 3 years of follow-up	TMAO level was a strong predictor of increased risk of myocardial infarction, stroke and death ($p < 0.001$)	Tang et al., 2013
1612 patients in the Swiss ACS Cohort	TMAO levels were associated with a graded increase (log rank $p < 0.001$) in risk of major adverse cardiac events	Li et al., 2017
1-year prospective trial of 608 patients from the "Prospective Cohort with Incident Stroke" study (PROSCIS) with first-ever stroke	TMAO level >4.86 μM was associated with an increased cardiovascular risk in patients aged over 66 years ($p < 0.01$)	Haghikia et al., 2018

an increased risk of cardiovascular disease development (Senthong et al., 2016a; Haghikia et al., 2018). **Table 1** summarizes the clinical studies on the association of TMAO with atherosclerosis and CVD.

The positive correlation of cIMT values with circulating TMAO levels, regardless of gender, age, and visceral fat mass was demonstrated in 220 participants of Tübingen Lifestyle Intervention Program. In this study, lifestyle intervention led to improvement of cardiovascular risk factor, but TMAO level did not change significantly, at the same time, cIMT decreased significantly only in participants with large TMAO decrease (Randrianarisoa et al., 2016). It was shown in another study in 175 CVD-free HIV-infected patients, who have a higher cardiovascular risk than non-infected individuals due to HIV infection, that cIMT was higher toward higher TMAO concentration (Montrucchio et al., 2020). There are some contradictions regarding the role of TMAO in early atherosclerosis, probably caused by insufficient data. In some studies, patients with atherosclerosis did not have an elevated serum TMAO level compared with healthy participants, but an increase in the content of its precursor, L-carnitine, was found (Skagen et al., 2016). Another clinical study in 817 patients aged 33–45 years showed that the concentration of TMAO was associated neither with cIMT nor with coronary calcium measured by computed tomography, which may indicate a slight effect of TMAO in the development of early atherosclerosis in comparison with other studies in older people (Meyer et al., 2016). In addition to studies on the association of the TMAO level and cIMT in patients with subclinical atherosclerosis, this indicator is also used for cardiovascular risk prognosis in patients with clinical manifestations of atherosclerosis, namely, coronary artery disease (CAD). In particular, it was shown in over 4000 participants mean aged 63 years old at least single-vessel CAD, that elevated plasma concentration of TMAO was a strong predictor of increased risk of adverse cardiovascular events such as myocardial infarction, stroke and death after 3 years of follow-up. At baseline, the addition of TMAO to traditional risk factors as a covariate significantly improved cardiovascular risk estimation (Tang et al., 2013). These data may indicate the effect of TMAO at later stages of the atherosclerosis development, in high-risk subgroups, as well as among older people.

In addition to TMAO, the association of microbiome composition and other microbiome characteristics with atherosclerosis and CVD are also being investigated in epidemiological studies. For example, sequencing of gut microbiome in 218 individuals with atherosclerosis-based cardiovascular disease and 187 healthy controls demonstrates that among major genera of the gut microbiome, there is a relative reduction in Bacteroides and Prevotella, and enrichment in Streptococcus and Escherichia in atherosclerotic patients. At the same time, the abundance of Enterobacteriaceae including *E. coli*, *Klebsiella* spp., *Enterobacter aerogenes*, *Eggerthella lenta*, and *Ruminococcus gnavus* was higher in atherosclerotic patients than in control samples (q-value < 0.05 for all) (Jie et al., 2017). The study in patients, who undergone surgery to excise an atherosclerotic plaque, in comparison with CVD-free participants, shows, that genus Collinsella was enriched in patients with symptomatic atherosclerosis and Eubacterium and Roseburia were enriched in controls ($p < 0.05$) (Karlsson et al., 2012). In another study, the composition of gut microbiota was compared in 39 patients with CAD, 30 patients with coronary risk factors and 50 healthy individuals; the order of Lactobacillales was increased ($p < 0.001$) and the phylum Bacteroidetes (Bacteroides + Prevotella) was decreased ($p < 0.001$) in CAD group (Emoto et al., 2016).

Besides gut microbiome, oral cavity is also an important source of bacteria which affect atherosclerosis and CVD development. So, it was demonstrated in almost 12,000 study participants that poor dental hygiene increases risk of cardiovascular disease events and cardiovascular disease death ($p = 0.001$, for trend) (de Oliveira et al., 2010). INVEST study demonstrates the association of periodontal bacterial burden with cIMT in 657 patients over 55 years old without history of stroke, myocardial infarction, or chronic inflammatory diseases. cIMT increased across tertiles of etiologic bacterial burden which summing the standardized values for the bacteria causative of periodontal disease (*P. gingivalis, Tannerella forsythensis, Aggregatibacter Actinomycetemcomitans,* and *Treponema denticola*) in the subgingival plaque ($p = 0.03$) as well as across predominance of causative bacteria over other health-associated bacteria ($p = 0.002$) (Desvarieux et al., 2005). It was also demonstrated in the study of oral microbiota in atherosclerotic patients that abundance of Anaeroglobus was significantly higher in patients with symptomatic atherosclerosis than in control group (Fak et al., 2015). Additionally, it was detected in the study in 492 participants, that median salivary levels of *A. actinomycetemcomitans* were significantly higher in a group of patients with stable coronary artery disease ($p = 0.014$) and in a group with ACS ($p = 0.044$) than in control group (Hyvärinen et al., 2012).

CONCLUSION

This review highlighted currently published data on the impact of microbiome in basic steps of the pathogenesis of atherosclerosis. Studies investigating the association of human microbiome with subclinical atherosclerosis and cardiovascular disease demonstrate prognostic value of TMAO in cardiovascular risk assessment and revealed several types of bacteria affecting the development of atherosclerosis and its clinical complications. Data on microbiome impact in atherosclerosis development open a wide perspective to develop new diagnostic and therapeutic approaches which can be especially relevant in case of subclinical atherosclerosis. Further comprehensive study of the mechanisms of microbiome impact in atherosclerosis development is still necessary.

AUTHOR CONTRIBUTIONS

AO and W-KW: conceptualization and design the review, and review final version approval. TK, VS, YM, and VK: bibliographic

research. TK, VS, and YM: writing – original draft preparation. TK, VS, and VK: table and figure design. W-KW and TK: supervision. AO: funding acquisition. All authors contributed to the article and approved the submitted version.

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Lactobacillus pentosus Increases the Abundance of *Akkermansia* and Affects the Serum Metabolome to Alleviate DSS-Induced Colitis in a Murine Model

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Lactobacillus pentosus has the beneficial function of regulating the host's immune system and plays an indispensable role in intestinal health. The purpose of this study was to investigate the specific mechanism by which *L. pentosus* relieves dextran sulfate sodium (DSS) induced ulcerative colon inflammation. We randomly divided 24 mice into three groups, which were administered either a basic diet, drinking water with 2.5% DSS (DSS), or drinking water with 2.5% DSS and intragastric administration of *L. pentosus* (DSS + *L. pentosus*). DSS was added to the drinking water on days 8 to 12, and *L. pentosus* was administered on days 12 to 19. Serum was collected for metabolomic analysis, colon length and weight were measured, and colon contents were collected to detect microbial structural composition. Compared with the DSS group, the DSS + *L. pentosus* group had significantly higher levels of indolepyruvate and pantothenic acid in the serum and significantly lower levels of 3,4-dimethyl-5-pentyl-2-furannonanoic acid and 5-oxo-6-trans-leukotriene B4. Moreover, compared with the other two groups, the DSS + *L. pentosus* group had a significantly greater abundance of *Akkermansia*. The abundance of *Akkermansia* was positively correlated with indolepyruvate and pantothenic acid levels. Therefore, *L. pentosus* can interact with *Akkermansia* to increase its abundance in the intestinal tract. This results in the production of metabolites that are beneficial for the regulation of intestinal immunity, thereby alleviating DSS-induced ulcerative colon inflammation.

Keywords: ulcerative colitis, *Lactobacillus pentosus*, *Akkermansia*, serum metabolomics, intestinal microbes

INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic disease caused by the interaction of environmental, immune, genetic, and other factors (Batura and Muise, 2018). It has a significant impact on human health, and its incidence continues to increase every year (Ventham et al., 2013; Miyoshi and Chang, 2017). Ulcerative colitis (UC) is a type of IBD characterized by inflammatory disease in the colon

and rectum, usually accompanied by inflammatory lesions of the colonic mucosa and submucosa (Danese and Fiocchi, 2011). In severe cases, it can cause diarrhea or even blood in the stool, and the occurrence of repeated illnesses eventually increases the risk of developing cancer (Ungaro et al., 2017). Although the specific cause of recurrent ulcerative colitis is not yet clear (Kaplan, 2015), approximately 200 genetic risk loci have been proposed for IBD. These susceptibility genes include those involved in autophagic regulation and microbial sensors that activate autophagy (Matsuoka and Kanai, 2015). Recent studies have shown that a lack of intestinal microbial protection signals promotes the expression of IBD susceptibility genes, thereby increasing inflammation (Chu et al., 2016). Therefore, we believe that remodeling the healthy gut microbial community structure will be an effective treatment strategy for colonic inflammation.

Clinical and animal model studies have shown that there are inextricable connections between the intestinal immune system and the intestinal flora (Abrams et al., 1963). For example, the transplantation of gut microbes from mice with inflammation induces inflammation in germ-free mice (Eun et al., 2014). This may occur because inflammation-related metabolites produced by specific microorganisms can activate dendritic cells and promote Th1-mediated inflammation (Devkota et al., 2012). The introduction of specific probiotics can also relieve the symptoms of inflammation and promote the development of a healthy intestinal microbial structure (Henker et al., 2008; Mardini and Grigorian, 2014). These probiotics mediate their effects by reshaping the structure of the intestinal flora, promoting the metabolic activities of beneficial flora, and improving intestinal immune activity (Marchesi et al., 2016).

An increasing number of probiotics are being used to improve human health (D'Mello et al., 2015; Park et al., 2018; Tsai et al., 2019). For example, *Clostridium butyricum CGMCC0313.1* has anti-diabetic effects, by increasing the proportion of butyric acid-producing bacteria (Jia et al., 2017). Similarly, *Bifidobacterium* strains can inhibit the accumulation of body fat, improve glucose tolerance, and ameliorate metabolic diseases (Aoki et al., 2017). However, there has been little research on the use of *Lactobacillus pentosus* to treat IBD induced by DSS. *L. pentosus* has been shown to have antifungal activity and inhibit the growth of pathogenic microorganisms (Lipińska et al., 2018). Other studies have shown that *L. pentosus* has a therapeutic effect on *Salmonella*-infected mice and it inhibits the growth of *Salmonella*, inhibits the damage caused by the invasion of intestinal epithelial cells, and improves the host's immune function (Liu et al., 2018). Even more importantly, *L. pentosus* induces Tr1 cells to inhibit systemic inflammation (Kim et al., 2019). In intestinal epithelial cells, *L. pentosus* promotes the expression of TLR inhibitors, such as A20, Tollip, SIGIRR, and IRAKM, thereby inhibiting downstream MAPK and NF-κB signaling pathways, and exerting an immunoregulatory effect on intestinal epithelial cells (Kanmani and Kim, 2019). Moreover, *L. pentosus* repairs intestinal barrier damage by promoting tight junction protein expression (Zeng et al., 2020). Therefore, *L. pentosus* plays an indispensable role in intestinal health. In this study, we assessed the ability of *L. pentosus* to regulate intestinal microbes and investigated the specific mechanism used to alleviate DSS-induced ulcerative colonic inflammation.

MATERIALS AND METHODS

Bacteria Preparation

The *L. pentosus* strain preserved in the Microbiology Laboratory of the College of Bioscience and Biotechnology, Hunan Agricultural University was activated using de Man, Rogosa and Sharpe medium. It was continuously cultivated for 2 days at 37°C and 120 r/min. The colonies of activated bacteria were counted using the dilution coating method to determine the concentration of the bacterial solution. The required volume of bacterial solution was centrifuged at 4,000 r/min for 10 min, the resulting supernatant was removed, and physiological saline was added to the precipitated bacteria to dilute the bacterial solution to 5×10^9 cfu/mL.

Animals and Experimental Design

Animal experiments were performed according to the Guidelines for Care and Use of Laboratory Animals of Hunan Agricultural University. Twenty-four 8-week-old ICR mice (Hunan Silaike Jingda Co, Changsha, China), with an average weight of 18 g, were acclimated for 7 days in a sterile environment. They were then randomly allocated to three groups, with eight mice in each group. The first group (CON) was treated with a basal diet, the second group was treated with 2.5% dextran sulfate sodium (DSS) to induce ulcerative colitis, and the third group was treated with *L. pentosus* and 2.5% DSS (DSS-*L. pentosus*). During the experiment, the mice were allowed to drink and eat freely. On days 8–12 of the experiment, 2.5% DSS was added to the drinking water of the mice in the DSS and DSS-*L. pentosus* groups. *L. pentosus* was intragastrically administered to the mice in the DSS-*L. pentosus* group on days 13–19 (**Figure 1**). The gavage volume for each mouse was 0.1 mL. All of the mice were sampled on day 20. Serum samples were collected for serum metabolite analysis, colon samples were collected and fixed in 4% formalin, and colon contents were rapidly frozen and stored at −80°C.

Colonic Histopathology

Colon samples fixed in 4% formaldehyde were cleared with a gradient of xylene concentrations, dehydrated with a gradient of ethanol concentrations, and finally embedded in paraffin. After sectioning, the tissue slices were placed in xylene solution I for 10 min, xylene solution II for 10 min, anhydrous ethanol solution I for 5 min, anhydrous ethanol solution II for 5 min, 95% alcohol for 5 min, 90% alcohol 5 min, 80% alcohol for 5 min, and 70% alcohol for 5 min, and then washed with distilled water. The tissue sections were then immersed in hematoxylin dye for 3–8 min; rinsed in running water; differentiated with 1% hydrochloric acid alcohol; rinsed again in running water; and finally immersed in an eosin dye solution for 1–3 min. Tissue sections were observed and photographed using a BX41 microscope (Olympus,

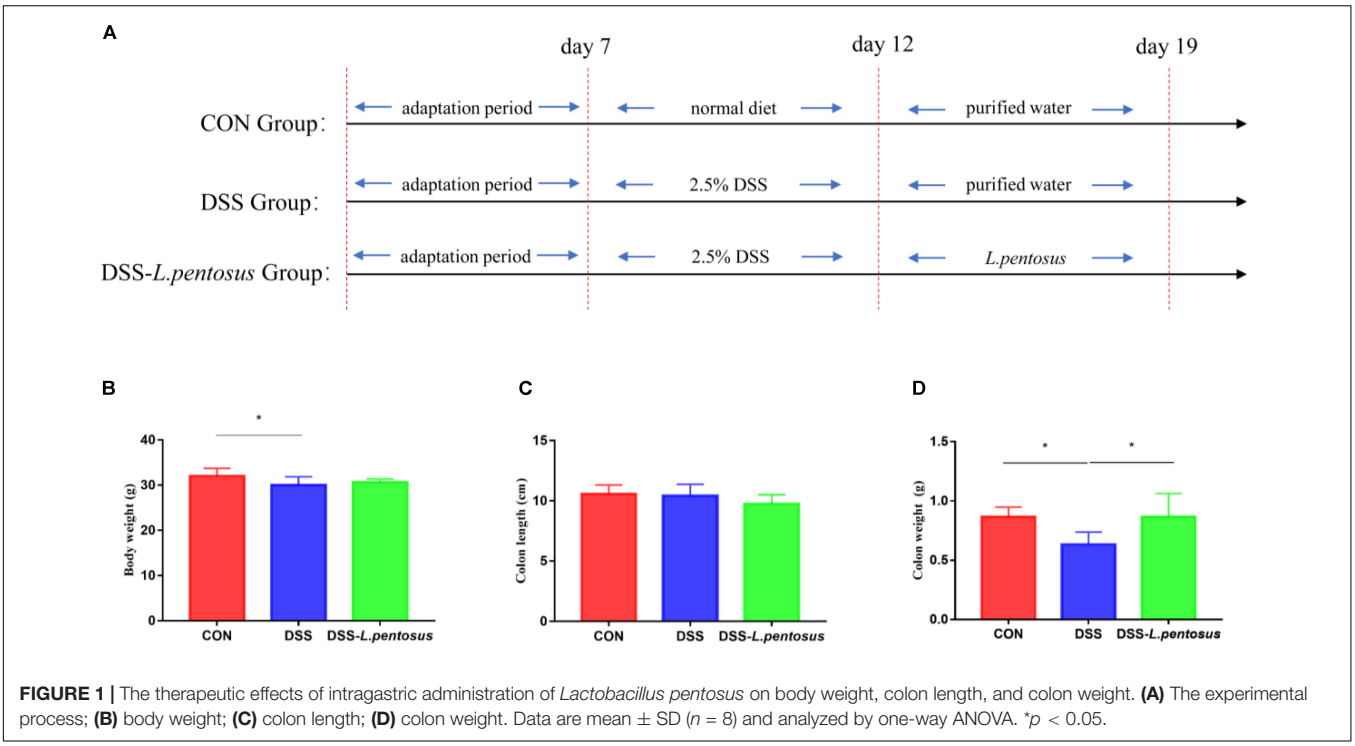

FIGURE 1 | The therapeutic effects of intragastric administration of *Lactobacillus pentosus* on body weight, colon length, and colon weight. **(A)** The experimental process; **(B)** body weight; **(C)** colon length; **(D)** colon weight. Data are mean ± SD (n = 8) and analyzed by one-way ANOVA. *$p < 0.05$.

Münster, Germany). The morphological analysis results were rated according to the scoring system presented in **Table 1**.

Serum Metabolomic Analyses

Thawed serum samples were extracted with methanol, mixed with dichlorophenylalanine, and then centrifuged. The resulting supernatant was transferred to a liquid phase bottle for testing. The analyses were performed using previously described analysis platforms, chromatographic columns, and chromatographic separation conditions (Ma et al., 2019). Compound Discoverer software (Thermo Fisher Scientific, Waltham, MA, United States) was used to extract and preprocess data from the instrument, and obtain information such as the retention time, molecular weight, sample name, and peak intensity. SIMCA-P 11 was used to analyze and plot principal component analysis (PCA), Partial Least Squares Discrimination Analysis (PLS-DA), and VIP values. The retention time and molecular weight data were compared with entries in the Human Metabolome Database (HMDB) to determine the metabolite composition of the serum samples.

TABLE 1 | The histologic scoring system.

Inflammation	Crypt Injury	Ulceration	Score
No significant inflammation	No injury	No ulceration	0
Neutrophilic inflammation in epithelium or lamina propria	Loss of basal one third of crypts	Two or fewer foci of ulceration	1
Inflammatory cells extending into submucosa	Loss of basal two thirds of crypts	Three or four foci of ulceration	2
Transmural inflammation	Loss of full thickness crypts	Diffuse/confluent ulceration	3

16S Ribosomal RNA Amplicon Sequencing

Microbial DNA was extracted from fecal samples using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany). The V3-V4 region sequence of the extracted DNA was determined using high-throughput sequencing technology. First, using purified DNA as the template, PCR amplification was performed using the universal primers, 357F (5′-ACTCCTACGGRAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) fused with Illumina (San Diego, CA, United States) sequencing primers. The amplified products were subjected to 1.2% agarose gel electrophoresis. Successfully amplified products were run on 2% agarose gels, and the electrophoretic bands were excised to recover the DNA. The recovered PCR products were used as templates for PCR amplification (8 cycles). The adaptors, sequencing primers, and tag sequences required for sequencing on the MiSeq platform (Illumina) were added to both ends of the target fragment. An AxyPrep DNA gel recovery kit (Axygen, Alachua, FL, United States) was used to recover all PCR products, and the FTC-3000^{TM} Real-Time PCR instrument was used for fluorescence quantification. After an equimolar ratio was mixed, the library was constructed and sequenced on a MiSeq platform (PE300). Sequencing data were analyzed off-line to determine α and β diversity, using mothur (version 1.33.3). The R language was used to map species composition at different taxonomic levels and determine the differences in species abundance between groups. The abundance of the sequenced microorganisms at different taxonomic levels was uploaded to http://huttenhower.sph.harvard.edu/galaxy/

for linear discriminant analysis (LDA) and LDA Effect Size (LEfSe) analysis.

Data Analysis

We have used SPSS 22.0 to perform bivariate Pearson correlation analysis on the data. All of the data in this study are expressed as mean ± standard deviation (SD). The data were analyzed using SPSS 22.0. The differences between the means of the experimental groups were analyzed using one-way analysis of variance and Tukey's multiple comparison test. A p-value < 0.05 was regarded as a significant difference.

RESULTS

Lactobacillus pentosus Improves Colonic Injury in Mice With DSS-Induced Colitis

After 5 days of 2.5% DSS treatment, mice were followed by 7 days of *L. pentosus* administration. The results demonstrated that the body weight of DSS group was reduced in comparison with CON group (**Figure 1B**, $p < 0.05$), there is no difference in colon length, but the weight of colon was decreased in DSS group in relative with CON and DSS-*L. pentosus* group (**Figure 1D**, $p < 0.05$).

Lactobacillus pentosus Inhibits the Development of Colitis Induced by DSS

Histomorphological analysis (**Figures 2A–C**) showed that the colon tissue of mice in the DSS group had obvious neutrophil aggregation, goblet cell disappearance, and infiltration with other inflammatory cells. When the mice in the DSS group received *L. pentosus*, inflammatory cell infiltration was alleviated. We developed a histological scoring system (**Table 1**), which was used to show that histological injury was significantly greater in the DSS group than the CON and DSS-*L. pentosus* groups (**Figure 2D**). Thus, the intragastric administration of *L. pentosus* can relieve colonic injury induced by DSS.

Lactobacillus pentosus Affects the Serum Metabolomic Profiles During Colitis

GC-MS was used to detect serum metabolites, and principal component analysis (PCA) to identify outliers and clusters of samples with high similarity. Partial least squares discriminant analysis (PLS-DA) was used to force the classification of each component, facilitating the identification of similarities and differences between groups. The results of PCA and PLS-DA analyses (**Figure 3**) showed that serum metabolite concentrations were similar within the three groups, but differed between the groups. Using PLS-DA analysis, we also identified 133 differential metabolites between the three groups, with VIP values > 1.5. These metabolites were mainly composed of lipids and lipid-like molecules (48.87%), organoheterocyclic compounds (14.29%), and organic acids and their derivatives (12.03%, **Figure 4**). The main metabolite differences between the three groups are shown in **Table 2**. Plasma levels of 3,4-dimethyl,5-pentyl,2-furannonanoic acid, 2,15-epoxy,13,14-dimethyleicosa-10,12,14-trienoic acid, alpha-dimorphecolic acid, and resolvin D5 were significantly higher in the DSS group than the CON group.

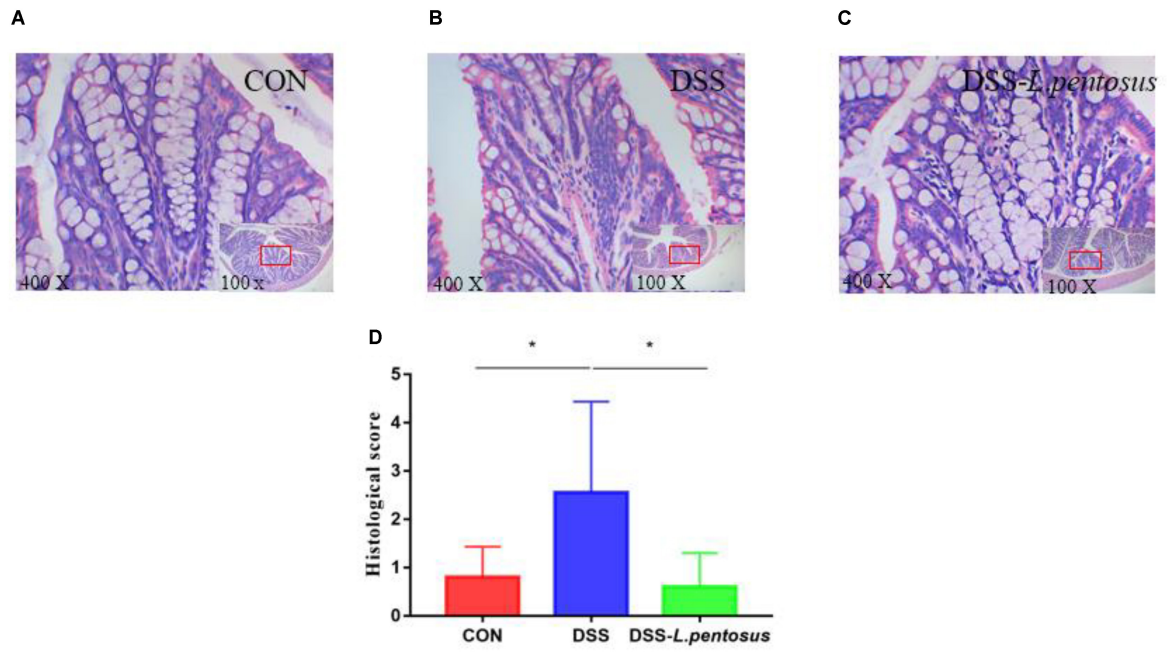

FIGURE 2 | The therapeutic effect of intragastric administration of *Lactobacillus pentosus* on colon tissue damage. Images of colon morphology in the CON **(A)**, DSS **(B)**, and DSS-*L. pentosus* **(C)** groups under 100 × and 400 × visual fields; **(D)** Histomorphological damage scores in the three groups. Data are mean ± SD ($n = 8$) and analyzed by one-way ANOVA. *$p < 0.05$.

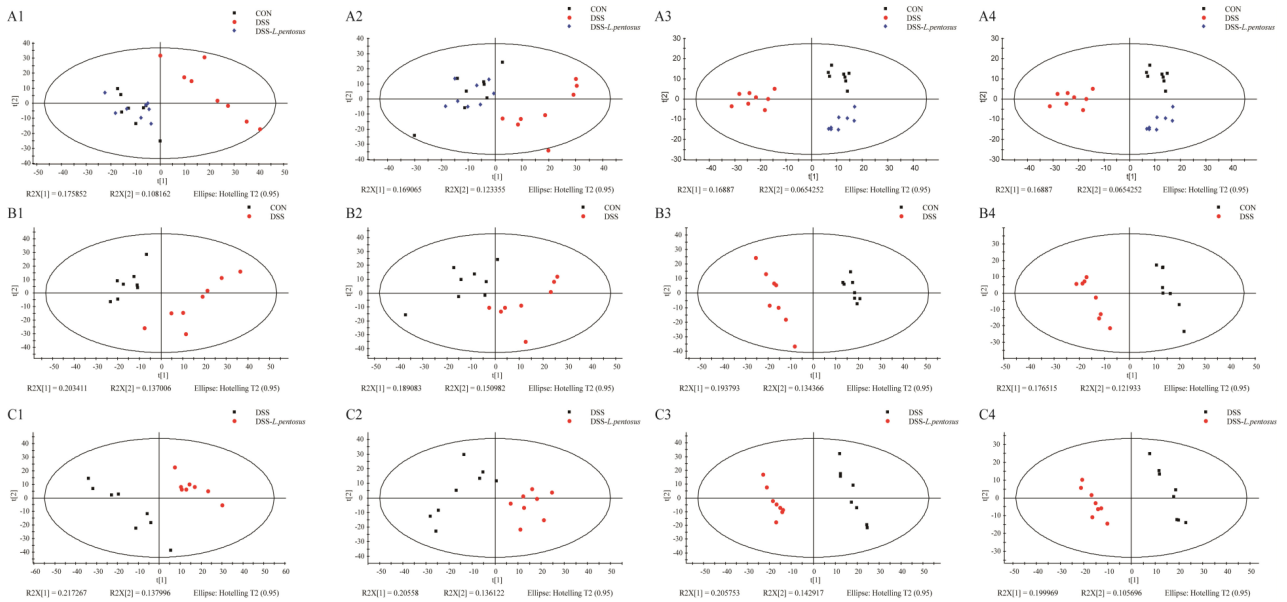

FIGURE 3 | Plots of the multivariate statistical comparisons between groups. **(A1)** PCA score plot of all samples (ESI+); **(A2)** PCA score plot of all samples (ESI-); **(A3)** PLS-DA score plot of all samples (ESI+); **(A4)** PLS-DA score plot of all samples (ESI-); **(B1)** PCA score plot of CON-DSS (ESI+); **(B2)** PCA score plot of CON-DSS (ESI-); **(B3)** PLS-DA score plot of CON-DSS (ESI+); **(B4)** PLS-DA score plot of CON-DSS(ESI-); **(C1)** PCA score plot of DSS-(DSS-*L. pentosus*) (ESI+); **(C2)** PCA score plot of DSS-(DSS- *L. pentosus*) (ESI-); **(C3)** PLS-DA score plot of DSS-(DSS-*L. pentosus*) (ESI+); **(C4)** PLS-DA score plot of DSS-(DSS-*L. pentosus*) (ESI-).

However, after *L. pentosus* administration, the levels of these metabolites decreased significantly. A volcanic map illustrating the differential metabolites between the DSS and DSS-*L. pentosus* groups is shown in **Figure 5A**. We found that *L. pentosus* significantly reduced the production of 3,4-dimethyl-5-pentyl-2-furannonanoic acid and 5-oxo-6-trans-leukotriene B4 and other harmful substances metabolized by mice in the DSS group, and increased the serum levels of indolepyruvate and pantothenic acid (**Figure 5B**).

Lactobacillus pentosus Regulates Intestinal Microbes in Mice With Colon Inflammation Induced by DSS

The 16S rRNA V3-V4 region sequences of colonic microbes were analyzed. Colonic microbe diversity was measured using the Chao index (**Figure 6A**), ACE index (**Figure 6B**), Shannon indices (**Figure 6C**), and Simpson index (**Figure 6D**). The values for all four indices were lower in the DSS group relative to the CON group (**Figure 6**, $p < 0.05$). Moreover, the Shannon and Simpson index values were higher in the DSS-*L. pentosus* group than the DSS group (**Figures 6C,D**, $p < 0.05$). Thus, *L. pentosus* administration recovered colonic microbial diversity after challenging with DSS.

The major bacterial phyla in the colon were Bacteroidetes, Firmicutes, Verrucomicrobia, and Proteobacteria, accounting for over 96% of colonic bacteria. In the CON, DSS, and DSS-*L. pentosus* groups, respectively, the proportions of Bacteroidetes were 64.17%, 41.09%, and 30.39%; the proportions of Firmicutes were 23.80%, 31.78%, and 31.96; the proportions of Verrucomicrobi were 3.49%, 16.45%, and 26.96%; and the proportions of Proteobacteria were 5.51%, 7.87%, and 7.56% (**Figure 7A**). The abundance of Bacteroidetes in the DSS-*L. pentosus* group relative to the DSS group was lower than the abundance of Bacteroidetes in the DSS group relative to the CON group (**Figure 7C**, $p < 0.05$). However, the abundance of Verrucomicrobia was higher in the DSS-*L. pentosus* group relative to the DSS group than in the DSS group relative to the CON group (**Figure 7B**, $p < 0.05$).

The ten most abundant bacterial orders are shown in **Figure 7D**. Bacteroidales, Verrucomicrobiales, Clostridiales, and Lactobacillales were the most abundant, accounting for more than 85% of colonic bacteria. In the CON, DSS, and DSS-*L. pentosus* groups, respectively, the proportions of Bacteroidales were 64.11%, 41.07%, and 29.85%; the proportions of Verrucomicrobiales were 3.49%, 16.45%, and 26.96%; the proportions of Clostridiales were 15.83%, 17.58%, and 19.43%; and the proportions of Lactobacillales were 5.16%, 11.16%, and 10.36%. The abundance of Bacteroidales was lower in the DSS-*L. pentosus* group relative to the DSS group than in the DSS group relative to the CON group (**Figure 7F**, $p < 0.05$). However, the abundance of Verrucomicrobiales was lower in the DSS-*L. pentosus* group relative to the DSS group than in the DSS group relative to the CON group (**Figure 7E**, $p < 0.05$).

The ten must abundant bacterial genera were selected and their percentage abundance was analyzed. The most abundant genera were *Akkermansia*, *Bacteroides*, *Lactobacillus*, and *Parasutterella*. In the CON, DSS, and DSS-*L. pentosus* groups, respectively, the proportions of *Bacteroides* were 10.04%,

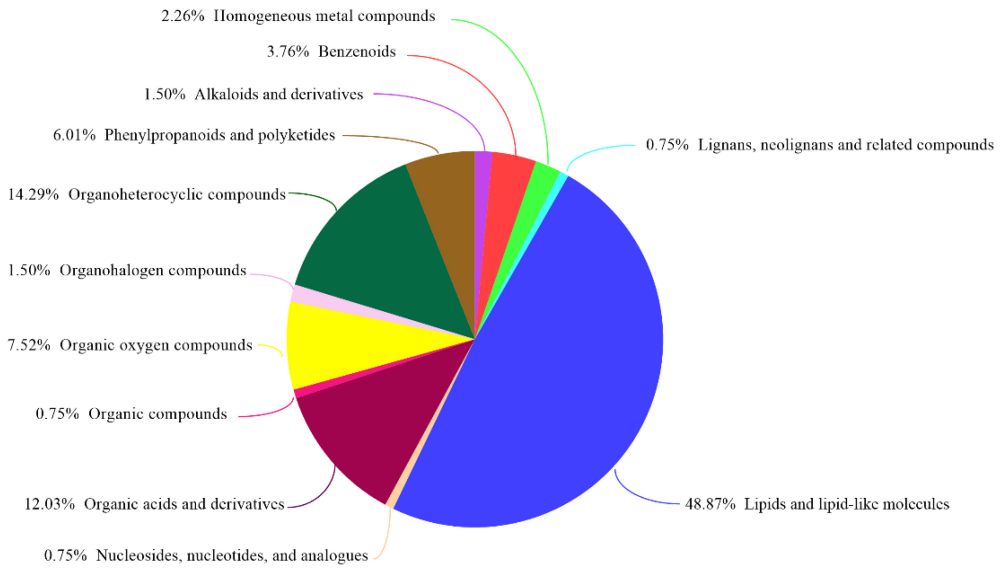

FIGURE 4 | HMDB compound classification. Based on the number of metabolites, the Human Metabolome Database (HMDB) level (Superclass, Class, or Subclass) classification and metabolite percentages are displayed. The different colors in each pie chart in the figure represent different HMDB classifications, and their area represents the relative proportion of metabolites in the classification.

TABLE 2 | Metabolomic changes in the plasma in the CON, DSS and DSS- *L. pentosus*.

			CON VS DSS			DSS VS DSS-*L. pentosus*		
	mz	RT (min)	VIP	*T*-TEST	Fold change	VIP	*T*-TEST	Fold change
3,4-Dimethyl-5-pentyl-2-furannonanoic acid	323.2581	7.917	2.2637	1.83195E-07	↑	2.11424	5.29157E-05	↓
12,15-Epoxy-13,14-dimethyleicosa-10,12,14-trienoic acid	313.2537	8.341	2.21032	1.18131E-06	↑	1.96774	0.010466241	↓
10,11-dihydro-20-dihydroxy-LTB4	371.2428	4.545	2.09009	2.07056E-05	↑	— —	— —	— —
11,12-Epoxyeicosatrienoic acid	321.2424	7.41	2.08799	2.2513E-05	↑	— —	— —	— —
Alpha-dimorphecolic acid	297.2424	7.272	1.95626	0.000177303	↑	1.79278	0.000741813	↓
Sorbose 1-phosphate	261.037	0.795	1.69084	0.002977137	↑	— —	— —	— —
Resolvin D5	295.2268	6.881	1.63705	0.004383114	↑	1.9099	0.02057	↓
Pantothenic acid	220.1179	2.013	— —	— —	— —	1.96618	0.000193157	↑
5-Oxo-6-trans-leukotriene B4	335.2217	6.189	— —	— —	— —	2.15637	0.0205647	↓
11,12-Epoxyeicosatrienoic acid	321.2424	7.665	— —	— —	— —	1.96516	3.77107E-05	↓
Indolepyruvate	204.0655	3.741	204.0655	3.741	↓	1.82665	0.000146027	↑
Phenylpyruvic acid	165.0546	3.662	— —	— —	— —	1.61367	0.010110291	↑

12.05%, and 9.15%; the proportions of *Akkermansia* were 3.49%, 16.45%, and 26.96%; the proportions of *Lactobacillus* were 5.04%, 10.89%, and 9.27%; and the proportions of *Parasutterella* were 2.32%, 3.67%, and 2.66% (**Figure 7G**). The abundance of *Akkermansia* was higher in the DSS-*L. pentosus* group than in the DSS group (**Figure 7H**, $p < 0.05$). There was no significant difference in the abundance of *Bacteroides* among the three groups (**Figure 7I**, $p < 0.05$).

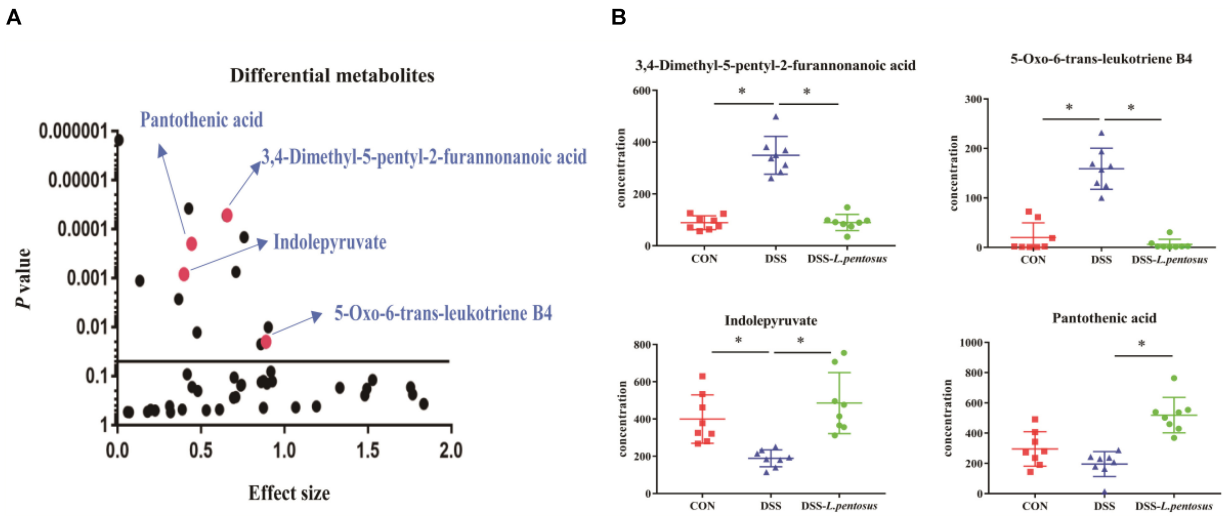

FIGURE 5 | Effects of different treatments on serum metabolite levels in mice. **(A)** Volcanic map of differential metabolite levels between the DSS and DSS-*L. pentosus* groups; **(B)**Serum levels of three group metabolic products.

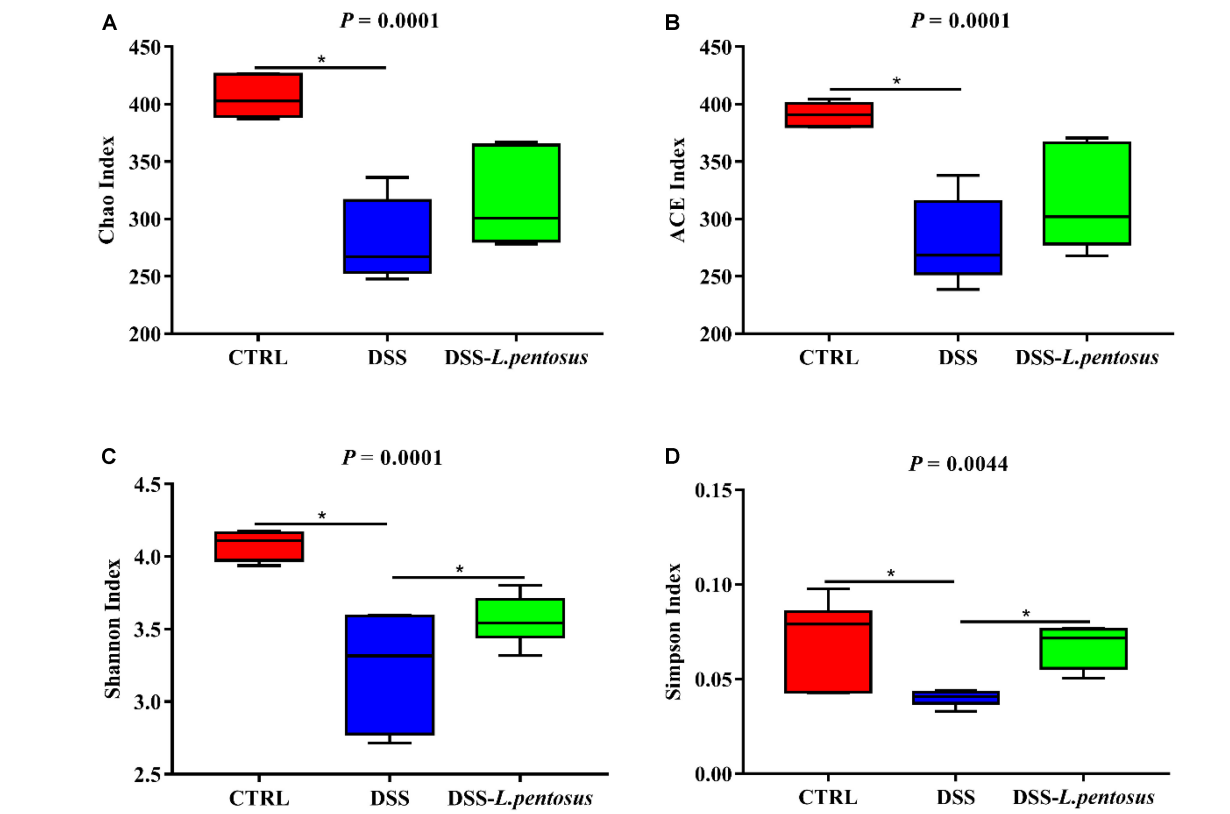

FIGURE 6 | Effects of different treatments on α-diversity of mouse intestinal microbiota. **(A)** Chao index; **(B)** ACE index; **(C)** Shannon index; **(D)** Simpson index. Data are mean ± SD ($n = 8$) and analyzed by one-way ANOVA. *$p < 0.05$.

LEfSe analysis showed that the order *Verrucomicrobiales*, the family *Verrucomicrobiaceae*, and the genus *Akkermansia* were significantly enriched in the DSS-*L. pentosus* group, and this had a significant effect on the differences between groups (**Figure 7J**, $p < 0.05$). LDA was then used to estimate the magnitude of the effect of species abundance on the effect of differences between each group. Species with differential abundance between groups were identified based on LDA scores greater than 2 (**Figure 7K**).

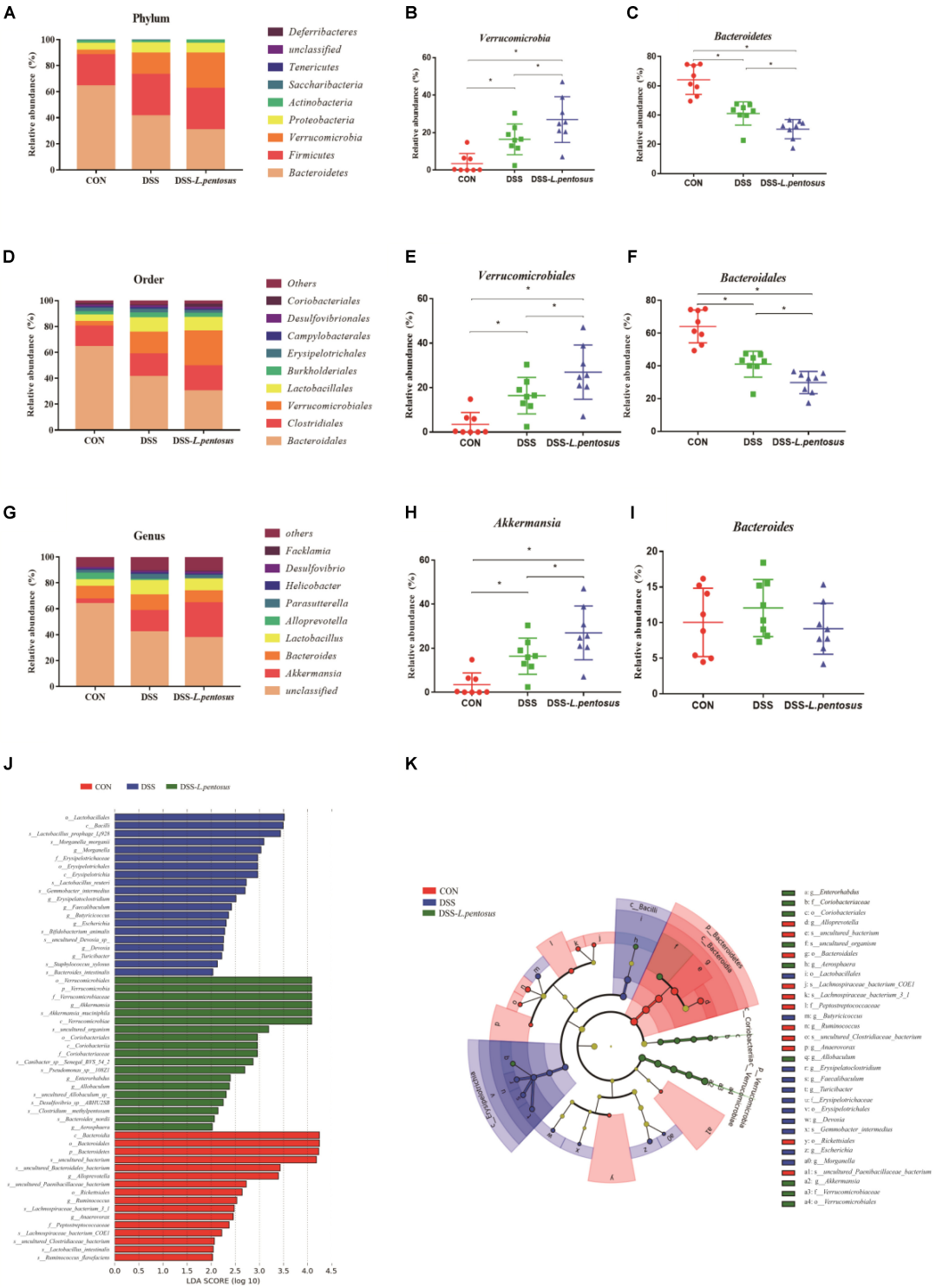

FIGURE 7 | Effects of different treatments on gut microbiota in mice. **(A)** Relative abundance of gut microbiota phyla; **(B)** percentage of *Verrucomicrobia* in each sample from the three groups; **(C)** percentage of *Bacteroidetes* in each sample from the three groups; **(D)** relative abundance of gut microbiota orders; **(E)** percentage of *Verrucomicrobiales* in each sample from the three groups; **(F)** percentage of *Bacteroidales* in each sample from the three groups; **(G)** relative abundance of gut microbiota genera; **(H)** percentage of *Akkermansia* in each sample from the three groups; **(I)** percentage of *Bacteroides* in each sample from the three groups. **(J)** LDA score, an LDA score greater than 4 was considered to indicate an important contributor to the model; **(K)** LEfSe taxonomic cladogram, with different colors indicating the enrichment of certain taxa in the control (red), DSS (blue), and DSS-*L. pentosus* (green) groups. Data are mean ± SD (n = 8) and analyzed by one-way ANOVA. *$p < 0.05$.

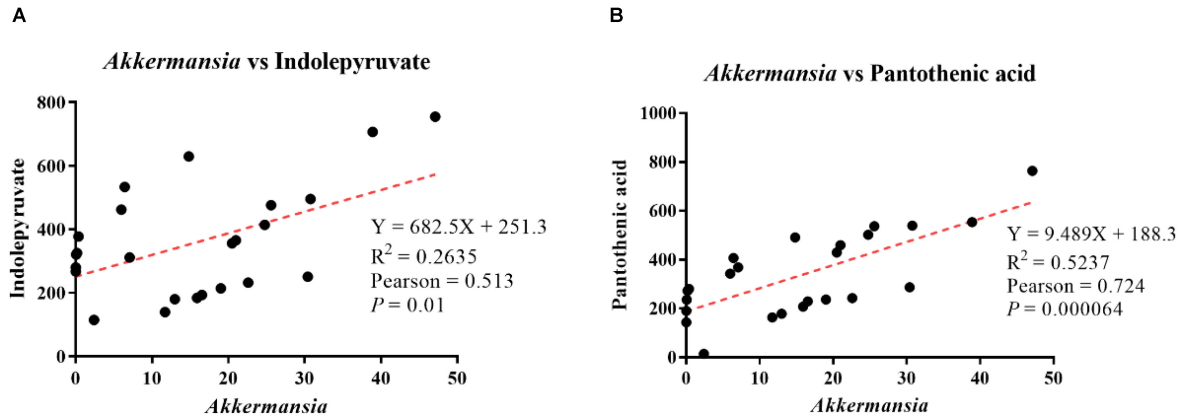

FIGURE 8 | Analysis of correlations between differential metabolite levels and genus-level intestinal microbe abundance. **(A)** Correlation analysis between *Akkermansia* abundance and indolepyruvate levels. **(B)** Correlation analysis between *Akkermansia* abundance and pantothenic acid levels.

The abundance of species in the order *Verrucomicrobiales*, the family *Verrucomicrobiaceae*, and the genus *Akkermansia* had the most significant effects on differential bacterial abundance in the DSS-*L. pentosus* group. However, in the CON group, the abundance of species in the phylum Bacteroidetes, the class *Bacteroidia*, the order *Bacteroidales*, and the genus *Bacteroides* had the most significant effects on differential abundance, with an LDA score greater than 4.

Correlation Between Key Microorganisms and Characteristic Differential Metabolites

Pearson's correlation analysis showed that *Akkermansia* abundance was positively correlated with indolepyruvate levels, with a correlation coefficient of 0.513 (p = 0.01, **Figure 8A**). Additionally, *Akkermansia* showed a positive correlation with pantothenic acid levels, with a Pearson's correlation coefficient of 0.724 (p = 0.00064, **Figure 8B**).

DISCUSSION

This study explored the protective effect of *L. pentosus* against DSS-induced colon damage. We also observed that *L. pentosus* significantly regulated the microbial community structure of the mouse intestine, making *Akkermansia* the dominant flora in the colon. Through analysis of serum metabolomics, differences in indolepyruvate and pantothenic acid levels were found to be the key metabolic differences between the DSS and DSS-*L. pentosus* groups. *L. pentosus* may relieve DSS-induced ulcerative colonic inflammation by increasing the abundance of *Akkermansia* and promoting the production of indolepyruvate and pantothenic acid.

Lactobacillus species have been widely used as probiotics to promote intestinal health. For instance, *L. plantarum TIFN101* regulates the transcriptional pathways of tight junction and adhesion proteins, including actinin alpha-4 and metalloproteinase-2 (Mujagic et al., 2017), thereby improving intestinal barrier function. Moreover, *L. plantarum strain WCFS1* activates Toll-like receptor 2 (TLR2) signaling to promote the expression of occludin and protect the intestinal epithelial cell barrier (Karczewski et al., 2010). In our study, *L. pentosus* also protected the integrity of the intestinal structure, thus improving intestinal barrier function. Foster KR et al. showed that colonic epithelial cells interact with intestinal microorganisms to promote the stability of the intestinal microecology and thus, improve the host's immune capacity (Foster et al., 2017). However, changes in the intestinal microbial community can disrupt the homeostasis of the colon, resulting in a loss of immune function in the intestinal epithelium (Byndloss and Baumler, 2018). These data suggest that the integrity of the intestinal structure and the stability of the internal environment of the intestinal microbial structure are key factors in maintaining immune function.

In our study, mice with DSS-induced colitis showed colon damage, and this loss of colonic barrier function caused structural changes in the gut microbiological environment. There is a dynamic balance between the host and intestinal microbes, and mechanical damage to the immune system can cause changes in the microbial community (Foster et al., 2017). Microorganisms in the colon benefit the host, because they can use complex fibers that cannot be digested by host enzymes, and fermentation can produce nutrients that the host can absorb (den Besten et al., 2013). *Bacteroides*, as the only determined sphingolipid producer among intestinal symbionts, can transport immunologically active metabolites to immune cells through outer membrane vesicles (Chu et al., 2016). Therefore, most studies have shown that IBD is associated with a significant decrease in the abundance of *Bacteroides* in the host colon (Nishino et al., 2018; Blandford et al., 2019; Brown et al., 2019). In our study, the percentage of *Bacteroides* was significantly lower in the DSS group than the CON group. However, the abundance of *Bacteroides* was significantly lower in the DSS-*L. pentosus* group than the other two groups, which may be because Verrucomicrobia became the dominant bacterial group *in vivo* after *L. pentosus* treatment, thus reducing the abundance of *Bacteroides*. *Akkermansia* is a

symbiotic genus of the phylum Verrucomicrobia (Ansaldo et al., 2019). A large number of studies have shown that a decrease in *Akkermansia* abundance is closely related to IBD (Png et al., 2010; Rajilić-Stojanović et al., 2013). *Akkermansia* species interact with intestinal epithelial cells to promote the expression of IL-8, which regulates intestinal immunity (Drell et al., 2015). *Akkermansia* species not only induce the intestinal adaptive immune response (Ansaldo et al., 2019), but also relieve colonic mucosal damage caused by inflammation (Everard et al., 2013), by inducing the expression of tight junction proteins, including zona occludens protein-1 and occludin, and reducing the internal circulation of endotoxins (Li et al., 2016). Specifically, the anti-inflammatory properties of *Akkermansia* are shown by inhibiting the expression of pro-inflammatory factors, such as TNF-α and IFN-γ, while also stabilizing the structure of intestinal microbes (Zhai et al., 2019). Thus, treatment with *L. pentosus* increases the abundance of *Akkermansia*, promotes intestinal immunity, and preserves intestinal barrier function.

Recent studies have shown that the alteration of serum metabolite levels can be used as a potential biological indicator of gut health (Uchiyama et al., 2017). The production of certain metabolites indicates that inflammation has occurred. Most commonly, inflammation induces neutrophil aggregation and promotes the formation of neutrophil extracellular traps, and furanoid fatty acids induce NADPH oxidase and the production of reactive oxygen species in the mitochondria (Khan et al., 2018). Chemotaxis neutrophils can also secrete leukotriene B4 substances, which increase the recruitment of neutrophils (Majumdar et al., 2016). LTB4 is also packaged in multivesicular bodies and then released by neutrophils as exosomes (Majumdar et al., 2016), thereby accumulating at the site of inflammation (Németh and Mócsai, 2016). Therefore, high levels of furanoid fatty acids and LTB4 indicate the occurrence of inflammatory cell infiltration. In our study, DSS treatment significantly increased the production of 3,4-dimethyl-5-pentyl-2-furannonanoic acid and 5-oxo-6-trans-leukotriene B4, but after intragastric administration of the probiotic *L. pentosus*, the levels of these two metabolites returned to normal. When the body is externally stimulated or when homeostasis is disrupted (such as after tissue damage or infection), tissues and immune cells produce corresponding metabolites to resolve inflammation and re-establish homeostasis (Gaber et al., 2017). When immune cells accumulate at the site of inflammation and are in a state of hypoxia, they transcribe *HIF-1α*. Indolepyruvate inhibits the HIF-1α signaling pathway induced by LPS, thereby reducing the expression levels of IL-1β (McGettrick et al., 2016). In our experiments, *L. pentosus* administration increased the production of indolepyruvate, thereby reducing the symptoms of inflammation. Ulcerative colitis is usually accompanied by the accumulation of colon stem cells and undifferentiated transit expanded cells at the base of the crypt (Papapietro et al., 2013), resulting in the inhibition of peroxisome proliferator-activated receptor (PPAR) synthesis in epithelial cells (Dubuquoy et al., 2003; Litvak et al., 2018). Pantothenic acid has been shown to continuously activate PPARs (Pourcel et al., 2020), thus reducing the risk of increased colon permeability (Ponferrada et al., 2007). However, pantothenic acid is mainly involved in metabolism in the form of coenzyme A. Coenzyme A is a ubiquitous cofactor that plays an irreplaceable role in carboxylic acid metabolism (Leonardi et al., 2005), and short-chain fatty acids have long been known to inhibit histone deacetylase and activate G-protein-coupled receptors to regulate the intestinal health (Sun et al., 2017). *L. pentosus* administration significantly increased the production of pantothenic acid, inhibited inflammation, and regulated the health of the intestine. Finally, through correlation analysis, we found that *Akkermansia* abundance was positively correlated with indolepyruvate and antothenic acid levels.

CONCLUSION

Our data indicate that *L. pentosus* improves intestinal barrier function by remodeling the intestinal microbial structure and increasing the abundance of *Akkermansia*. Moreover, *L. pentosus* regulates components of serum metabolites; reduces the harmful metabolism of 3,4-dimethyl-5-pentyl-2-furannonanoic acid and 5-oxo-6-trans-leukotriene B4 caused by DSS; and promotes the metabolism of beneficial metabolites, such as indolepyruvate and pantothenic acid. Therefore, *L. pentosus* may be used as an auxiliary bacterial agent to regulate intestinal health clinically, due to its indispensable role in intestinal barrier repair.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/bioproject, PRJNA655588.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Care and Use Committee of Hunan Agricultural University.

AUTHOR CONTRIBUTIONS

YM performed the study and conducted data analysis. GL designed the research. WY provided assistance for the study. HJ and CH prepared the first draft of the manuscript. All authors read and revised the manuscript.

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Gut Microbiota-Related Evidence Provides New Insights Into the Association Between Activating Transcription Factor 4 and Development of Salt-Induced Hypertension in Mice

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Activating transcription factor 4 (ATF4), which regulates genes associated with endoplasmic reticulum stress, apoptosis, autophagy, the gut microbiome, and metabolism, has been implicated in many diseases. However, its mechanistic role in hypertension remains unclear. In the present study, we investigated its role in salt-sensitive hypertensive mice. Wild-type (WT) C57BL/6J mice were used to establish *Atf4* knockout (KO) and overexpression mice using CRISPR-Cas9 and lentiviral overexpression vectors. Then, fecal microbiota transplantation (FMT) from *Atf4*$^{\pm}$ mice and vitamin K_2 (VK2) supplementation were separately carried out in high-salt-diet (8% NaCl)-induced mice for 4 weeks. We found that *Atf4* KO inhibited and *Atf4* overexpression enhanced the increase in blood pressure and endothelial dysfunction induced by high salt intake in mice, while regulating the gut microbiota composition and VK2 expression. It was further verified that ATF4 is involved in the regulation of salt-sensitive hypertension and vascular endothelial function, which is achieved through association with gut microbiota and may be related to VK2 and different bacteria such as *Dubosiella*. In addition, we found that VK2 supplementation prevents the development of salt-sensitive hypertension and maintains vascular endothelial function; moreover, VK2 supplementation increases the abundance of intestinal *Dubosiella* and downregulates the relative expression of *Atf4* in the thoracic aorta of mice. We conclude that ATF4 plays an important role in regulating gut microbiota and VK2 production, providing new insights into the association between ATF4 and development of salt-induced hypertension in mice, meanwhile contributing to the development for a new preventive strategy of hypertension.

Keywords: hypertension, gene regulation, gut microbiota, vitamin K_2, mice

Abbreviations: ANGI, angiotensin I; ATF4, activating transcription factor 4; ELISA, enzyme-linked immunosorbent assay; ET-1, endothelin-1; IBD, inflammatory bowel disease; NO, nitric oxide; OTUs, operational taxonomic units; SBP, systolic blood pressure; TEM, transmission electronic microscope; VEGF, vascular endothelial growth factor; VK2, vitamin K_2.

INTRODUCTION

Hypertension is a systemic disease characterized by elevated arterial pressure and can be accompanied by damage to the heart, brain, kidney, blood vessels, and other target organs (Wang et al., 2018). According to a previous study, in China, the prevalence rate of hypertension in adults over 18 years of age is 23.2%, and the number of patients is 245 million; the prevalence rate of high blood pressure is 41.3%, and the number of patients is 435 million (Wang et al., 2018). Hypertension has become the main risk factor for the incidence of and death due to cardiovascular and cerebrovascular diseases such as stroke and coronary heart disease in China (Wang et al., 2018). Hypertension is a complex disease affected by both genetic and environmental factors, and salt intake is one of the important environmental factors. Individual blood pressure responses to salt load or salt limitation differ within the population, and the phenomenon called salt sensitivity is observed (Wilck et al., 2017; Paczula et al., 2019). Excessive salt intake leads to vascular endothelial dysfunction, which promotes and sustains the occurrence and development of hypertension—one of the pathogenesis of salt-sensitive hypertension (Wilck et al., 2017; Bier et al., 2018). Therefore, study of the occurrence and development of hypertension and effective prevention and treatment of hypertension constitute an important issue.

Activating transcription factor 4 is a member of the ATF/CREB transcription factor family and is associated with endoplasmic reticulum stress, apoptosis, and autophagy (Tameire et al., 2019; Xu et al., 2019; Wu et al., 2020). A previous study found that blocking the expression of ATF4 could promote cancer cells to produce much proteins and die (Tameire et al., 2019). A multi-group analysis of transcriptome, proteome, and metabolomics suggested that ATF4 was a key regulator of mitochondrial stress response in mammals, which provided a theoretical basis for the study of mitochondrial dysfunction and other related diseases (Quirós et al., 2017). Another study found that ATF4 directly regulated the expression of SLC1A5, which affected the levels of glutamine and expression of antimicrobial peptides in intestinal cells; thus, ATF4 may regulate the mechanism of IBD (Hu et al., 2019). In recent years, the research done at our research center has mainly focused on the relationship between vascular endothelial dysfunction and hypertension, including endothelial inflammatory response, endoplasmic reticulum stress, apoptosis, and miRNA mechanism. A study of cases of hypertension showed that ATF4 was the target of miR-1283—an miRNA associated with hypertension—and significant differences were observed between patients with hypertension and healthy volunteers (He et al., 2016a,b). However, the specific mechanism of action of ATF4 in the development of hypertension remains unclear. Therefore, we use a novel approach to investigate the association between ATF4 and hypertension and the mechanism of action of ATF4.

Previous studies have shown that gut microbiota disorders are closely associated with hypertension (Jing et al., 2017; Touyz and Camargo, 2019). Gut microbiota not only plays a role in regulating blood pressure but also regulates immune, neurological, and endocrine functions through their metabolites (Hendrik et al., 2019; Tang et al., 2019). For example, propionate can effectively reduce inflammation, atherosclerosis, and hypertensive cardiac remodeling and protect target organs (Hendrik et al., 2019). VK2—a gut microbial metabolite—suppresses inflammation, inhibits vascular smooth muscle cell apoptosis and prevents vascular calcification, and it is also associated with several cardiovascular diseases (Bhalerao and Clandinin, 2012; Vos et al., 2012; Ren et al., 2020). Bentley et al. illustrated the VK2 biosynthesis pathway related to bacteria in 1971 (Bentley and Meganathan, 1982; Ren et al., 2020). Ponziani et al. (2017) found gut microbiota were the main source of VK2 in humans, and small-intestinal bacterial overgrowth was associated with altered VK2 metabolism. Moreover, a higher intake of VK2 produced by gut microbiota was associated with lower risk of coronary heart disease (Haugsgjerd et al., 2020). Studies have shown that gut microbiota may interact with ATF4 through metabolites, and intestinal bacteria-related proteins are associated with ATF4 (Hodin et al., 2011; Sakai et al., 2019). Hu et al. (2019) found that ATF4 directly regulates the transcriptional activation of glutamine in intestinal epithelial cells, thus maintaining the function of Paneth cells secreting antimicrobial peptides, suggesting a role of ATF4 in maintaining the intestinal microenvironment. Therefore, it is of great importance to study the mechanisms of ATF4 involvement in the development of hypertension from the perspective of gut microbiota. On the basis of previous studies, we hypothesize that ATF4 regulates the balance of gut microbiota and mediates VK2 expression, which leads to endothelial dysfunction, thus participating in the development of hypertension.

In the present study, wild-type (WT) C57BL/6J mice were used to construct *Atf4* knockout (KO) ($Atf4^{\pm}$) and *Atf4* overexpression mice using CRISPR-cas9 and lentiviral vectors. High-salt (8% NaCl)-induced mice were subjected to fecal microbiota transplantation (FMT) from $Atf4^{\pm}$ mice and VK2 intervention. The cartoon of experimental design to investigate the potential mechanism of ATF4 participating in high-salt diet-induced hypertension is shown in **Figure 1**.

MATERIALS AND METHODS

Animals and Study Design

All mice used in the study were of the C57BL/6J background. WT male mice (10–12-week-old) were purchased from the Experimental Animal Center of Guangzhou University of traditional Chinese Medicine. *Atf4* KO mice were established using theCas9/RNA system gene targeting technology by the Nanjing Biomedical Research Institute of Nanjing University (item no: T002330, Nanjing, China). Using the Cas9/RNA system gene targeting technology, sgRNA targeting ATF4 Exon2–3 gene was constructed, and Cas9 protein was directed to cut DNA double strand at specific sites of crRNA guided sequence target, thus resulting in sequence deletion. After microinjection of gRNA and cas9 into fertilized eggs of WT mice, F0 mice were produced

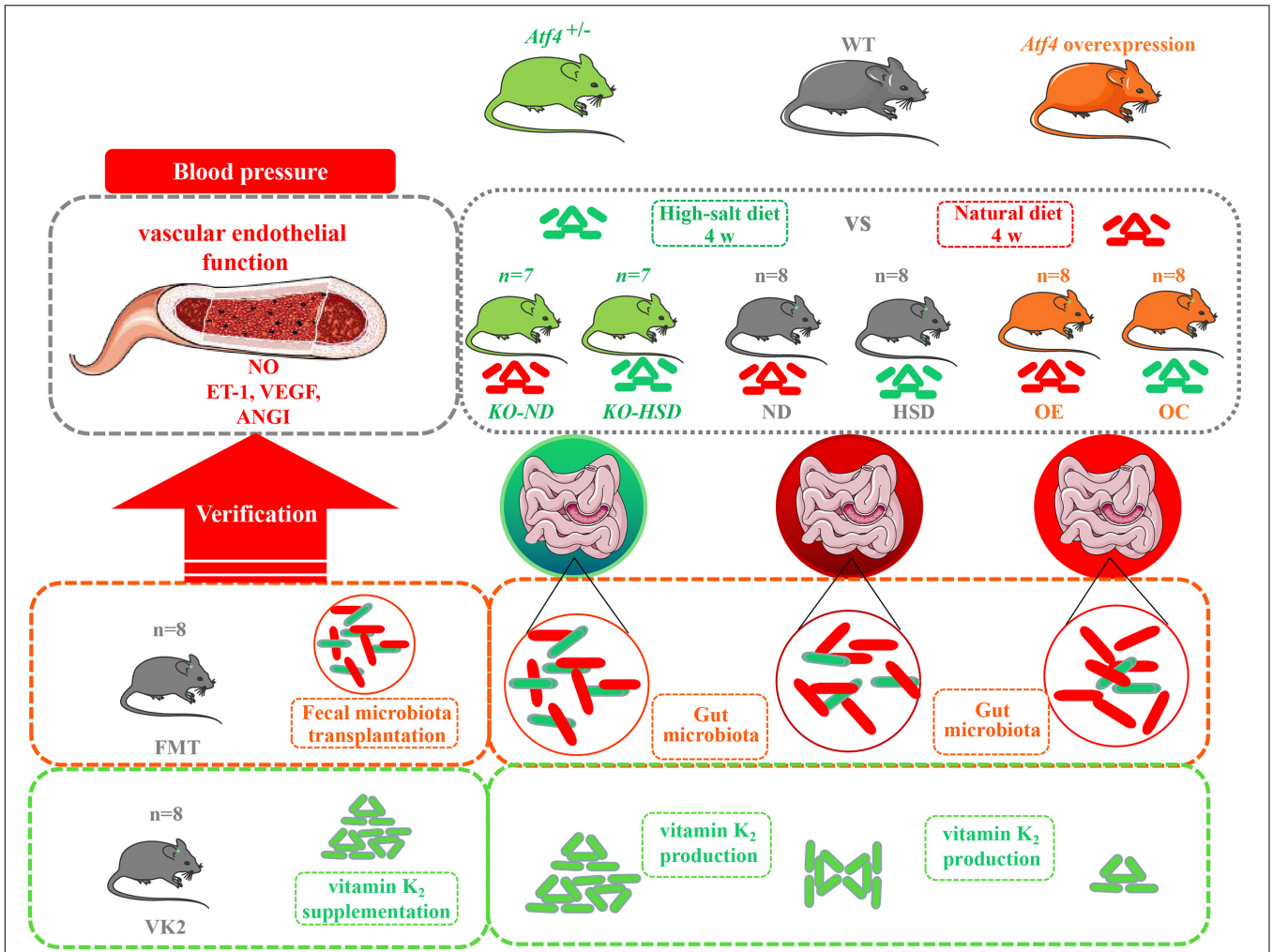

FIGURE 1 | The cartoon of experimental design to investigate the potential mechanism of ATF4 participating in high-salt diet-induced hypertension. Wild-type (WT) C57BL/6J mice, *Atf4* knockout (*Atf4*$^{\pm}$) and *Atf4* overexpression mice were used and high-salt-diet (8% NaCl) for 4 weeks was used to induce hypertensive mice. Then, fecal microbiota transplantation from *Atf4*$^{\pm}$ mice and vitamin K_2 supplementation were separately carried out to verify the gut microbiota-related evidence of the association between ATF4 and blood pressure. ATF4, activating transcription factor 4; KO, *Atf4*$^{\pm}$ mice; WT, wild-type mice; NO, nitric oxide; VEGF, vascular endothelial growth factor; ANGI, angiotensin I; ET-1, endothelin-1; KO-ND, *Atf4*$^{\pm}$ mice with natural diet; KO-HSD, *Atf4*$^{\pm}$ mice with high-salt diet; ND, WT mice with natural diet; HSD, WT mice with high-salt diet; OE, *Atf4* overexpression with high-salt diet; OC, *Atf4* overexpression control with high-salt diet; FMT, fecal microbiota transplantation; VK2, vitamin K2.

after transplantation. The F1 generation mice were obtained by mating male and female of F0 generation mice and identified as positive. The F2 generation was obtained by mating male and female of F1 generation mice and identified as positive. Finally, male F2 heterozygous mice (10–12-week-old) were used for subsequent experiments. The use of the *Atf4*$^{\pm}$ heterozygous mice was based on the fact that *Atf4*$^{-/-}$ null mice have been also found to be mostly neonatal lethal, and even surviving mice are dwarf and immunocompromised (Masuoka and Townes, 2002; Wang et al., 2009; Cornejo et al., 2013). All mice were housed at the Experimental Animal Center of Jinan University in a pathogen-free environment under 12 h dark and 12 h light conditions. After being housed for 10 days to allow for adaptation, the KO mice were randomly grouped into two groups: *Atf4*$^{\pm}$ mice with natural diet (KO-ND) and *Atf4*$^{\pm}$ mice with high-salt diet (KO-HSD). The WT mice were randomly divided into the following groups: natural diet (ND), high-salt diet (HSD), *Atf4* overexpression with high-salt diet (OE), *Atf4* overexpression control with high-salt diet (OC), FMT, and VK2.

Establishment of *Atf4* Overexpression Mice by Lentiviral Injection

Recombinant lentivirus containing *Atf4* was purchased from GeneChem (production license: GOSL0206520, Shanghai, China). WT mice in the OE group were infected with lentivirus containing *Atf4* (1×10^6 infectious units per mice) by injection through the tail vein, whereas WT mice in the OC group were infected with the lentivirus (LVCON238) which provided by GeneChem (production license: 1341609, Shanghai, China)

by equivalent injection into the tail vein to act as the negative control (Huang et al., 2014).

Hypertension Induced by High-Salt Diet in Mice

The mouse model of hypertension was induced by a high-salt (8% NaCl) diet for 4 weeks (Zhang et al., 2018). The 8% high-salt feed was purchased from Teluofei (production license: [2014] 06092, Nantong, China) and sterilized by Nantong Michael Irradiation Co., Ltd. (sterilization batch: 19072705, Nantong, China).

Fecal Microbiota Transplantation

Fresh feces (0.5 g) were collected from *Atf4*$^{\pm}$ mice fixed to the operating table and were placed into a 50 mL centrifuge tube. After weighing the collected feces, normal saline was added in the ratio of 1:10 (Liu et al., 2017; Yan et al., 2018). After sufficient mixing, the fecal samples were centrifuged at 3000 r/min for 5 min. The fecal bacterial suspension was transferred to a sterile centrifuge tube with a pipette and then was administered by gavage to mice in the FMT group, within 2 h of preparation (1 mL/day per mice) (Liu et al., 2017; Yan et al., 2018).

VK2 Diet

The mice in the VK2 group were fed a high-salt diet containing 0.036% VK2 (100 mg/kg/day), which was provided by Nantong Teluofei Feed Technology Co., Ltd. (production license: [2014] 06092, Nantong, China) and sterilized by Nantong Michael Irradiation Co., Ltd. (sterilization batch: 19072705). VK2 was purchased from Sanitary (China) Pharmaceutical Co., Ltd. (registration certificate: h20100462).

Systolic Blood Pressure Measurements

Systolic blood pressure of the caudal artery was measured manometrically using a blood pressure measurement system. When the baseline body temperature of all mice to be tested was stable at 37°C, the mice were fixed on the test platform. The measurements were taken when the waveform was stable and the measurement results were constant. The mice were placed in the mouse fixator on the test platform to warm up for approximately 3 min to allow them to adapt to the environment of the test platform, and then the tail of the mice was placed through the sensor at the bottom of the V-groove. The blood pressure analysis program (Visitech Systems, BP-2000-M) was used for the measurements. Measurements were performed 15 times per mouse. The average value was recorded and measured at 3 p.m. The blood pressure measurements were taken once a week, and all measurements were taken by the same operator.

Evaluation of Serum Levels of Endothelial Function-Related Factors by Enzyme-Linked Immunosorbent Assay (ELISA)

After 4 weeks of feeding, the mice were placed under sodium pentobarbital anesthesia to minimize suffering. Blood was collected from the eyeballs of the mice in a 5 mL blood collection tube. After 2–4 h, blood samples were centrifuged at 3000 r/min for 5 min, and the serum was collected in a sterilized centrifuge tube. The serum levels of NO, ET-1, ANGI, and VEGF were measured according to the instructions provided with the respective kits. The NO ELISA kit (production license: MM-0658M1/96T), ET-1 ELISA kit (production license: MM-0561M1/96), ANGI ELISA kit (production license: MM-0128M1/96T), and VEGF ELISA kit (production license: MM-0397M1/96T) were purchased from MEIMIAN (Yancheng, China).

Examination of Endothelial Morphology by Transmission Electronic Microscope (TEM)

After being fixed in 2.5% glutaraldehyde at 4°C for 2–4 h to minimize the mechanical injury such as traction, contusion, and extrusion, the mouse aortic tissue was fixed and rinsed three times using 0.1 M phosphate buffer Pb (pH7.4). After dehydration and infiltration, the tissue of mice was cut into 60–80 nm ultrathin sections. Then these sections were stained with uranium and lead (2% uranium acetate saturated alcohol solution, lead citrate), each for 15 min. Finally, three images were collected and analyzed from each group under a transmission electron microscope (HT7700; Hitachi; Tokyo, Japan).

Genomic DNA Extraction, 16S rRNA Gene Sequencing, and Gut Microbiota Composition Analysis

Genomic DNA was extracted from the fecal samples of mice (six mice randomly selected from each group) using the E.Z.N.A.® soil DNA Kit (Omega Bio-tek, Norcross, GA, United States) according to manufacturer's protocol. The DNA concentration and purity were determined by NanoDrop 2000 UV–vis spectrophotometer (Thermo Scientific, Wilmington, DE, United States), and DNA quality was checked using 1% agarose gel electrophoresis. The V3–V4 hypervariable regions of the bacterial 16S rRNA gene were amplified with primers 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) using a thermocycler PCR system (GeneAmp 9700, ABI, Carlsbad, CA, United States). PCR was conducted using the following program: 3 min of denaturation at 95°C; 27 cycles of 30 s at 95°C, 30 s for annealing at 55°C, and 45 s for elongation at 72°C; and a final extension for 10 min at 72°C. The PCR reactions were performed in triplicate. The reaction mixture (20 μL) contained 4 μL of 5x FastPfu buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL of FastPfu polymerase, and 10 ng of template DNA. The resulting PCR products were extracted from a 2% agarose gel and further purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, United States) and quantified using QuantiFluor™-ST (Promega, Madison, WI, United States) according to the manufacturer's protocol.

Purified amplicons were pooled in equimolar quantities and subjected to paired-end sequencing (2 × 300) on an Illumina

MiSeq platform (Illumina, San Diego, CA, United States), according to the standard protocols, by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). OTUs were clustered with 97% similarity cutoff using UPARSE (version 7.1[1]) with a novel "greedy" algorithm that performs chimera filtering and OTU clustering simultaneously. The taxonomy of each 16S rRNA gene sequence was analyzed by RDP Classifier algorithm[2] against the Silva (SSU123) 16S rRNA database using confidence threshold of 70%. Principal component analysis (PCA) and non-metric multidimensional scaling (NMDS) were performed using R package (MathSoft, Inc., United States) to display the main distribution characteristics and similarity of samples. The data of 16S rRNA gene sequencing were analyzed using the free online Majorbio I-Sanger Cloud Platform[3].

Evaluation of VK2 by ELISA

Serum VK2 was quantified using a VK2 ELISA kit provided by MEIMIAN (production license: MM-44662M1/96T, Yancheng, China) according to manufacturer's instructions.

Expression of ATF4 in Aortic Tissue

Real-Time PCR

RNA from the aortic and intestinal tissues of mice (three mice randomly selected from each group) were isolated using TRIzol (Invitrogen), and reverse transcription was carried out using the first-strand cDNA synthesis kit (TaKaRa). Real-time PCR (RT-PCR) was performed using EvaGreen dye (Biotium) in an ABI PRISM® 7500 sequence detection system. Primer sequences are summarized in **Supplementary Table 1**. The relative expression level of ATF4 mRNA was calculated using the $2^{-\Delta\Delta CT}$ method with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal reference.

Western Blotting

Total protein was extracted from the aortic and intestinal tissue of mice (three mice randomly selected from each group). Phenylmethylsulfonyl fluoride (10 μL; 100 mM) and cocktail (10 μL) were added to 1 mL cracking solution. The protein concentration was determined using the BCA protein assay kit (KGPBCA, KeyGenBiotech). Equal quantities of protein were subjected to western blot analysis. Then, the membranes were washed with Tris-HCl-Tween buffer salt solution (TBST), and the signal was enhanced by chemiluminescence. The expression of ATF4 protein was observed using the gel imager. ATF4 Monoclonal Antibody (CST 11815S), GAPDH Loading Control Antibody (CST 51332S), and Goat Anti-Mouse IgG Antibody (CST 14709S) were used. ATF4 protein expression is presented as the ratio of ATF4 protein/GAPDH.

Statistical Analysis

All data are presented as mean ± standard deviation (SD). Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software Inc., United States). Statistical comparisons were performed using Student *t*-test or one-way analysis of variance (ANOVA). $p < 0.05$ was considered as statistically significant.

[1]http://drive5.com/uparse/

[2]http://rdp.cme.msu.edu/

[3]www.i-sanger.com

RESULTS

ATF4 Altered Systolic Blood Pressure and Endothelial Function in High-Salt-Induced Mice

To validate and verify ATF4 expression, we investigated the protein and mRNA levels of ATF4 in mouse aortic and intestinal tissues using western blotting and RT-PCR, respectively. As shown in **Supplementary Figure 1**, ATF4 protein expression in *Atf4*$^{\pm}$ mice was indeed lower than that in WT mice (**Supplementary Figures 1A,B,D,E**). A high-salt diet significantly increased the ATF4 protein expression in WT mice, and overexpression of *Atf4* also significantly increased the ATF4 protein expression in WT mice (**Supplementary Figures 1A,B,D,E**). Moreover, ATF4 mRNA expression in *Atf4*$^{\pm}$ mice was lower than that in WT mice (**Supplementary Figures 1C,F**). A high-salt diet significantly increased the expression of ATF4 mRNA in WT mice. Overexpression of *Atf4* also significantly increased the expression of ATF4 mRNA in WT mice (**Supplementary Figures 1C,F**). These data suggested that ATF4 expression in *Atf4*$^{\pm}$ mice was suitable for use in the current study and ATF4 plays a pivotal role in the pathogenesis of high-salt diet in mice.

In order to investigate the association between ATF4 and blood pressure, we observed the variations in blood pressure in mice for 4 weeks. It was found that the blood pressure of WT mice increased significantly after 4 weeks of high-salt diet; the blood pressure increased to a higher degree in *Atf4* overexpression mice but did not increase in *Atf4*$^{\pm}$ mice, indicating that ATF4 contributes to the development of hypertension induced by high salt intake in mice (**Figure 2A**). A previous study showed that mechanisms of salt-sensitive hypertension were primarily derived from vascular dysfunction and characterized by endothelial dysfunction (Kurtz et al., 2016). To investigate whether the increase in blood pressure induced by a high-salt diet in mice was linked to endothelial dysfunction, we examined the endothelial function by analyzing the serum levels of NO, VEGF, ANGI, and ET-1. High-salt diet significantly reduced the serum levels of NO in WT mice; *Atf4* overexpression also significantly lowered the serum levels of NO (**Figure 2B**). High-salt diet significantly increased the serum levels of VEGF, ANGI, and ET-1 in WT mice; after overexpression of *Atf4*, high-salt diet also significantly increased higher (**Figures 2C–E**). The serum levels of VEGF and ET-1 in the *Atf4*$^{\pm}$ mice induced by high-salt diet were less than that in WT mice induced by high-salt diet, whereas the serum levels of NO in the *Atf4*$^{\pm}$ and WT mice induced by high-salt diet were not significant (**Figures 2B–E**). In addition, significantly decreased serum levels of ANGI were found in the *Atf4*$^{\pm}$ mice induced by high-salt diet and the serum levels of ANGI were less than that in WT mice induced by high-salt diet (**Figure 2D**).

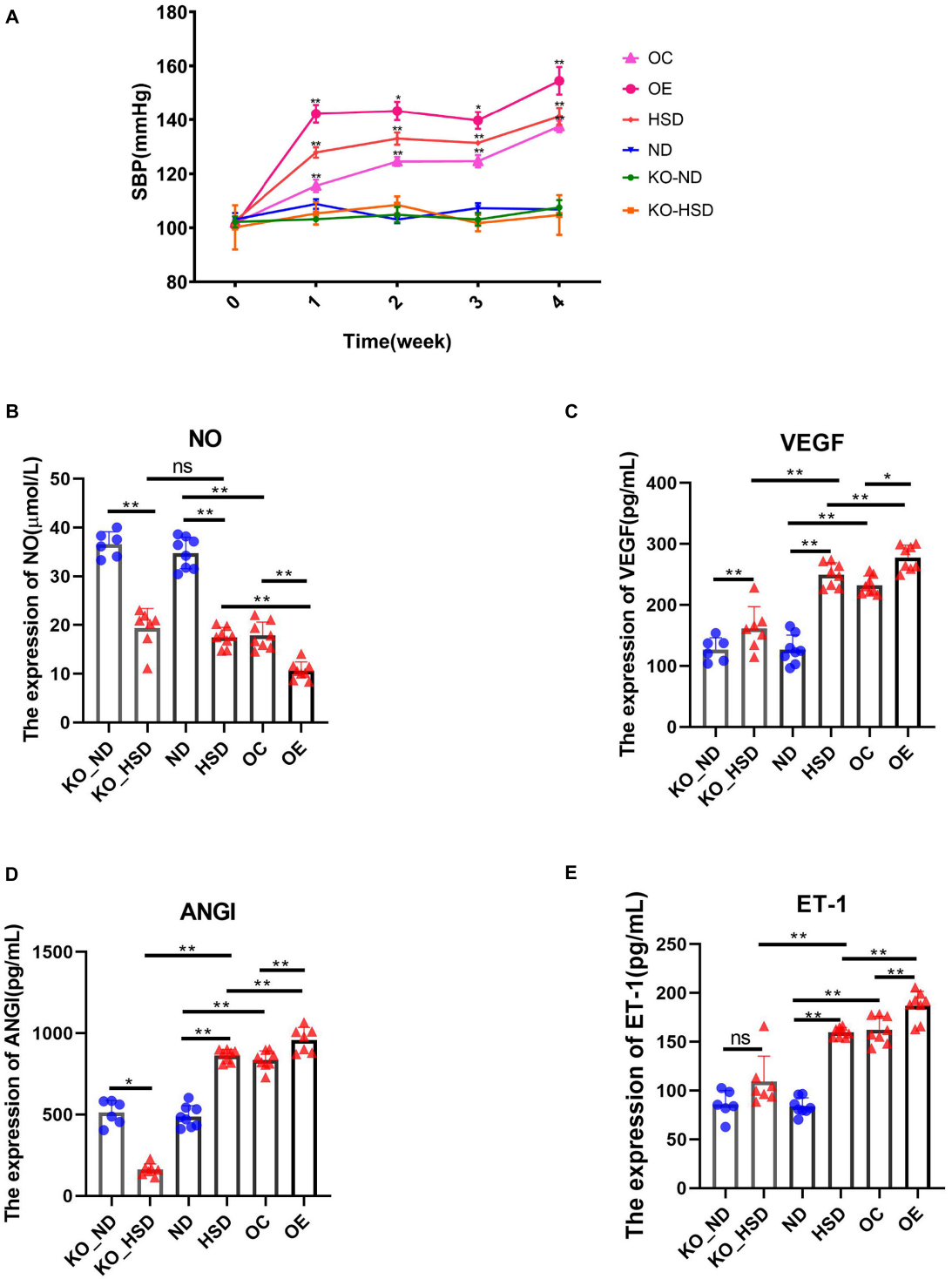

FIGURE 2 | ATF4 altered SBP and endothelial function-related factors in high-salt-induced hypertensive mice. **(A)** ATF4 altered SBP in high-salt-induced hypertensive mice. ND vs HSD, HSD vs OE, OE vs OC, $*p < 0.05$ and $**p < 0.01$. **(B)** ATF4 altered the expression of NO in high-salt-induced hypertensive mice. **(C)** ATF4 altered the expression of VEGF in high-salt-induced hypertensive mice. **(D)** ATF4 altered the expression of ANGI in high-salt-induced hypertensive mice. **(E)** ATF4 altered the expression of ET-1 in high-salt-induced hypertensive mice. Data are presented as mean ± SD; ns, no significance, $*p < 0.05$ and $**p < 0.01$, $n = 6–8$; statistical comparisons were performed using Student *t*-test or one-way analysis of variance (ANOVA). ATF4, activating transcription factor 4; KO, *Atf4*$^{\pm}$ mice; WT, wild-type mice; SBP, systolic blood pressure; NO, nitric oxide; VEGF, vascular endothelial growth factor; ANGI, angiotensin I; ET-1, endothelin-1; KO-ND, *Atf4*$^{\pm}$ mice with natural diet; KO-HSD, *Atf4*$^{\pm}$ mice with high-salt diet; ND, WT mice with natural diet; HSD, WT mice with high-salt diet; OE, *Atf4* overexpression with high-salt diet; OC, *Atf4* overexpression control with high-salt diet.

ATF4 Altered the Gut Microbiota Composition and VK2 Levels in High-Salt-Induced Hypertensive Mice

To exhibit the mechanisms of action of ATF4 in the development of hypertension induced by high salt in mice, we analyzed composition of the gut microbiota and serum levels of the microbial metabolite VK2 in high-salt-induced hypersensitive mice. As shown in **Figures 3A,B**, the species of dominant phyla and genera of ATF4 participation in the development of hypertension induced by high salt in mice did not change, but the relative abundance of dominant phyla and genera changed.

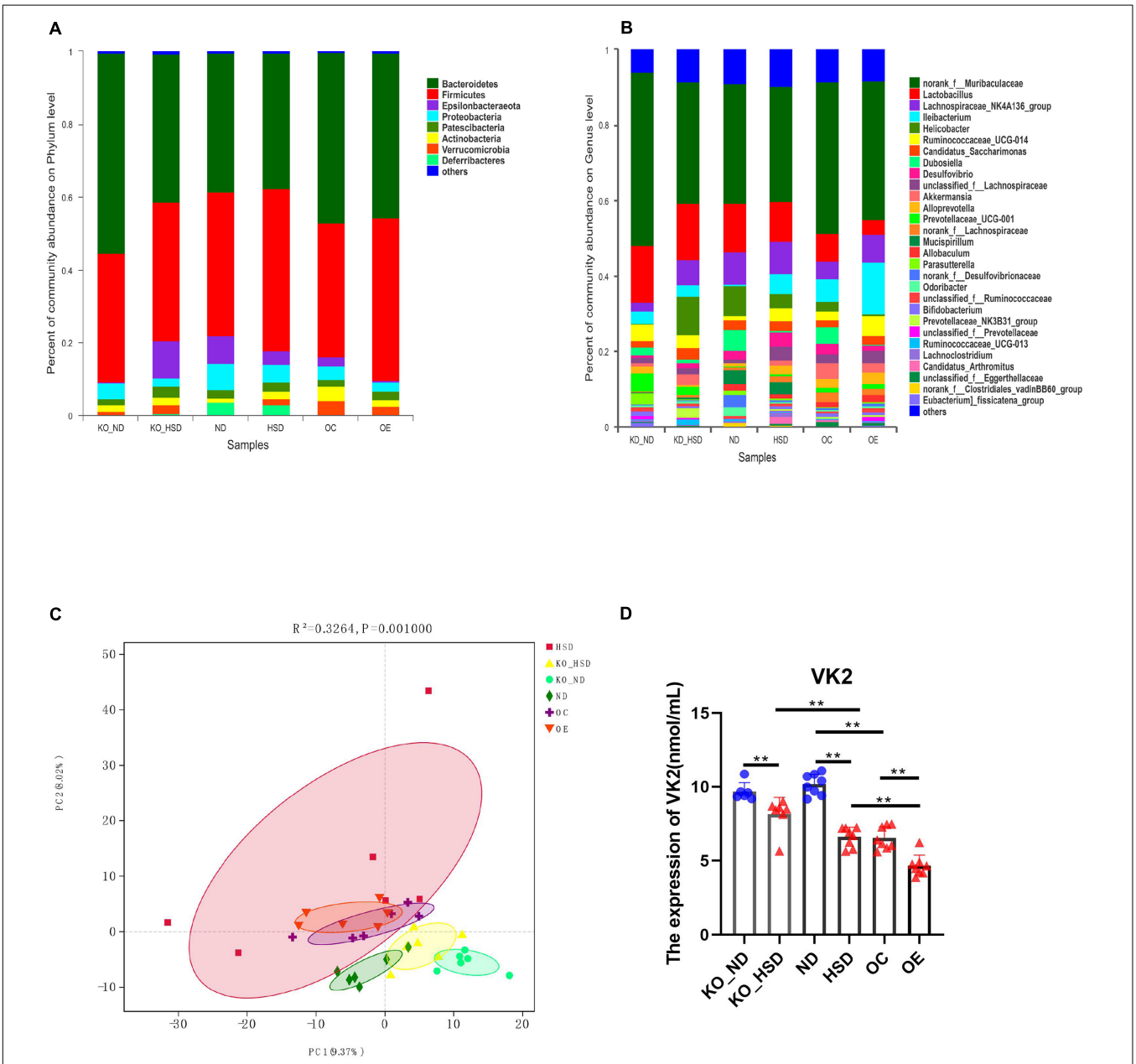

FIGURE 3 | ATF4 altered the composition of gut microbiota and expression levels of the microbial metabolite VK2 in high-salt-induced hypertensive mice. **(A)** Relative abundance plots displaying the differences in the microbial community structure at the phylum level. **(B)** Relative abundance plots displaying the differences in the general microbial community structure. **(C)** PCA plots were used to visualize differences in weighted UniFrac distances of samples of OTUs from different groups ($R^2 = 0.3264$, $p = 0.001$). **(D)** ATF4 altered the expression of VK2. Data are presented as mean ± SD; $^{**}p < 0.01$, $n = 6–8$; statistical comparisons were performed using Student t test or one-way analysis of variance (ANOVA). ATF4, activating transcription factor 4; KO, *Atf4*$^{\pm}$ mice; WT, wild-type mice; SBP, systolic blood pressure; NO, nitric oxide; VEGF, vascular endothelial growth factor; ANGI, angiotensin I; ET-1, endothelin-1; KO-ND, *Atf4*$^{\pm}$ mice with natural diet; KO-HSD, *Atf4*$^{\pm}$ mice with high-salt diet; ND, WT mice with natural diet; HSD, WT mice with high-salt diet; OE, *Atf4* overexpression with high-salt diet; OC, *Atf4* overexpression control with high-salt diet.

The PCA plot revealed significant differences in gut microbial community structure of ATF4 participation in the development of hypertension induced by high salt in mice ($R^2 = 0.3264$, $p = 0.001$; **Figure 3C**). Using ELISA, we examined whether the serum levels of the microbial metabolite VK2 differed among groups. It was found that a high-salt diet significantly reduced the expression of VK2 in WT and *Atf4*$^{\pm}$ mice after 4 weeks. Moreover, a high-salt diet significantly reduced the expression of VK2 in the *Atf4* overexpression mice. In addition, the decrease of VK2 in the WT mice induced by high salt intake was lower than that in the *Atf4*$^{\pm}$ mice (**Figure 3D**). These results suggest that ATF4 is involved in the regulation of gut microbiota in high-salt-induced hypertensive mice, and the regulation of blood pressure by ATF4 may be associated with gut microbiota and VK2.

Microbial Correlation

Spearman correlation analysis of the factors associated with vascular endothelial function and gut microbiota as well as VK2 indicated that the top 20 genera, such as *Akkermansia*, *Allobaculum*, *Dubosiella*, *Ileibacterium*, *Lactobacillus*, *Mucispirillum*, *Parasutterella*, and *Ruminococcaceae_UCG-014*, showed a positive or negative correlation with levels of NO, VEGF, ANGI, ET-1, and VK2 (**Figure 4A**). Additionally, RDA correlation analysis showed a positive correlation between VK2 and NO levels and a negative correlation between VK2 levels and VEGF, ANGI, and ET-1 levels (**Figure 4B**).

Fecal Microbiota Transplantation From *Atf4*$^{\pm}$ Mice Improved Systolic Blood Pressure and Altered Endothelial Function-Related Factor Expression in High-Salt-Induced Hypertensive Mice

To verify whether involvement of ATF4 in BP and endothelial function is dependent on the regulation of gut microbiota in high-salt-induced hypertensive mice, we transplanted the fecal microbiota of *Atf4*$^{\pm}$ mice into WT mice fed with a high-salt diet. The results showed that blood pressure of the WT mice induced by the high-salt diet increased over time, whereas blood pressure of the fecal microbiota transplanted mice did not significantly increase (**Figure 5A**). The serum levels of NO in the WT mice induced by the high-salt diet significantly decreased after 4 weeks and significantly increased after FMT (**Figure 5B**). In addition, the serum levels of VEGF, ANGI, and ET-1 in the WT mice induced by the high-salt diet significantly increased after 4 weeks and significantly decreased after FMT (**Figures 5C–E**). Moreover, the results of ultrastructural changes by TEM were shown in **Figure 5F**. Compared with the ND group, the results showed that the vascular endothelial cells in HSD had obvious edema, the intracellular matrix was obviously lightened, the electron density of large area was decreased, and the internal elastic lamina was partially broken; the rough endoplasmic reticulum was obviously expanded and degranulated; the nucleus showed local depression; the number of mitochondrion was small and swollen, and the mitochondrial cristae became shorter and disappeared; the intercellular space was significantly widened; there were tight junctions and autophagy. After FMT, the cell membrane of vascular endothelial cells was relatively intact; the nucleus was irregular and heterochromatin was edge gathered; the mitochondrion and the internal elastic lamina were not obviously broken (**Figure 5F**). All these findings suggested that the involvement of ATF4 in the regulation of blood pressure and endothelial function is a result of the gut microbiota in salt-sensitive hypertensive mice.

Fecal Microbiota Transplantation From *Atf4*$^{\pm}$ Mice Altered the Gut Microbiota Composition and VK2 Levels in High-Salt-Induced Hypertensive Mice

To ensure successful transplantation, a gut microbiota analysis was performed by 16S rRNA gene sequencing. The results showed that there were 638 OTUs in the feces of *Atf4*$^{\pm}$ mice, 713 OTUs in the WT mice, 854 OTUs in the high-salt-diet-induced WT mice, and 750 OTUs in mice in the FMT group (**Figure 6A**). Thus, the gut microbiota composition differed before and after transplantation (**Figures 6B–D**), which could be significantly distinguished at the genus level (**Figure 6D**). Furthermore, we found 11 different genera based on the higher relative abundance and greater difference; the relative abundance of six of these differed significantly among groups (**Figure 6E** and **Table 1**; $p < 0.05$). The findings suggested that FMT from *Atf4*$^{\pm}$ mice could decrease the relative abundance of *norank_f__Muribaculaceae*, *Lactobacillus*, *Dubosiella*, *Alloprevotella*, *Allobaculum*, and *[Eubacterium]_xylanophilum_group* and increase the relative abundance of *Lachnoclostridium*, *norank_f__F082*, *Lachnospiraceae_UCG-006* and *GCA-900066575* in high-salt-induced hypertensive mice. Additionally, *Dubosiella* was selected according to the higher relative abundance, the higher fold change and the p-value < 0.05 at the same time, which suggest that *Dubosiella* may be associated with the development of salt-sensitive hypertension in which ATF4 is involved. Moreover, FMT significantly upregulated the serum levels of VK2 in the WT mice induced by the high-salt diet after 4 weeks (**Figure 6F**), indicating that the changes in VK2 resulted from the observed differences in the microbiota associated with ATF4.

VK2 Maintained Systolic Blood Pressure and Endothelial Function-Related Factors in High-Salt-Induced Hypertensive Mice

To assess whether VK2 was a target metabolite that may be associated with the regulation of blood pressure and endothelial function, VK2 was added into the high-salt diet to induce the mice. The results confirmed that VK2 supplementation could significantly maintain blood pressure in high-salt-induced mice (**Figure 7A**). Moreover, VK2 supplementation significantly increased the serum levels of NO in the high-salt-induced hypertensive mice (**Figure 7B**) and significantly decreased the serum levels of VEGF, ANGI, and ET-1 in hypertensive mice induced by the high-salt diet after 4 weeks

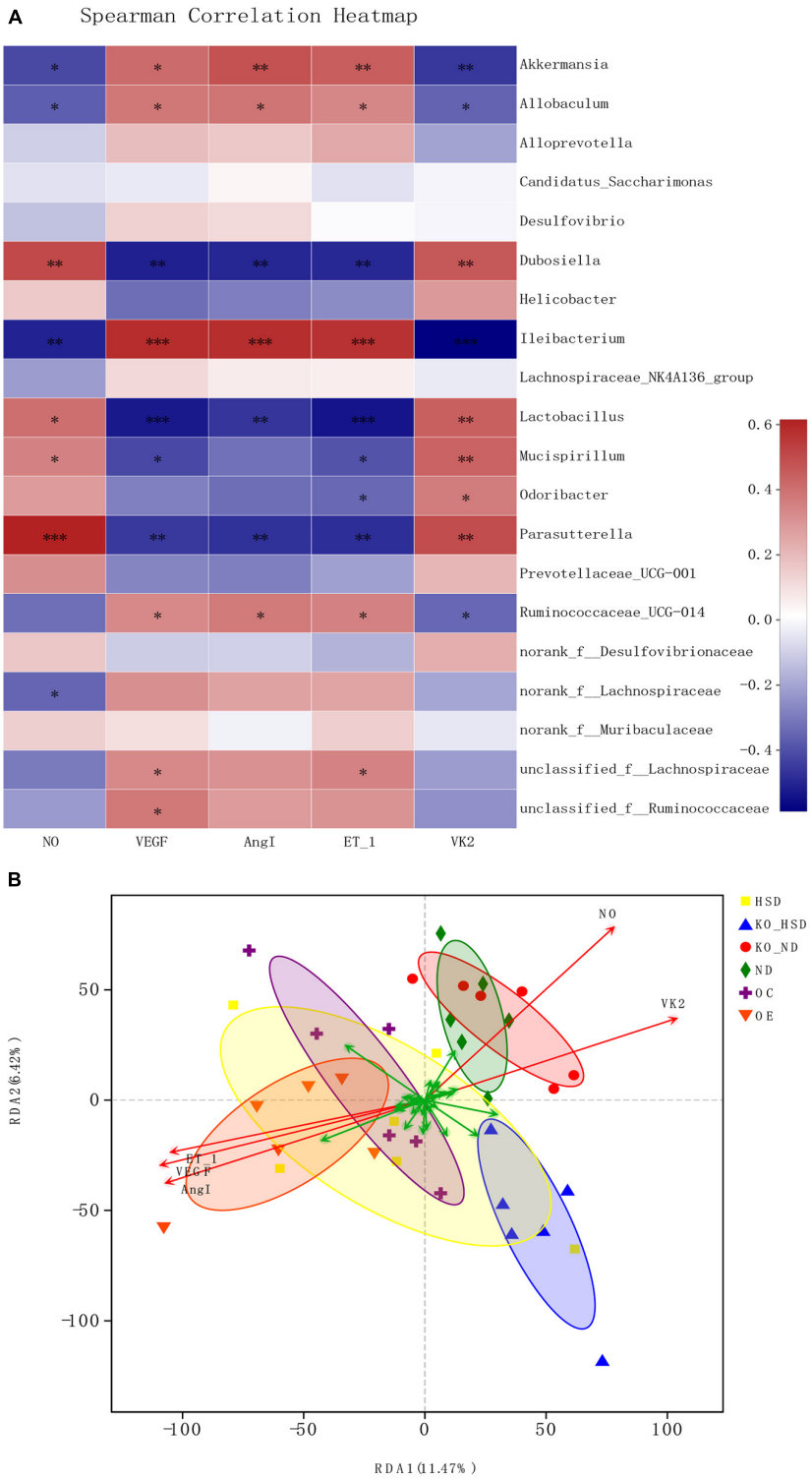

FIGURE 4 | Microbial correlation in high-salt-induced hypertensive mice. **(A)** Spearman correlation analysis between top 20 genera and environmental factors. Red represents positive correlation, whereas blue represents negative correlation; the darker the color, the stronger the correlation. $^{*}0.01 < p \leq 0.05$, $^{**}0.001 < p \leq 0.01$, $^{***}p \leq 0.001$. **(B)** RDA analysis of environmental factors. The angle between the arrows of different environmental factors represents positive and negative correlations (acute angle: positive correlation; obtuse angle: negative correlation; right angle: no correlation). ATF4, activating transcription factor 4; KO, *Atf4*$^{\pm}$ mice; WT, wild-type mice; SBP, systolic blood pressure; NO, nitric oxide; VEGF, vascular endothelial growth factor; ANGI, angiotensin I; ET-1, endothelin-1; KO-ND, *Atf4*$^{\pm}$ mice with natural diet; KO-HSD, *Atf4*$^{\pm}$ mice with high-salt diet; ND, WT mice with natural diet; HSD, WT mice with high-salt diet; OE, *Atf4* overexpression with high-salt diet; OC, *Atf4* overexpression control with high-salt diet.

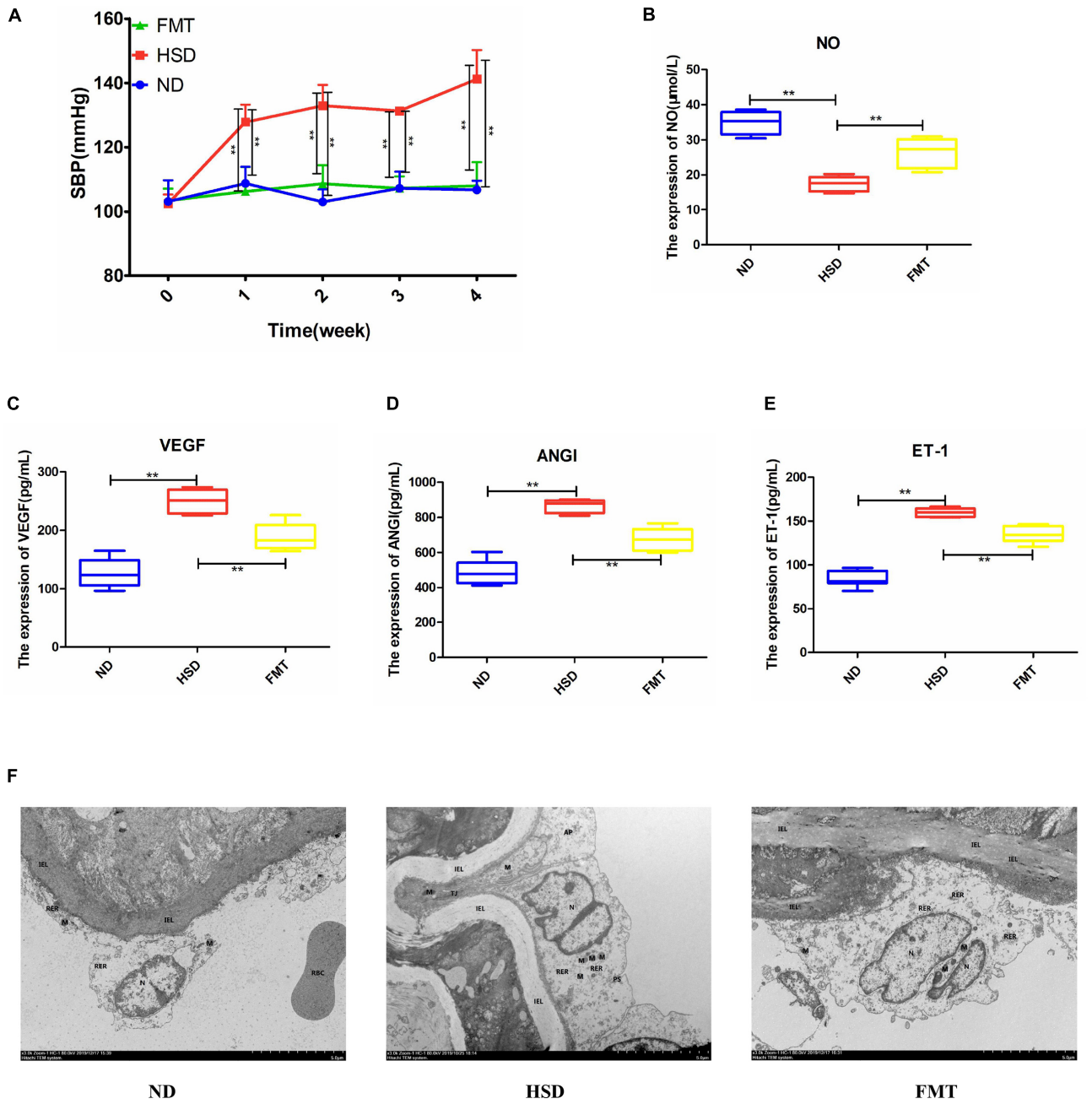

FIGURE 5 | FMT using *Atf4*$^{\pm}$ mice fecal microbiota improved SBP and altered expression of endothelial function-related factors in high-salt-induced hypertensive mice. **(A)** FMT maintained SBP. **(B)** FMT upregulated the expression of NO. **(C)** FMT downregulated the expression of VEGF. **(D)** FMT downregulated the expression of ANGI. **(E)** FMT downregulated the expression of ET-1. **(F)** Examination of endothelial morphology by transmission electronic microscope (TEM). IEL, internal elastic lamina; N, nucleus; M, mitochondrion; RER, rough endoplasmic reticulum; TJ, tight junction; AP, autophagy. Data are presented as mean ± SD; $^{**}p < 0.01$, $n = 6$–8; statistical comparisons were performed using Student *t*-test or one-way analysis of variance (ANOVA). FMT, fecal microbiota transplantation; SBP, systolic blood pressure; NO, nitric oxide; VEGF, vascular endothelial growth factor; ANGI, angiotensin I; ET-1, endothelin-1; ND, WT mice with natural diet; HSD, WT mice with high-salt diet.

(**Figures 7C–E**). Further analysis of serum VK2 confirmed that higher VK2 levels could be detected in the serum after high-salt diet containing VK2 was fed to mice for 4 weeks (**Supplementary Figure 2**). Furthermore, VK2 supplementation significantly increased the abundance of *Dubosiella*, which may be associated with the alterations in blood pressure and endothelial function due to ATF4 (**Supplementary Figure 2**). In addition, VK2 supplementation significantly decreased ATF4 expression (protein and mRNA levels) in high-salt-induced hypertensive mice (**Supplementary Figure 3**).

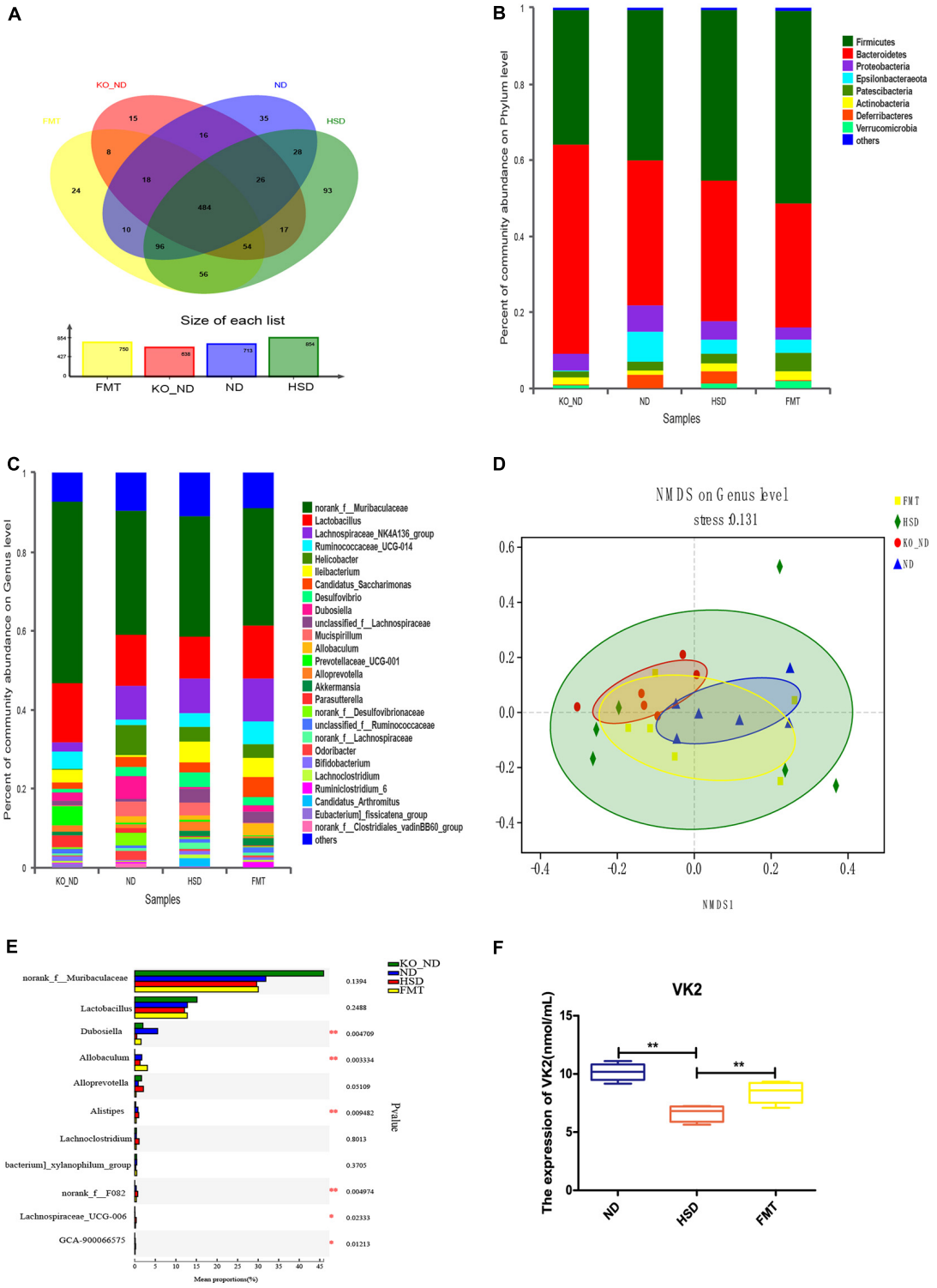

FIGURE 6 | FMT using *Atf4*$^{\pm}$ mice fecal microbiota altered the composition of gut microbiota and expression of the microbial metabolite VK2 in high-salt-induced hypertensive mice. **(A)** Venn diagram of OTUs. Different colors represent different groups. **(B)** Relative abundance plots displaying the differences in the microbial community structure at the phylum level. **(C)** Relative abundance plots displaying the differences in the general microbial community structure. **(D)** NMDS plots were used to visualize differences in weighted UniFrac distances at the genus level of samples from different groups. **(E)** The 11 taxonomic groups associated with FMT. $^{*}p < 0.05$ and $^{**}p < 0.01$. **(F)** FMT upregulated the expression of VK2. Data are presented as mean ± SD; $^{**}p < 0.01$, $n = 6$–8; statistical comparisons were performed using Student *t*-test or one-way analysis of variance (ANOVA). FMT, fecal microbiota transplantation; VK2, vitamin K_2; KO-ND, *Atf4*$^{\pm}$ mice with natural diet; ND, WT mice with natural diet; HSD, WT mice with high-salt diet.

TABLE 1 | Relative abundance of fecal microbiota transplantation-related taxonomic groups (Mean ± SD; $n = 6$).

Name	KO_ND (%)	ND (%)	HSD (%)	FMT (%)	*p* value	Corrected *p*-value
norank_f__Muribaculaceae	45.93 ± 10.81	31.86 ± 12.8	29.63 ± 17.38	30.03 ± 9.48	0.1394	0.2993
Lactobacillus	15.14 ± 11.82	12.79 ± 6.729	12.1 ± 23.25	12.76 ± 10.61	0.2488	0.4555
Dubosiella	1.985 ± 0.5682	5.578 ± 2.641	0.4698 ± 0.4571	1.533 ± 0.6933	0.004709	0.03755
Allobaculum	0.005346 ± 0.005733	1.726 ± 1.028	1.292 ± 1.076	3.058 ± 2.642	0.003334	0.03396
Alloprevotella	1.672 ± 0.6219	0.8547 ± 0.3379	2.112 ± 2.561	0.327 ± 0.2928	0.05109	0.157
Alistipes	0.2481 ± 0.1307	0.7732 ± 0.2805	0.9667 ± 0.656	0.351 ± 0.1823	0.009482	0.06099
Lachnoclostridium	0.4284 ± 0.3213	0.3939 ± 0.1745	1.058 ± 1.364	0.3555 ± 0.2004	0.8013	0.8143
[Eubacterium]_xylanophilum_group	0.4528 ± 0.2188	0.4992 ± 0.3265	0.2361 ± 0.3438	0.4957 ± 0.496	0.3705	0.466
norank_f__F082	0.005244 ± 0.008202	0.3672 ± 0.2363	0.7269 ± 1.189	0.398 ± 0.322	0.004974	0.03755
Lachnospiraceae_UCG-006	0.027 ± 0.02584	0.06006 ± 0.03032	0.364 ± 0.3993	0.1974 ± 0.2518	0.02333	0.1019
GCA-900066575	0.006198 ± 0.004577	0.1095 ± 0.2425	0.2101 ± 0.3947	0.1041 ± 0.07371	0.01213	0.06937

KO-ND, Atf4$^{\pm}$ mice with natural diet; ND, WT mice with natural diet; HSD, WT mice with high-salt diet; FMT, fecal microbiota transplantation.

DISCUSSION

In the present study, we investigated the gene *Atf4* on the basis of previous studies and found it to be associated with hypertension (He et al., 2016a,b). Research showed vascular endothelial dysfunction could promote and sustain the occurrence and development of hypertension (Wilck et al., 2017; Bier et al., 2018). A previous study showed that mechanisms of salt-sensitive hypertension were primarily derived from vascular dysfunction and characterized by endothelial dysfunction (Kurtz et al., 2016). Studies have shown that NO, VEGF, ANGI, and ET-1 were related to vascular endothelial function (Xu et al., 2004; Rufaihah et al., 2017; Binder et al., 2020). To investigate whether the increase in blood pressure induced by a high-salt diet in mice was linked to endothelial dysfunction, we examined the endothelial function by analyzing the serum levels of NO, VEGF, ANGI, and ET-1. Moreover, gut microbiota could play a role in regulating blood pressure (Hendrik et al., 2019; Tang et al., 2019). Previous studies have not reported whether ATF4 is associated with endothelial function and blood pressure. Therefore, we sought to use *Atf4*$^{\pm}$ and WT mice to determine whether ATF4 regulates the blood pressure and endothelial function in high-salt diet-induced mice and to investigate the microbiota-related mechanism of action of ATF4 in hypertension. Our major findings are as follows: First, ATF4 was involved in the development of salt-sensitive hypertension accompanied by changes in blood pressure and endothelial function in mice. Second, the gut microbiota was the associated intermediary between ATF4 and salt-sensitive hypertension, indicating that ATF4-related changes in blood pressure and endothelial function are due to gut microbiota. Third, the microbial metabolite VK2 had beneficial effects on salt-sensitive hypertension, which may be a microbiota-related molecular mechanism.

Recent studies have suggested that ATF4 is a target for the prevention and treatment of a variety of diseases. For example, it is believed that inhibitors of ATF4 protein synthesis may help to find new approaches to prevent cancer and that ATF4 may become a novel potential target for the treatment of IBD, mitochondrial dysfunction, and other related diseases (Quirós et al., 2017; Hu et al., 2019). Moreover, the studies revealed the relationship between gene and blood pressure regulation. For example, G-protein-coupled estrogen receptor 1 (Gper1) is increasingly considered to be playing a role in blood pressure regulation because it acts as a receptor for microbial metabolites (Waghulde et al., 2018). Renal medullary interstitial cell (RMIC) cyclooxygenase-2 (COX-2) deficiency was found to cause salt-sensitive hypertension and papillary damage in response to chronic salt loading (Zhang et al., 2018). The results showed that the blood pressure of WT mice increased significantly after 4 weeks of high-salt diet; the blood pressure increased to a higher degree in ATF4 overexpression mice but did not increase in ATF4 knockdown mice, indicating that ATF4 contributes to the development of hypertension induced by high salt intake in mice. The present study is the first to suggest that ATF4 functions as a blood pressure regulator.

The development of microbiota-targeted emergence and therapy has become a new trend of thought for hypertension. Using high-salt-induced hypertensive mice, Wilck et al. (2017) showed that gut microbiota may serve as a potential target to counteract salt-sensitive conditions. Moreover, Bier et al. (2018) demonstrated a significant association between gut microbiota composition and blood pressure regulation; even the metabolic short-chain fatty acids (SCFAs) may highlight another aspect of the complex interaction between diet, gut, and blood pressure. Additionally, it has been suggested that gut microbiota composition and microbial metabolites are the potential mediators between a single gene associated with blood pressure regulation and hypertension (Faulkner et al., 2018; Waghulde et al., 2018; Yang et al., 2018). In the present study, we found that ATF4 could regulate the gut microbiota composition and the expression of VK2 in hypersensitive mice induced by high-salt diet, suggesting that the mechanisms of ATF4 involved in blood pressure regulation and the vascular endothelial function may be related to gut microbiota and VK2.

Fecal microbiota transplantation has been widely used in several studies to verify microbiota-related mechanisms (Bárcena et al., 2019; Liao et al., 2019; Stebegg et al., 2019; Viennois et al., 2019). In the present study, FMT was performed to verify whether ATF4 involvement in the regulation of blood pressure and endothelial function is associated with

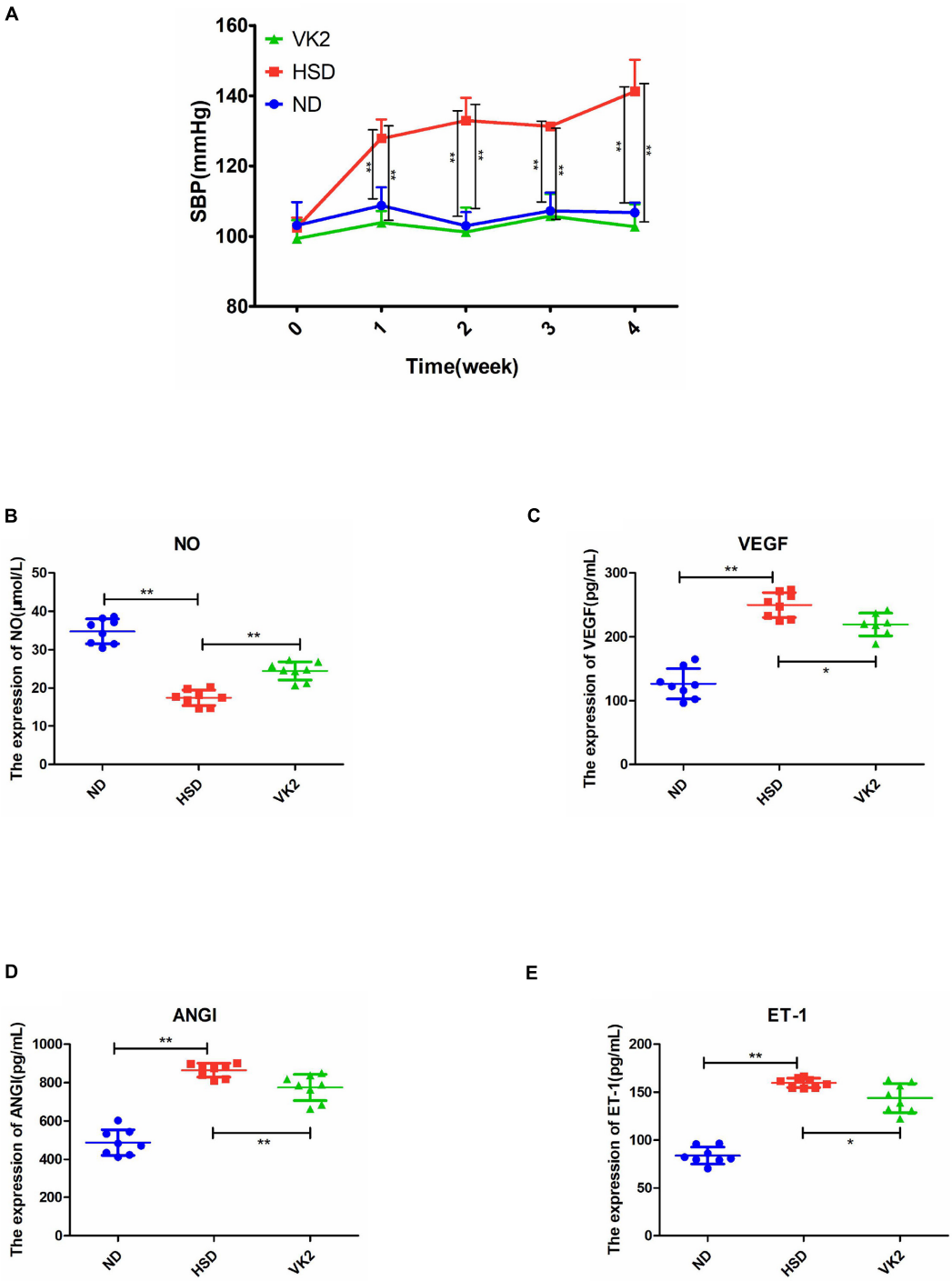

FIGURE 7 | VK2 maintained SBP and altered endothelial function-related factors in high-salt-induced hypertensive mice. **(A)** VK2 altered SBP. **(B)** VK2 upregulated the expression of NO. **(C)** VK2 downregulated the expression of VEGF. **(D)** VK2 downregulated the expression of ANGI. **(E)** VK2 downregulated the expression of ET-1. Data are presented as mean ± SD; $^{*}p < 0.05$ and $^{**}p < 0.01$, $n = 6$–8; statistical comparisons were performed using Student *t*-test or one-way analysis of variance (ANOVA). SBP, systolic blood pressure; VK2, vitamin K_2; NO, nitric oxide; VEGF, vascular endothelial growth factor; ANGI, angiotensin I; ET-1, endothelin-1; ND, WT mice with natural diet; HSD, WT mice with high-salt diet.

the gut microbiota in high-salt-induced hypertensive mice. Our data suggested that the regulation of blood pressure and endothelial function by ATF4 resulted from the gut microbiota composition in salt-sensitive hypertensive mice. Moreover, gut microbiota composition analysis showed that FMT could lower the relative abundance of *norank_*

f__Muribaculaceae, *Lactobacillus*, *Dubosiella*, *Alloprevotella*, *Allobaculum*, and *[Eubacterium]_xylanophilum_group* and increase the relative abundance of *Lachnoclostridium*, *norank_f__F082*, *Lachnospiraceae_UCG-006*, and *GCA-900066575* in high-salt-induced hypertensive mice. Furthermore, *norank_f__Muribaculaceae*, *Lactobacillus*, *Dubosiella*, *Alloprevotella*, *Allobaculum*, and *[Eubacterium]_xylanophilum_group* may function as probiotics, whereas *Lachnoclostridium*, *norank_f__F082*, *Lachnospiraceae_UCG-006*, and *GCA-900066575* may function as pathogens. Additionally, *Dubosiella* was considered as a core microbial mediator owing to its high relative abundance (Bai et al., 2019; Fastrès et al., 2020). And the significant difference in abundance among groups was found in current study, which may be associated with ATF4-regulated blood pressure. A previous study indicated that *Dubosiella* was associated with obesity (Bai et al., 2019). A high-fat diet reduced the relative abundance of *Dubosiella*, which may be an important genus for therapeutic effects (Bai et al., 2019). Moreover, the FMT study also indicated that the effects of VK2 might result from the observed changes in the gut microbiota composition associated with ATF4.

Recently, small quantities of VK2 derived from gut microbiota were shown to have a significant impact on health (Bentley and Meganathan, 1982; Bhalerao and Clandinin, 2012; Vos et al., 2012; Ponziani et al., 2017; Haugsgjerd et al., 2020; Ren et al., 2020). A case-control study revealed that an increase in arterial stiffness was associated with an increase in markers of VK2 deficiency (Mansour et al., 2019). Our data confirmed that VK2 was a target metabolite that regulated blood pressure and endothelial function. Moreover, we confirmed the VK2 was absorbed in the serum, as shown in **Supplementary Figure 2B**. Our data also suggested that VK2 was associated with *Dubosiella*, which was consistent with the previous correlation analysis (**Figure 4**), indicating that *Dubosiella* may play a role in VK2 metabolism. However, this function needs to be verified through further studies. Our data showed that VK2 significantly decreased ATF4 expression in salt-sensitive hypertensive mice. Therefore, the significant changes in gut microbiota composition and VK2 levels not only confirmed the microbiota-related mechanisms of ATF4 in regulating blood pressure but also suggested that *Dubosiella*-VK2-associated pathways may be the key biomarkers of formative and therapeutic effects in salt-sensitive hypertension. Host genetics and gut microbiota interactions contribute to the pathogenesis of a disease (Chu et al., 2016; Wang et al., 2016). In the present study, we investigated the role of ATF4 in blood pressure regulation in salt-sensitive hypertensive mice. In this study, vascular endothelial function-related factors such as NO, ET-1, ANGI, and VEGF were used to evaluate the vascular endothelial function, and the TEM was used to reflect the structural changes of vascular endothelial cell, which further confirmed the regulation of blood pressure by ATF4. Therefore, our study shows that gut microbiota and VK2 play a role in the participation of ATF4 in hypertension, and these interactions are the possible potential mechanism of ATF4 participating in high-salt diet-induced hypertension. Of course, the detailed mechanism remains to be further studied.

CONCLUSION

In summary, ATF4 plays an important role in regulating gut microbiota composition and VK2 expression, thus participating in the development of salt-sensitive hypertension, providing new insights into the association between ATF4 and development of salt-induced hypertension in mice, meanwhile contributing to the development for a new preventive strategy of hypertension.

DATA AVAILABILITY STATEMENT

The datasets generated in this study are available from the corresponding author upon request. 16S sequencing data have been uploaded to NCBI SRA with accession number PRJNA660089.

ETHICS STATEMENT

The Animal Experiment Protocol listed has been reviewed and approved by the Laboratory Animal Ethics Committee of Jinan University.

AUTHOR CONTRIBUTIONS

T-hL and L-gC designed the experiments and analyzed the data. T-hL, W-cT, and Q-eL performed the experiments. T-hL, L-gC, YX, and W-qT wrote the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.585995/full#supplementary-material

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Responses of Intestinal Microbiota and Immunity to Increasing Dietary Levels of Iron Using a Piglet Model

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Iron is an essential metal for both animals and microbiota. In general, neonates and infants of humans and animals are at the risk of iron insufficiency. However, excess dietary iron usually causes negative impacts on the host and microbiota. This study aimed to investigate overloaded dietary iron supplementation on growth performance, the distribution pattern of iron in the gut lumen and the host, intestinal microbiota, and intestine transcript profile of piglets. Sixty healthy weaning piglets were randomly assigned to six groups: fed on diets supplemented with ferrous sulfate monohydrate at the dose of 50 ppm (Fe50 group), 100 ppm (Fe100 group), 200 ppm (Fe200 group), 500 ppm (Fe500 group), and 800 ppm (Fe800), separately, for 3 weeks. The results indicated that increasing iron had no significant effects on growth performance, but increased diarrheal risk and iron deposition in intestinal digesta, tissues of intestine and liver, and serum. High iron also reduced serum iron-binding capacity, apolipoprotein, and immunoglobin A. The RNA-sequencing analysis revealed that iron changed colonic transcript profile, such as interferon gamma-signal transducer and activator of transcription two-based anti-infection gene network. Increasing iron also shifted colonic and cecal microbiota, such as reducing alpha diversity and the relative abundance of *Clostridiales* and *Lactobacillus reuteri* and increasing the relative abundance of *Lactobacillus* and *Lactobacillus amylovorus*. Collectively, this study demonstrated that high dietary iron increased diarrheal incidence, changed intestinal immune response-associated gene expression, and shifted gut microbiota. The results would enhance our knowledge of iron effects on the gut and microbiome in piglets and further contribute to understanding these aspects in humans.

Keywords: iron overload, pig, diarrhea, immunity, microbiota, *Lactobacillus*

Abbreviations: ALB, albumin; ALT, alanine aminotransferase; APOA, apolipoprotein; AST, aspartate aminotransferase; BUN, ureal; Ca, calcium; CER, ceruloplasmin; CHHOL, cholesterol; CREA, creatinine; DEG, differential expression gene; *E. coli, Escherichia coli*; GLU, glucose; GO, Gene Ontology; HDL, high-density lipoprotein; IDA, iron-deficiency anemia; IFN-γ, interferon gamma; IgA, immunoglobin A; KEGG, Kyoto Encyclopedia of Genes and Genomes; *L.amy*, *Lactobacillus amylovorus*; *L.reu*, *Lactobacillus reuteri*; LDL, low-density lipoprotein; LPA, lipoprotein; Mg, magnesium; MNPs, iron-containing micronutrient powders; OTBA, total bile acids; OTU, operational taxonomic unit; PPIs, protein–protein interactions; STAT2, signal transducer and activator of transcription 2; STFR, soluble transferrin receptors; TG, triglyceride; Th1, T helper -type 1; TP, total protein; TSFR, transferrin; UA, uric acid; UIBC, unsaturated iron-binding capacity.

INTRODUCTION

Iron is an essential metal for humans and animals, requiring an iron-containing bioactive such as heme protein, enzyme, and iron–sulfur cluster proteins, to maintain essential functions such as sensing, storing, and transporting oxygen, energy metabolism, DNA synthesis, intermediate metabolism and detoxification, and host defense. Iron inadequacy usually causes IDA, and iron deficiency in early life would lead to growth retardation and cognitive disorder (Hershko and Camaschella, 2014). Iron overload, commonly due to the genetic disorder, would induce iron accumulation, contributing to hemochromatosis, cancer, cardiovascular disease, metabolism syndrome, and neurodegenerative disease (Lonnerdal, 2017). Iron is also an indispensable growth factor for microbiota, which complicates with the host to acquire iron for survival (Barton and Acton, 2019). Thus, it is crucial to maintain iron homeostasis, dependent on iron supplementation, absorption, cycling, storage, and interaction between the host and microbiota.

Neonates and infants of humans and animals, such as piglets, are commonly at the risk of iron insufficiency because of high iron requirements, low iron stores, and inadequate dietary supplementation or poor absorptive efficiency. However, excess iron accumulation causes oxidative pressure and further damage to DNA and proteins and peroxide lipids. Prolonged iron supplementation also causes gastroenteric disorder and intestinal microbiota dyshomeostasis and would increase the risk of numerous diseases. Increased body iron stores were recognized as a feature of metabolic syndrome, and high dietary iron levels increased blood glucose levels but decreased HDL cholesterol levels. Excess dietary iron in the high-fat diet increased glucose, insulin, insulin resistance, and liver fat deposition (Choi et al., 2013). Moreover, iron-rich diets, such as red meat, promote the risk of colorectal cancer. In a randomized controlled trial, iron-containing MNP supplementation to 6-month infants for 4 months increased intestinal inflammation and gut pathogen abundance, such as pathogenic *Escherichia coli (E. coli)*, and iron at a dose of 12.5 mg/day in MNPs increased diarrhea risk than that of 2.5 mg/day (Jaeggi et al., 2015). The following controlled intervention trial revealed that MNPs given to 8- to 10-month infants interfered with antibiotic efficiency and promoted the risk of diarrhea (Paganini et al., 2019). As an excellent model for human studies of nutrition, metabolism, neurodevelopment, gut, and microbiota, pigs share many physiological structures and function with humans (Roura et al., 2016; Mudd and Dilger, 2017; Kleinert et al., 2018). Like humans, piglets born with limited iron stores, which will be deficient without exogenous sources, and weaning piglets also suffer from anemic and iron deficiency (Council, 2012; Perri et al., 2016). The dietary iron requirement for post-weaning piglets is recommended at a dose of 80 mg/kg dry matter; however, the dietary iron in commercial feed usually exceed the suggested dose up to three times (Flohr et al., 2016). High dietary iron impairs the gut by increasing intestinal permeability, malondialdehyde abundance, neutrophil infiltration, diarrheal incidence, and reducing villus height of duodenum in weanling pigs; excess iron also disrupts intestinal microbiota, such as enriching coliform bacillus and reducing *Bifidobacterium* spp. in weanling pigs (Lee et al., 2008; Li et al., 2016b). Furthermore, iron uptake is essential to maintain the virulence of pathogens; thus, sequestration of iron is a vital strategy of the host to infection. However, excess iron deposition in the gut bins host's iron sequestration strategy, leading to pathogen burst to increase the risk of infection and inflammation (Nairz and Weiss, 2020). For example, dietary iron aggravates dextran sulfate sodium-induced colonic inflammation and activates IL-6/IL-11-Stat3 signaling-induced colonic cancer development in mice (Carrier et al., 2006; Chua et al., 2013). Although these studies demonstrated the adverse effects of iron overload on the metabolism of glucose and lipid metabolism, inflammation, and microbiota, few studies revealed the deposition of dietary iron in different intestinal anatomical regions and the region-specific response of the microbiota and intestine.

This study aimed to analyze the effect of dietary gradient iron supplementation on the distribution pattern of iron in the gut lumen and body, intestinal microbiota, and intestine gene expression profile. The results would enhance our knowledge of iron distribution and its effects on the gut and microbiome in piglets and further contributes to the understanding these aspects in humans.

MATERIALS AND METHODS

Animals and Experiment Design

Sixty healthy weaning piglets (Duroc × Landrace × Landrace, 21 days old, average body weight 6.58 kg), purchased from a commensal farm (Hunan New Wellful, Co., Ltd., Changsha, China), were randomly assigned to six groups: Fe50, Fe100, Fe200, Fe500, and Fe800 groups (**Figure 1A**). The piglets in the Fe50 group were fed on a corn and soybean meat-based diet [**Supplementary Table 1**, a formula modified according to NRC 2012 (Council, 2012)], according to our previous study (Zhou et al., 2017), supplemented with 50 ppm ferrous sulfate, while the diets of piglets in Fe100, Fe200, Fe500, and Fe800 were fed our formula supplemented with 100, 200, 500, and 800 ppm ferrous sulfate monohydrate, separately. The piglets were housed individually in an environmentally controlled facility with hard plastic slatted flooring and maintained at an ambient temperature of 25 ± 2°C with free access to diets and drinking water. This experiment was last for 21 days. The body weight, feed intake, and fecal score were detected weekly, and the feed conversation ratio was calculated. The fecal consistency score was assessed by a modified method according to our previous study (Wang et al., 2019): 1 = solid, 2 = pasty, and 3 = liquid. The piglets were sacrificed on day 21 to collect blood, liver tissue, intestinal tissue (including duodenum, jejunum, ileum, and colon), and digesta of cecum and colon. The blood was centrifuged at 3,000 rpm for 10 min for serum separation. All the samples were snap-frozen in liquid nitrogen and stored at −80°C before further processing. The Animal Welfare Committee of the Institute of Subtropical Agriculture, Chinese Academy of Sciences, approved all the animal experimental procedures.

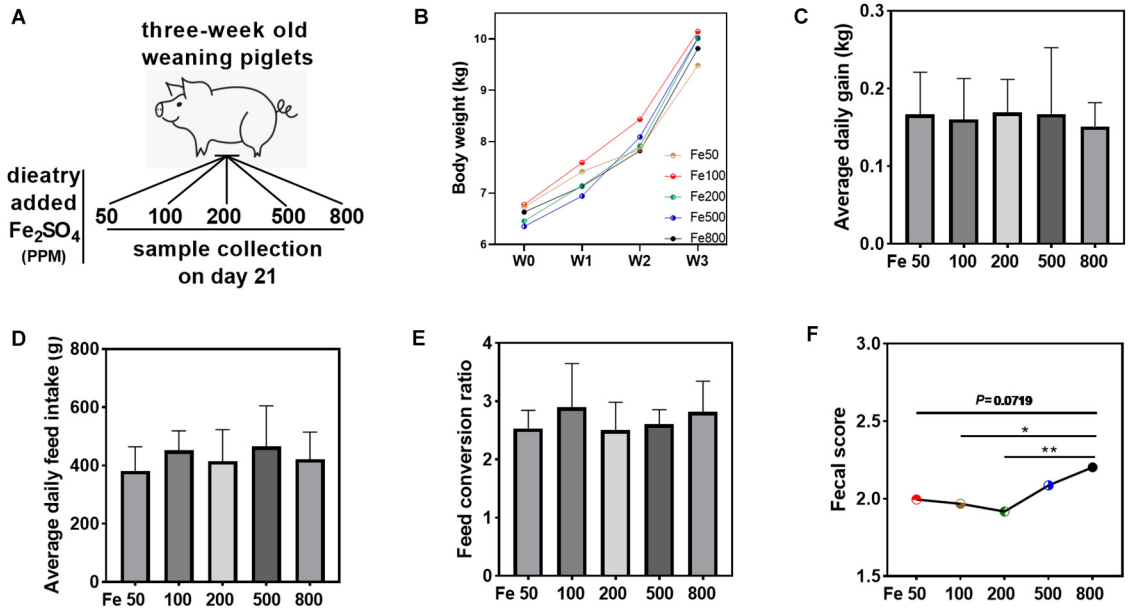

FIGURE 1 | Dietary iron had no significant effects on the growth performance but increased fecal consistency score. Growth performance and fecal consistency scores were evaluated in each group. Schematic showing dietary iron treatment of each group **(A)**. Body weight **(B)**, average daily gain **(C)**, average daily feed intake **(D)**, and feed conversion ratio **(E)** were similar among the five groups. The average fecal score of the 21 days **(F)** in the Fe800 group was also higher than that in the other groups. Ordinary one-way ANOVA or Kruskal–Wallis test was used for the statistical analysis among different groups. Data were presented as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$.

Iron Status Analysis

Iron levels in duodenal, jejunal, ileal, colonic, liver tissues, and the digesta of jejunum, ileum, cecum, colon, and feces were determined by inductively coupled plasma optical emission spectrometry (ICP-OES; ICP 720 ES; Agilent, United States) according to our previous study (Zhang et al., 2017). The intestinal digesta was freeze dried before assessed. Briefly, the samples were weighed in triplicate, subjected to acid digestion, dried at 260°C, and redissolved in 5 ml of 1% HNO_3. Then, the samples were diluted with 1% HNO_3 and subjected to ICP analyses after dilution.

Conventional Biochemical Measurements

Blood hemoglobin level was tested by the BeneCheck Hemoglobin Test System (BeneCheck, Taiwan, China). Several blood parameters related to the glucose, lipid, and iron metabolism and liver and kidney function were analyzed by Cobas c 311 analyzers (Roche Analytic Instruments, Nutley, NJ, USA.) according to the manufacturer's instructions. The detected blood parameters were as follows: iron, calcium, magnesium, unsaturated iron-binding capacity, transferrin, soluble transferrin receptors, ceruloplasmin, glucose, total bile acids, triglyceride, cholesterol, HDL, LDL, apolipoprotein, lipoprotein, total protein, alanine aminotransferase, aspartate aminotransferase, albumin, immunoglobin A, IgM, IgG, ureal, uric acid, and creatinine.

16S rDNA Sequencing Analysis of Cecal and Colonic Microbiota

16S rDNA sequencing of cecal and colonic microbiota was used for intestinal microbiota analysis according to our previous study (Wu et al., 2020). Briefly, the DNA was extracted from cecal and colonic digesta by Qiagen QIAamp DNA Stool Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendation. The V3 to V4 regions of bacterial 16S rDNA were selected to analyze using Illumina MiSeq sequencing. The library was generated and sequenced to produce 400 base pair/600 base pair single-end reads. Single-end reads were assigned to the samples based on their unique barcode, and then their barcode and the primer sequence were removed. Sequence analysis was following quality filtering. The sequences with more than 97% similarity were clustered to the same OTU by UPARSE software (v7.0.1001[1]). RDP Classifier (V2.2, Michigan State University Board of Trustees, East Lansing, MI, United States) was used for species annotations, based on the GreenGene database[2]. The MUSCLE software (Version 3.8.31) was used to analyze the phylogenetic relationships of different OTUs to reveal differences among samples and groups and for multiple-sequence alignments. The OTUs were normalized for subsequent analysis of the alpha diversity, beta diversity, and the environmental-factor correlation analysis. The 16S rDNA sequencing and data analysis were performed by a commercial company (Novogene, Co., Ltd., Beijing, China).

[1]https://drive5.com/usearch/

[2]https://greengenes.secondgenome.com

RNA-Seq-Based Reference Transcriptome Analysis of Colonic Tissue

RNA isolation was processed according to our previous study (Chen et al., 2014). Briefly, total RNA was isolated from the liquid nitrogen-frozen colon by TRIZOL regents (Invitrogen, United States) and then treated with DNase I (Invitrogen, United States) according to the manufacturer's instructions. After quantification and qualification, the RNA was used to generate sequencing libraries by NEBNext® UltraTM RNA Library Prep Kit for Illumina® (NEB, United States) according to the manufacturer's protocol and then index coded. The index-coded samples were clustered using TruSeq PE Cluster Kit v3-cBot-HS (Illumia) on a cBot Cluster Generation System following the manufacturer's recommendation and then sequenced on an Illumina Novaseq platform. One hundred fifty base pair paired-end reads were generated for the following data analysis. Hisat2 (v2.0.5) was used for read mapping to the reference genome. FPKM of each gene was calculated after counting the read numbers of each mapped gene using feature Counts (v1.5.0-p3). After gene expression level quantification, DEG analysis was performed by the DESeq2 R package (1.16.1), and P-value < 0.05 was assigned as differential expression. The clusterProfiler R package was used to implement Gene Ontology (GO) enrichment analysis and KEGG enrichment analysis of DEGs. PPI analysis of DEGs was based on the STRING database and further analyzed and visualized by Cytoscape (v3.8.0). The whole transcriptome sequencing and data analysis were performed by a commercial company (Novogene, Co., Ltd., Beijing, China).

Statistical Analyses

The data were preprocessed with Excel 2019 (Microsoft, Redmond, United States). Word 2019 software (Microsoft, Redmond, United States) was used to prepare tables, and GraphPad Prism 8.0 (GraphPad Software, Inc., La Jolla, CA, United States) was used in statistical analysis and figure generation. Ordinary one-way ANOVA or Kruskal–Wallis test was used for the statistical analysis among different groups. Unpaired t-test was used to statistically analyze between two groups if the data followed a normal distribution; otherwise, the Wilcoxon signed-rank test was used for the analysis. The results were presented as mean ± standard deviation (SD) or mean ± standard error of the mean (SEM), and P-value < 0.05 was considered as statistically significant.

RESULTS

Dietary Iron Had No Significant Effects on the Growth Performance but Increased Fecal Consistency Score

Growth performance and fecal consistency scores were evaluated to assess the effects of iron on the growth and diarrheal incidence of the piglets. The results indicated that growth parameters, including body weight, average daily gain, average daily feed intake, and feed conversion ratio, were similar among the five groups (**Figures 1B–E**). The daily fecal score was shown in **Supplementary Figure 1A**, and the fecal score on day 21 was significantly higher in the Fe800 group than in other groups (**Supplementary Figure 1B**). Meanwhile, the average fecal score on the 21 days in the Fe500 group was also higher than in the other groups (**Figure 1F**).

Dietary Iron Increased Iron Deposition in Intestinal Digesta, Intestine, Liver, and Serum

The iron concentration in the jejunal digesta was similar among different groups (**Figure 2A**). Iron levels of ileal, cecal, and colonic digesta and feces in the Fe800 group was significantly higher than in the Fe50, Fe100, Fe200 groups, and iron abundance of cecal and colonic digesta and feces in the Fe500 group was also higher compared to Fe50, Fe100, and Fe200 groups (**Figures 2B–E**). The iron content in duodenum, jejunum, colon, liver, and serum went up from the Fe50 to Fe800 group (**Figures 2F,J**), but not in the ileum (data not shown). Jejunal iron in the Fe800 and Fe500 groups was significantly higher than those of the other three groups and also higher in the Fe500 group than in the Fe50 and Fe100 groups (**Figure 2G**). Colonic iron in the Fe800 group was higher than that of the other four groups (**Figure 2H**). Liver iron in the Fe800, Fe500, and Fe200 groups was significantly increased than in the Fe50 group (**Figure 2I**). Serum iron in the Fe800 group was much higher than in the Fe50–200 groups (**Figure 2J**).

Dietary Iron Downregulated Serum Unsaturated Iron-Binding Capacity, Apolipoprotein, and Immunoglobin A

Serum UIBC went down in the Fe50 to Fe800 group, and UIBC in the Fe50 group was higher than in the Fe800 group (**Figure 3T**). HB, STFR, and CER were similar among all the groups (**Figures 3Q–S**). Serum GLU, OTBA, TG, CHOL, HDL, LDL, TP, ALT, AST, ALB, IgG, and IgM were the same among all the groups (**Figures 3A–F,H–K,M,N**), but APOA (**Figure 3G**) and IgA (**Figure 3L**) were downregulated with dietary iron increasing. Kidney function-related serum BUN and CREA were also similar among all the groups (**Figures 3O,P**). TSFR and LPA were not detectable in this study.

Dietary Iron Shifted Gut Microbiology

The data, including Raw reads, Clean Reads, Base, AvgLen, Q20, GC%, and Effective%, were generated from 16S RNA sequencing of 38 colonic digesta samples (**Supplementary Table 2**) for following OUT clustering and taxonomy annotation analysis. 1464 OTUs were detected, and 640 OTUs were the core OUTs shared among the five groups, while 96, 111, 64, 33, and 37 OTUs were specifically enriched in the Fe50, Fe100, Fe200, Fe500, and Fe800 groups, individually (**Supplementary Figure 2A**). The rarefaction curve (**Supplementary Figure 2B**) and Good's-coverage (**Table 1**) indicated that the sequencing data met the demand for further analysis. Alpha diversity indexes, including Chao, ACE, Shannon, and Simpson, were decreased with increasing iron supplementation, revealing that both microbial community richness and diversity were reduced

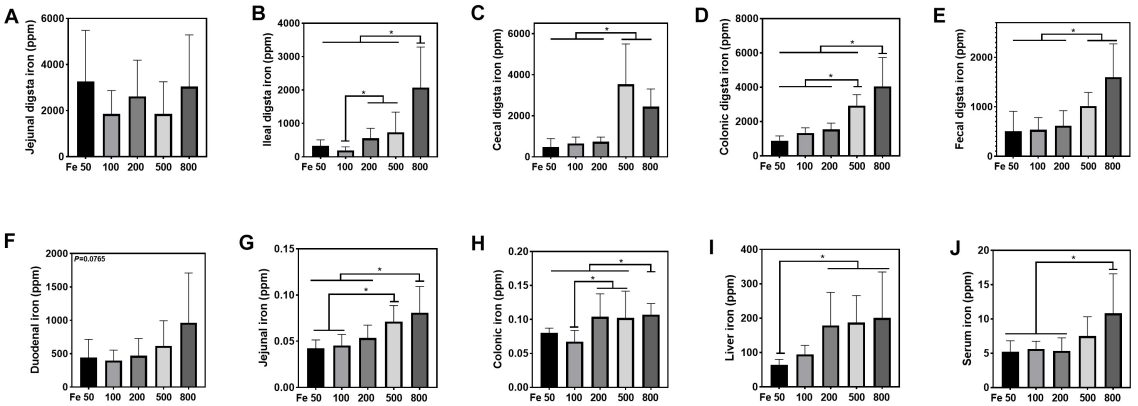

FIGURE 2 | Dietary iron increased iron deposition in intestinal digesta, intestine, liver, and serum. The iron concentration in the jejunal digesta **(A)** was similar among different groups. Iron levels of ileal **(B)**, cecal **(C)**, and colonic digesta **(D)** and feces **(E)** in the Fe800 group were significantly higher than those in the Fe50, Fe100, and Fe200 groups, and iron abundance of cecal and colonic digesta and feces in the Fe500 group was also higher compared with that in the Fe50, Fe100, and Fe200 group. Tissue iron content in the duodenum **(F)**, jejunum **(G)**, colon **(H)**, liver **(I)**, and serum **(J)** went up from Fe50 to Fe800. Ordinary one-way ANOVA or Kruskal–Wallis test was used for the statistical analysis among different groups. Data were presented as mean ± SD. $^*P < 0.05$.

by high dietary iron (**Table 1**). The top 10 taxa at each level of colonic microbiota were listed in **Table 2**. The relative abundances of Bacilli (class level), Lactobacillales (order level), Lactobacillaceae (family level), and *Lactobacillus* (genus level) were upregulated with dietary iron increasing, while Clostridia (class level), Clostridiales (order level), Ruminococcaceae, Lachnospiraceae, Christensenellaceae (family level), and *Ruminococcaceae_UCG-005* (genus level) were downregulated by dietary iron increasing. In the species level, the relative abundance of colonic *Lactobacillus amylovorus (L.amy)*, and *Lactobacillus reuteri (L.reu)* were dominant (**Figure 4A**). The relative abundance of *L.amy* went down with iron supplementation increasing, while *L.reu* went up (**Figures 4B,C**). *L.amy* was significantly higher in the other groups than in the Fe50 group and was more enriched in the Fe200, Fe500, and Fe800 groups than that in the Fe100 group (**Figure 4B**). *L.reu* was significantly augmented in the Fe500 group than in the Fe50 group (**Figure 4C**), and *E. coli* was not changed among the five groups (**Figure 4D**). Correlation analysis indicated that the abundance of *L.amy*, *Lactobacillus coleohominis*, and *Lactobacillus iners* were positively correlated with the iron concentration of colonic digesta, while the abundance of *L.reu*, *Streptococcus gallolyticus*, and *Dorea longicatena* were negatively correlated with the colonic digesta iron concentration (**Figure 4E**). The cecal microbiota in the Fe50, Fe500, and Fe800 groups was also analyzed to support the findings from colonic microbiota analysis, and the changes in microbiota of the cecum was similar with the results from colonic microbiota. Compared with those in the Fe50 group, alpha diversity indexes were significantly decreased in the Fe500 and Fe800 groups, and *Lactobacillus* was also dominant and higher in the Fe800 group than in the Fe50 group (**Supplementary Table 4**, **Supplementary Figure 2C**). Compared with those in the Fe50 group, *L.amy* was higher in the Fe500 and Fe800 groups, and *L.reu* was lower in the Fe500 group (**Figures 4F,G**). And *E. coli* was also similar among the three groups (**Supplementary Figure 2D**). Correlation analysis also demonstrated that iron concentration in cecal digesta positively and negatively correlated with *L.amy* and *L.reu*, separately (**Figure 4H**).

Dietary Iron Changed Colonic Transcript Profiles

We used an RNA-Seq-based reference transcriptome analysis method to evaluate the effect of dietary iron on colonic gene expression. Data preprocessing statistics and quality control of RNA-sequencing were shown in **Supplementary Table 5**. More than 25,000 gene expressions were detected and quantitated, and 424 genes were DEGs, including 254 upregulated and 170 downregulated (**Figure 5A**). The top 20 up/downregulated genes, including IFIT1, ITGBL1, IFNG, TLR8, IFIT5, SERPINB1, and CCL22, were presented in **Table 3**. GO enrichment analysis showed that G-protein-coupled receptor activity and chemokine activity were significantly enriched (**Figure 5B**), while KEGG enrichment analysis indicated that the cytokine–cytokine receptor interaction pathway was significantly enriched (**Figure 5C**). The largest connected component of the PPI network, as shown in **Figure 5D**, was related to immunity and inflammation.

DISCUSSION

This study explored excess dietary iron on the growth performance and iron deposition in the host and gut lumen, intestine gene expression profile, and intestinal microbiota. The results demonstrated increasing iron supplementation increased diarrheal risk, increased iron deposition (in intestinal digesta, intestine, liver, and serum), and reduced serum UIBC, APOA, and IgA. Excess iron also shifted gut microbiota and colonic gene expression profiles.

Iron is essential for neonates and infants, and adequate iron supplementation contributes to the growth and development

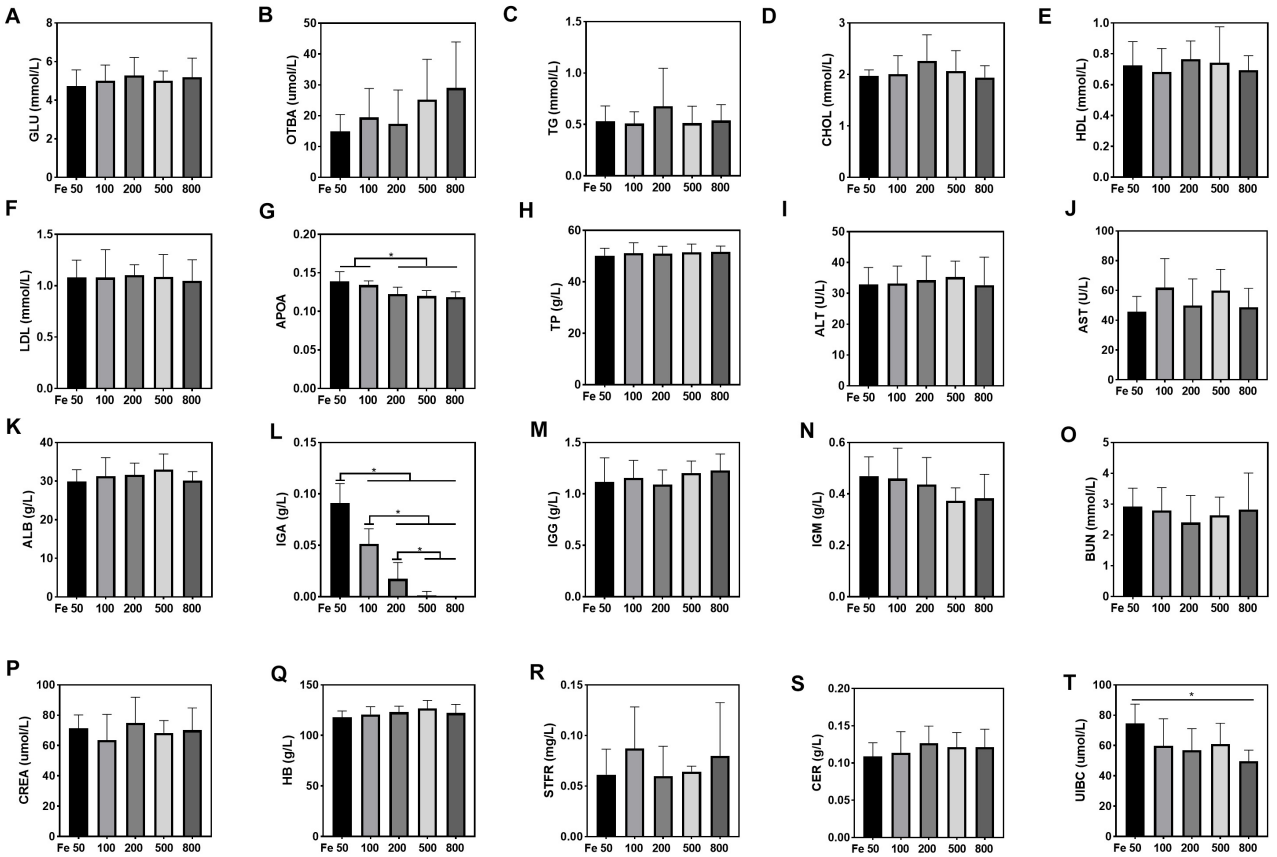

FIGURE 3 | Dietary iron downregulated serum unsaturated iron-binding capacity (UIBC), apolipoprotein (APOA), and immunoglobin A (IGA). Serum glucose (GLU), total bile acids (OTBAs), triglyceride (TG), CHOL, high-density lipoprotein (HDL), low- density lipoprotein (LDL), total protein (TP), alanine aminotransferase (ALT), aspartate amino transferase (AST), albumin (ALB), IgG, and IgM were the same among all the groups **(A–F,H–K,M,N)**. Serum APOA **(G)** and IgA **(L)** were downregulated with dietary iron increasing. Blood HB and serum soluble transferrin receptor (STFR) and ceruloplasmin (CER) were similar among all the groups **(Q–S)**. Serum UIBC **(T)** went down from the Fe50 to Fe800 groups, and UIBC in the Fe50 group was higher than in the Fe800 group. Serum BUN **(O)** and CREA **(P)** were also similar among all the groups. Ordinary one-way ANOVA or Kruskal–Wallis test was used for the statistical analysis among different groups. Data were presented as mean ± SD. $^*P < 0.05$.

TABLE 1 | Alpha diversity indices of the colonic microbiota of piglets.

	Fe50	Fe100	Fe200	Fe500	Fe800	SEM	*P*-value
Goods_coverage	0.999	0.999	0.999	0.999	0.999	0.0001	-
Shannon	5.493^{a}	5.455^{a}	5.000^{a}	4.589^{ab}	3.983^{b}	0.390	0.007
Simpson	0.904^{a}	0.887^{a}	0.861^{ab}	0.823^{ab}	0.790^{b}	0.0384	0.073
Chao1	513.465^{a}	438.437^{ab}	443.219^{ab}	443.013^{ab}	360.0693^{b}	39.849	0.056
ACE	523.693^{a}	450.541^{ab}	487.018^{ab}	454.794^{ab}	371.591^{b}	38.282	0.024

Goods_coverage, richness estimator (Chao1 and ACE), and diversity estimator (Shannon and Simpson) were analyzed by mothur software of the piglets from the Fe50 (n = 8), Fe100 (n = 8), Fe200 (n = 8), Fe500 (n = 6), and Fe800 (n = 6) groups. Ordinary one-way ANOVA or Kruskal–Wallis test was used for the statistical analysis among the different groups. Data were presented as mean ± SEM. Means in a row that have no superscript shared are significantly different from each other (P < 0.05).

in early life, especially in IDA individuals (Black et al., 2004). However, excess iron supplementation is also harmful to host and microbiota (Paganini et al., 2016). O'donovan et al. (1963) reported that a basal diet containing 65 ppm iron with 15 ppm iron supplementation promoted growth rate and feed consumption, and the basal diet supplemented with excess iron, up to 4,000 ppm but not less than 3,000 ppm, reduced the growth rate of pigs. Coincidently, we found that increasing dietary iron supplementation (up to 800 ppm) had no significant effects on growth performance. However, inconsistent results were reported that 4 weeks of dietary supplemented with iron (250 ppm) reduced ADG of piglets, although it did not affect ADFI and ADG to ADFI ratio (Lee et al., 2008). The difference between the results might come from the treatment time or feed formulation. Although it did not affect growth performance, increasing iron increased

TABLE 2 | The top 10 taxa at the phylum, class, order, family, and genus level of colonic microbiota.

Taxonomy	Fe50	Fe100	Fe200	Fe500	Fe800	SEM	*P*-value
Phylum							
Firmicutes	0.9001	0.9020	0.9034	0.9127	0.9409	0.0158	0.6079
Bacteroidetes	0.0592	0.0614	0.0571	0.0527	0.0342	0.0128	0.5260
Proteobacteria	0.0024	0.0032	0.0085	0.0015	0.0134	0.0005	0.1113
Spirochaetes	0.0127	0.0114	0.0061	0.0088	0.0029	0.0012	0.4731
Actinobacteria	0.0040	0.0072	0.0065	0.0053	0.0015	0.0003	0.4461
Tenericutes	0.0143	0.0078	0.0148	0.0146	0.0044	0.0018	0.1412
Cyanobacteria	0.0043	0.0059	0.0025	0.0018	0.0011	0.0006	0.3213
Saccharibacteria	0.0021	0.0009	0.0007	0.0023	0.0016	0.0002	0.5717
Verrucomicrobia	0.0005	0.0000	0.0001	0.0000	0.0000	0.0000	0.2362
Chlamydiae	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.9279
Class							
Bacilli	0.3610^{c}	0.4572^{b}	0.4856^{b}	0.4452^{b}	0.7503^{a}	0.0413	0.0031
Clostridia	0.5104^{a}	0.3989^{b}	0.3729^{b}	0.4374^{b}	0.1780^{c}	0.0332	0.0010
Erysipelotrichia	0.0268	0.0427	0.0409	0.0284	0.0122	0.0017	0.7686
Bacteroidia	0.0591	0.0612	0.0571	0.0527	0.0342	0.0128	0.7187
Gammaproteobacteria	0.0014	0.0022	0.0063	0.0013	0.0127	0.0006	0.1493
Epsilonproteobacteria	0.0007	0.0007	0.0015	0.0000	0.0006	0.0000	0.6812
Unidentified_Spirochaetes	0.0127	0.0114	0.0061	0.0088	0.0029	0.0012	0.4731
Mollicutes	0.0143	0.0078	0.0148	0.0146	0.0044	0.0018	0.1412
Unidentified_Actinobacteria	0.0002	0.0019	0.0029	0.0024	0.0004	0.0001	0.6667
Coriobacteriia	0.0038	0.0053	0.0036	0.0029	0.0011	0.0002	0.2964
Order							
Lactobacillales	0.361^{c}	0.4571^{b}	0.4855^{b}	0.4452^{b}	0.7501^{a}	0.0413	0.0032
Clostridiales	0.5100^{a}	0.3987^{b}	0.3729^{b}	0.4374^{ab}	0.1779^{c}	0.0331	0.0009
Erysipelotrichales	0.0268	0.0427	0.0409	0.0284	0.0122	0.0017	0.5534
Bacteroidales	0.0591	0.0612	0.0571	0.0527	0.0342	0.0128	0.7187
Enterobacteriales	0.0008	0.0013	0.0053	0.0004	0.0085	0.0003	0.4038
Campylobacterales	0.0007	0.0007	0.0015	0.0000	0.0006	0.0000	0.6812
Spirochaetales	0.0127	0.0114	0.0061	0.0088	0.0029	0.0012	0.4731
Mollicutes_RF9	0.0142	0.0077	0.0148	0.0146	0.0043	0.0018	0.1406
Coriobacteriales	0.0038	0.0053	0.0036	0.0029	0.0011	0.0002	0.2964
Pasteurellales	0.0005	0.0006	0.0008	0.0009	0.0041	0.0003	0.8905
Family							
Lactobacillaceae	0.3521^{c}	0.4554^{b}	0.4834^{b}	0.4422^{b}	0.7451^{a}	0.0428	0.0033
Clostridiaceae_1	0.0923	0.0270	0.0196	0.0904	0.0094	0.0039	0.2508
Ruminococcaceae	0.2544^{a}	0.2635^{a}	0.2479^{a}	0.2353^{a}	0.121^{b}	0.0201	0.0303
Erysipelotrichaceae	0.0268	0.0427	0.0409	0.0284	0.0122	0.0017	0.5534
Peptostreptococcaceae	0.0034	0.0020	0.0014	0.0092	0.0013	0.0006	0.2217
Lachnospiraceae	0.1018^{a}	0.0784^{ab}	0.0813^{ab}	0.0797^{ab}	0.0341^{b}	0.0052	0.0255
Enterobacteriaceae	0.0008	0.0013	0.0053	0.0004	0.0085	0.0003	0.4038
Christensenellaceae	0.0501^{a}	0.0215^{ab}	0.0152^{ab}	0.0170^{ab}	0.0090^{b}	0.0039	0.0154
Bacteroidales_S24-7_group	0.0258	0.0297	0.0220	0.0271	0.0181	0.0073	0.9648
Streptococcaceae	0.0088	0.0017	0.0021	0.0029	0.0048	0.0005	0.3046
Genus							
Lactobacillus	0.3521^{c}	0.4554^{b}	0.4834^{b}	0.4422^{b}	0.7451^{a}	0.0428	0.0033
Clostridium_sensu_stricto_1	0.0907	0.0265	0.0181	0.0887	0.3768	0.0039	0.2551
Subdoligranulum	0.0184	0.0557	0.0393	0.0276	0.0130	0.0038	0.1211
Romboutsia	0.0007	0.0002	0.0001	0.0035	0.0091	0.0000	0.3648
Faecalibacterium	0.0111	0.0294	0.0275	0.0352	0.0061	0.0028	0.4615
Ruminococcaceae_UCG-014	0.0553	0.0566	0.0506	0.0698	0.0243	0.0105	0.6604
Lachnospiraceae_XPB1014_group	0.0446	0.0125	0.0243	0.0370	0.0232	0.0031	0.2903

(Continued)

TABLE 2 | Continued

Taxonomy	Fe50	Fe100	Fe200	Fe500	Fe800	SEM	*P*-value
Escherichia–Shigella	0.0008	0.0013	0.0053	0.0004	0.0089	0.0003	0.4043
Ruminococcaceae_UCG-005	0.0472^{a}	0.0188^{ab}	0.0146^{ab}	0.0168^{ab}	0.0088^{b}	0.0046	0.0154
Streptococcus	0.0088	0.0017	0.0021	0.0029	0.0070	0.0005	0.3034

The top 10 microbial population at different taxonomy levels in the colon of piglets from the Fe50 (n = 8), Fe100 (n = 8), Fe200 (n = 8), Fe500 (n = 6), and Fe800 (n = 6) groups. Ordinary one-way ANOVA or Kruskal–Wallis test was used for the statistical analysis among the different groups. Data were presented as mean ± SEM. Means in a row that have no superscript shared are significantly different from each other (P < 0.05).

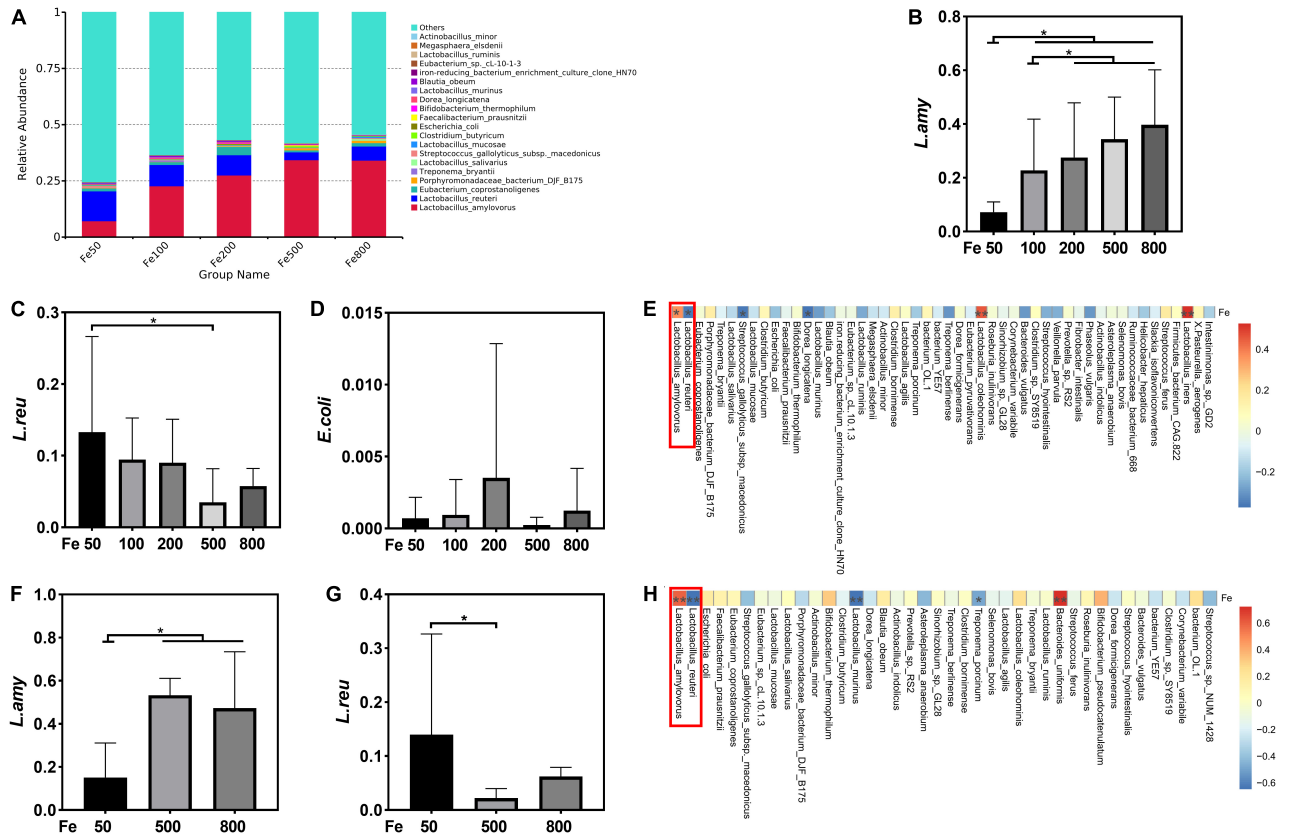

FIGURE 4 | Dietary iron shifted gut microbiology. The relative abundance of *Lactobacillus amylovorus* (*L.amy)* and *Lactobacillus reuteri* (*L.reu)* were dominant in the colon at the species level **(A)**. *L.amy* was significantly higher in the other groups than in the Fe50 group and was more enriched in the Fe200, Fe500, and Fe800 groups than that of the Fe100 group **(B)**. *L.reu* was significantly augmented in the Fe500 group than in the Fe50 group **(C)**, and *Escherichia coli* was not changed among the five groups **(D)**. Correlation analysis indicated that the abundance of *L.amy*, *Lactobacillus coleohominis*, and *Lactobacillus iners* were positively correlated with the iron concentration of colonic digesta, while the abundance of *L.reu*, *Streptococcus gallolyticus*, and *Dorea longicatena* were negatively correlated with the colonic digesta iron concentration **(E)**. Compared with that in the Fe50 group, *L.amy* was higher in the Fe500 and Fe800 groups **(F)**, and *L.reu* was lower in the Fe500 group **(G)**. Correlation analysis also demonstrated that cecal digesta iron positively and negatively correlated with *L.amy* and *L.reu*, separately **(H)**. Ordinary one-way ANOVA or Kruskal–Wallis test was used for the statistical analysis among different groups. Data were presented as mean ± SD. *$P < 0.05$.

diarrheal risk; the result was consistent with previous studies in humans and animals (Rincker et al., 2004; Paganini and Zimmermann, 2017). It is still inconclusive of the mechanism of excess iron induced-diarrhea, and the current consensus is that overloaded iron promotes oxidative stress and disrupts intestinal and microbiota function, further leading to diarrhea (Paganini et al., 2016).

Because it excretes very little, iron absorption and metabolism is finely regulated. The bioavailability of iron is limited, ranging from 2–20% for non-heme iron and 15–35% for heme iron, and is inversely related to iron stores (South et al., 2000). In this study, intestinal digesta iron deposition in the highest iron group is up to six times than that in the lowest iron group. Furthermore, the iron content of the high-iron groups excessed up to two times in the intestinal tissue and serum, and three times in the liver. A large amount of iron accumulation would generate free radical species via catalyzing the Fenton reaction, which might be further leading to oxidative stress, inflammation, and microbiota disruption (Nairz and Weiss, 2020). Dietary supplement with 25 g/kg of carbonyl iron

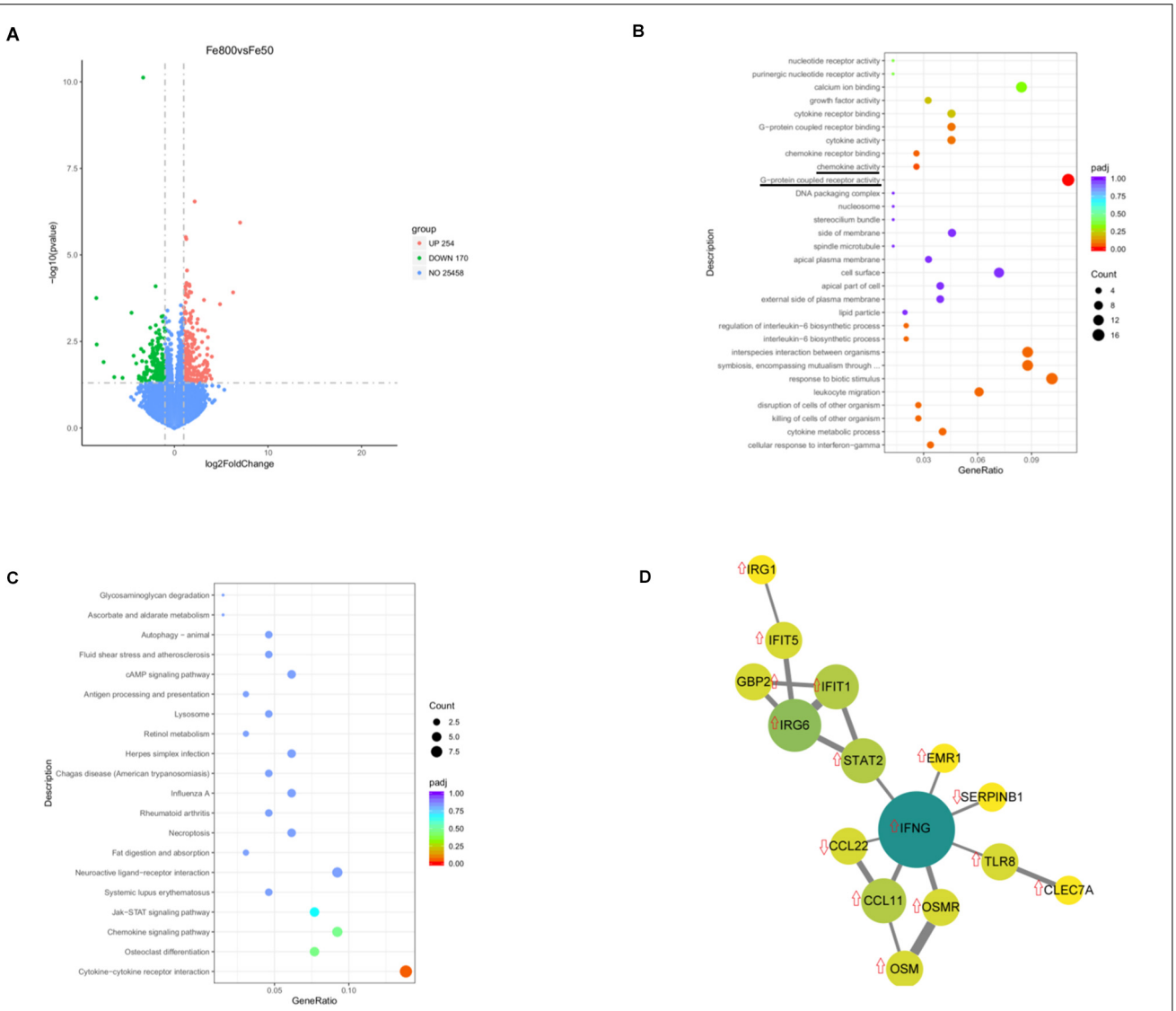

FIGURE 5 | Dietary iron changed colonic transcript profiles. Volcano diagram shows that more than 25,000 gene expressions were detected and quantitated and 424 genes were differential expression genes (DEGs), including 254 upregulated DEGs and 170 downregulated genes **(A)**. Gene Ontology (GO) enrichment analysis showed that G-protein-coupled receptor activity and chemokine activity were significantly enriched **(B)**, while Kyoto Encyclopedia of Genes and Genomes (KEGGs) enrichment analysis indicated that the cytokine–cytokine receptor interaction pathway was significantly enriched **(C)**. The largest connected component of the protein–protein interactions (PPIs) network is shown in **(D)**. Fe50 group (n = 5) and Fe800 (n = 5) group.

induced oxidative stress in the mitochondria, and long-term excess dietary iron damaged liver mitochondrial function (Volani et al., 2017; Atarashi et al., 2018). Serum creatinine and uric acid were increased in iron-overload thalassemia patients, indicating iron overload corrected with kidney dysfunction (Rasool et al., 2016). However, no significant changes were observed in the serum parameter relation with liver and kidney functions in this study. The inconsistent findings may be related to different animal models or iron handing. Increasing iron also did not affect serum Glu and lipid-related metabolites except for APOA, which was coincident with the previous study that iron status was negatively associated with APOA (Zhou et al., 2020).

Previous studies demonstrated that dietary iron (229.2 ± 1.9 μg/g) increased fecal calprotectin, a biomarker of intestinal inflammation, in Kenyan infants (Jaeggi et al., 2015). Dietary iron promoted colonic inflammation and accelerated tumorigenesis through activating the IL-6/IL-11/Stat3 signaling pathway in mice (Chua et al., 2013). Dietary iron (520 mg/kg, Fe_2SO_4) promoted duodenal neutrophil counts and inflammation-related genes in pigs (Li et al., 2016a). In this study, RNA-sequencing analysis of the colon revealed that the cytokine–cytokine receptor interaction pathway was enriched. PPI network analysis revealed the relation of many DEGs, such as interferon-gamma (IFN-γ) and signal transducer and activator of transcription 2 (STAT2). IFN-γ is well-recognized

TABLE 3 | The top 20 up/downregulatory colonic gene expressions.

gene_name	Fe800	Fe50	log2FoldChange	*P*-value
Upregulated				
NEURL3	254.0	56.6	2.2	2.86354E-07
PTPRO	106.3	43.8	1.3	3.4817E-06
MS4A7	248.0	97.3	1.3	2.8176E-05
IFIT1	1,451.6	592.2	1.3	6.44434E-05
ITGBL1	250.9	111.0	1.2	8.15475E-05
OSCAR	73.4	32.3	1.2	9.8907E-05
ACOD1	72.6	21.0	1.8	0.0001
OXTR	63.4	0.8	6.3	0.0001
IFNG	57.1	21.2	1.4	0.0001
PON3	17.4	4.4	2.0	0.0004
cC1QA	4,308.3	1,945.4	1.1	0.0004
FBXO39	163.9	79.3	1.0	0.0005
TLR8	363.8	164.3	1.1	0.0005
THRSP	533.7	165.6	1.7	0.0005
MS4A8	159.4	46.3	1.8	0.0006
TOPAZ1	65.7	19.5	1.7	0.0007
P2RY12	53.0	25.3	1.1	0.0008
IFIT5	1,280.4	609.9	1.1	0.0009
ADGRE1	69.6	20.4	1.8	0.0009
S100A12	17.3	2.2	3.0	0.0015
Downregulated				
GIF	9.2	93.0	−3.3	7.53398E-11
RPS14	17.8	45.9	−1.4	0.0006
ESPN	38.3	88.6	−1.2	0.0008
NTNG2	10.8	41.9	−2.0	0.0018
SERPINB1	10.2	27.6	−1.4	0.0024
TAPBPL	5.0	17.1	−1.8	0.0032
APELA	1.5	8.0	−2.4	0.0038
CCL22	75.4	206.2	−1.5	0.0040
LAMP3	6.1	20.4	−1.7	0.0051
KCNK10	2.4	10.6	−2.2	0.0066
ACTA1	16.5	101.2	−2.6	0.0086
KCTD16	2.5	12.2	−2.2	0.0097
ANKRD1	2.2	7.6	−1.9	0.0103
ECEL1	2.6	9.5	−1.8	0.0116
LSR	0.2	2.9	−3.5	0.0117
CCDC114	72.4	174.0	−1.3	0.0129
TXK	26.6	59.7	−1.2	0.0148
PYGM	11.5	35.4	−1.6	0.0161
KCNQ1OT1_1	0.4	3.0	−3.0	0.0172
CYP4F22	7.2	23.8	−1.7	0.0185

The top 20 up/downregulatory differential expression genes (DEGs) of colon from the Fe50 (n = 5) and Fe800 (n = 5) groups.

for its antivirus role and is also essential in resistance to bacteria infection (Shtrichman and Samuel, 2001). CLEC7A, a member of C-type lectin receptors, is produced by myeloid cells to sense pathogens and bacterial infections and could activate immune cells in diarrhea-predominant irritable bowel syndrome (Chi et al., 2020). CLE7A and toll-like receptors (TLRs) could be independent or together to activate immunocytes, such as dendritic cells and T-helper-type 1 (Th1) cells, further inducing IFN-γ production (Loures et al., 2015; Perkins and Vogel, 2015; Zimara et al., 2018). IFN-induced proteins with tetratricopeptide repeats (IFITs) could be generated through the IFN-γ–JAK–STAT1/2 pathway to fight against pathogen infection, and IFIT1-deficiency mice are more sensitive to *Burkholderia cenocepacia* (Van Treuren and Dodd, 2020; Zheng et al., 2020). Oncostatin M treatment promoted peptide-pulsed HepG2 cell-induced IFN-γ production by cytotoxic T cells (Larrea et al., 2009). Th1-associated chemokines CCL11 and Th17-associated chemokines CCL22 are elevated in the eye and lymph nodes of IFN-γ-deficiency mice (Su et al., 2007). Th1-immnue response is vital to resist intracellular pathogens, and IFN-γ could limit iron export out of enterocyte by inhibiting ferroportin-1 (Cassat and Skaar, 2013). In macrophage, IFN-γ could stimulate hepatic hepcidin expression through STAT1 (Sow et al., 2009). These reports and our data suggested that the changes of IFN-γ-STAT2 based gene network induced by high level of iron might be the response to limit iron absorption or anti-infection. Our results also revealed that increasing iron reduced serum IgA, which was also observed in children with beta-thalassemia, indicating that iron overload might impair humoral immunity (Hagag et al., 2016). Collectively, high dietary iron changed intestinal gene expression profile, especially for immune genes.

A complex microbial community inhabits the intestine, perceives metal ion limitation or excess via metalloregulatory proteins, and acquires and stores metal in starvation or efflux them while in excess, which would induce cell growth arrest or even death (Chandrangsu et al., 2017). The host tightly regulates pathogen iron access to restrict infection by iron regulatory systems such as hepcidin and lactoferrin. The competition of bacteria with the host for iron is essential for the maintenance of indigenous microbial populations and host health (Chandrangsu et al., 2017). Overload oral iron causes excess iron deposition in the intestine and changes intestinal microbiota in humans and animals (Paganini and Zimmermann, 2017; Ding et al., 2020). Some gram-negative bacteria, such as *Salmonella*, *Shigella*, and pathogenic *E. coli*, require iron for virulence and colonization (Kortman et al., 2014). However, some gram-positive bacteria, such as *Lactobacillus acidophilus*, and unclassical bacteria, such as *Borrelia burgdorferi*, do not need iron (Sabine and Vaselekos, 1967; Posey and Gherardini, 2000). A review by Paganini and Zimmermann (2017) demonstrated that both iron fortification and supplementation in infants and children could disrupt intestinal flora by decreasing bifidobacteria and lactobacilli and increasing enterobacteria such as enteropathogenic *E. coli*. Dietary iron supplemented with iron (up to 250 mg/kg) for 2 weeks linearly increased diarrheal incidence and fecal coliform bacteria in piglets, while the total anaerobic bacteria population was reduced, and coliform bacteria was increased, in diarrheal feces compared with those in normal feces (Lee et al., 2008). A recent study also reported that dietary iron, at a dose of 3,000 ppm, but not 300 ppm, for 4 weeks decreased Clostridiales, Faecalibacterium, and Prevotellaceae, and increased *Desulfovibrio* and *Anaerovibrio* in the cecum of piglets, compared with those of controls (Ding et al., 2020).

These studies indicate the regulatory role of dietary iron in intestinal bacteria. Our results also indicated that increasing dietary iron changed the intestinal microbiota, such as decreasing community richness and diversity, increasing Lactobacillus and *L.amy*, and reducing Clostridiales and *L.reu*. However, other populations, such as *E. coli* and bifidobacteria, were unchanged. *Lactobacillus* species, such as *L.reu*, was reported as the main sensors to monitor the gut iron level and were robust in response to intestinal iron deficiency and arrest host iron absorption by its metabolites, for example, reuterin-producing *L.reu* inducing the inhibition of HIF-2a gene expression (Das et al., 2020). *L.amy* is isolated from swine and a potential probiotic bacterium that could produce lactate, acetate, amylovorin, and phytase (Moreno et al., 2008). Together, these data suggest that increasing dietary iron could shift intestinal flora, such as reducing alpha diversity and augmenting the relative abundance of *Lactobacillus*. However, how iron deposition affect the absolute abundance of total bacteria and *Lactobacillus* remains unclear that requires further study.

CONCLUSION

In summary, this study reveals the effects of increasing dietary iron on the distribution pattern of iron in the host and gut lumen, intestinal microbiota, and intestine gene expression profile. The results demonstrate that high dietary iron increases diarrheal incidence, changes intestinal immune response-associated gene expression, and shifts gut microbiota. Further studies are needed to analyze the interaction of *Lactobacillus* and the host under conditions of iron excess.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Welfare Committee of the Institute of Subtropical Agriculture, Chinese Academy of Sciences.

AUTHOR CONTRIBUTIONS

HY, SC, and YY designed the experiments, which was performed by SC, XWu, and XWa. SC, QT, and JY analyzed the data. JY and YS prepared the tables and figures. SC and XWu prepared the manuscript. HY, QT, and YY revised the manuscript. All the authors reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.603392/full#supplementary-material

Supplementary Figure 1 | Dietary iron had no significant effects on the growth performance but increased fecal consistency score. The daily fecal score was shown **(A)**. The fecal score on d21 was significantly higher in the Fe800 group than in other groups **(B)**. Ordinary one-way ANOVA or Kruskal–Wallis test was used for the statistical analysis among different groups. $^{**}P < 0.01$ and $^{***}P < 0.001$.

Supplementary Figure 2 | Dietary iron shifted gut microbiology. Venn diagram showed colonic shared and unique OTUs among different groups **(A)**. The rarefaction curve of colonic microbial in each group **(B)**. The relative abundance of species in the cecum at the species level was shown **(C)**. Cecal *E. coli* was similar among each group **(D)**. Ordinary one-way ANOVA or Kruskal–Wallis test was used for the statistical analysis among different groups. $^{*}P < 0.05$.

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Structural and Functional Dysbiosis of Fecal Microbiota in Chinese Patients With Alzheimer's Disease

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Increasing evidence suggests that gut dysbiosis plays vital roles in a variety of gut–brain disorders, such as Alzheimer's disease (AD). However, alterations of the gut microbiota as well as their correlations with cognitive scores and host immunity have remained unclear in well-controlled trials on Chinese AD patients. In this study, samples from 100 AD patients, and 71 age- and gender-matched, cognitively normal controls were obtained to explore the structural and functional alterations of the fecal microbiota targeting the V3–V4 region of the 16S rRNA gene by MiSeq sequencing, and to analyze their associations with clinical characteristics. Our data demonstrated a remarkably reduction in the bacterial diversity and alterations in the taxonomic composition of the fecal microbiota of the AD patients. Interestingly, the abundant butyrate-producing genera such as *Faecalibacterium* decreased significantly, where this was positively correlated with such clinical indicators as the MMSE, WAIS, and Barthel scores in the AD patients. On the contrary, abundant lactate-producing genera, such as *Bifidobacterium,* increased prominently, and were inversely correlated with these indicators. This shift in the gut dysbiosis of the microbiota, from being butyrate producers to lactate producers, contributed to immune disturbances in the host that could be used as non-invasive biomarkers to distinguish the controls from the AD patients. Moreover, several predicted functional modules, including the biosynthesis and the metabolism of fatty acids, that were altered in the microbiota of the AD patients could be utilized by the bacteria to produce immunomodulatory metabolites. Our study established the structural and functional dysbiosis of fecal microbiota in AD patients, and the results suggest the potential for use of gut bacteria for the early, non-invasive diagnosis of AD, personalized treatment, and the development of tailor-made probiotics designed for Chinese AD patients.

Keywords: Alzheimer's disease, *Bifidobacterium*, *Faecalibacterium*, gut-brain axis, sequencing

INTRODUCTION

Alzheimer's disease (AD) is an age-related neurodegenerative disease characterized by slowly progressive memory decline and cognitive dysfunction, and no preventative or disease-modifying treatments are available for it at present (Gaugler et al., 2019). AD is a leading cause of dementia, and its prevalence is increasing drastically among aging populations worldwide. According to the World Alzheimer Report 2015, almost 47 million people worldwide were expected to be affected by dementia in 2015, with 9.9 million new diagnoses each year. This number is estimated to exceed 130 million by 2050, with the greatest increase expected in low- and middle-income countries. In 2017, 121,404 deaths caused by AD were recorded in USA, making it the sixth leading cause of death among Americans of all ages and the fifth leading cause among elderly Americans (age ≥65 years) (Gaugler et al., 2019). In addition, the economic costs of the disease are formidable due to the care needed by the growing number of patients with AD and other dementias. Thus, addressing the rapidly growing incidence of AD should be regarded as a global public health priority.

Numerous studies in recent decades have focused on elucidating the etiopathology of AD, but its pathogeneses remain unclear, and no therapeutic strategy is available to cure this disease. The most important risk-related factor for AD is advancing age, and as lifespans increase and demographic aging occurs worldwide, the number of AD patients is expected to increase drastically. The depositions of amyloid-beta peptide, a product of the cleavage of the amyloid-beta protein precursor, and the abnormal tau protein can be used as diagnostic markers for AD (Holtzman et al., 2011). However, whether they are the causes of AD or its consequences remains unknown. Many recent studies have noted that several infectious agents, such as *Chlamydia pneumoniae*, herpes simplex virus type 1, and several types of spirochaete and fungi, are involved in the pathogenesis of late-onset AD (De Chiara et al., 2012; Alonso et al., 2014; Balin and Hudson, 2014; Itzhaki, 2014; Miklossy, 2015; Pisa et al., 2015). This has prompted the suggestion that long-term, largely subclinical pathogenic infection might contribute to the characteristic neurodegeneration that occurs due to AD (Balin and Hudson, 2014). Based on these findings, Reis et al. (2010) considered AD itself as an infectious disease. Recent advances have revealed that microbiota of the human gut have numerous beneficial functions, such as immune development and resistance to pathogens, and serve as an important reservoir of pathogenic bacteria, viruses, and fungi. Previous work has clarified the role of gut microbiota in regulating multiple neuro-chemical pathways through the gut–brain axis (Bonfili et al., 2017). The dysbiosis of intestinal microbiota might impair intestinal mucosal integrity, increase intestinal permeability, and then disturb the intestinal homeostasis, where this can contribute to spreading potential pathogens to the target organs, such as the brain. Several studies have found altered gut microbiota in AD patients, which suggests that gut microbiota may be involved in AD pathogenesis (Vogt et al., 2017; Zhuang et al., 2018; Liu P. et al., 2019). Our group has observed that the transplantation of probiotics, prebiotics, and even fecal microbiota can ameliorate cognitive deficits and neurodegeneration in a model of mice with AD through the modulation of the gut microbiota (Sun et al., 2019a,b; Sun et al., 2020). Thus, the eubiosis of gut microbiota might have beneficial roles in preventing the occurrence and development of AD and other gut–brain disorders. However, the changing patterns of the composition and diversity of the gut microbiota are not always uniform in AD patients, but vary with population, geographical location, diet, and habits. The difference in the genetic background of hosts and dietary constitutions between Western and Chinese populations might contribute to the baseline disparity in the composition of the microbiota between them, which might in turn influence the roles of specific bacteria in the etiopathology of AD.

Lishui is a city that features dense mountains with a vegetation coverage of 80.79%, and has ranked second on China's ecological index behind Zhejiang for each of the past 13 years. It is also called the longevity town of China, with nearly 200 centenarians. The average life expectancy of the residents of Lishui is 80.06, 2.76 years higher than the national average. Its heredity, dietary patterns, and natural geographical environment influence the health of and incidence of diseases among the population, and may also influence the overall structure and function of the people's gut microbiota. The higher depth of sequencing and coverage with the advent of advanced sequencing techniques have made it possible to decipher key unknown functional taxa in Chinese AD patients. In this study, the fecal microbiota associated with AD are analyzed in a large AD cohort and matched healthy controls from Lishui by using the 16S rRNA high-throughput gene MiSeq platform, and are correlated with clinical indicators to provide novel targets for the early, non-invasive diagnosis and personalized treatment of AD as well as the development of tailor-made probiotics designed for Chinese AD patients.

Abbreviations: ACE, abundance-based coverage estimator; AD, Alzheimer's disease; AUC, an area under the curve; Aβ, amyloid-β; BMI, body mass index; CNS, central nervous system; FDR, false discovery rate; F/B, *Faecalibacterium*/*Bifidobacterium*; IFN-γ, interferon gamma; IL, interleukin; IP-10, interferon gamma-inducible protein 10; KEGG, Kyoto Encyclopedia of Genes and Genome; LDA, linear discriminant analysis; LEfSe, linear discriminant analysis effect size; MCP-1, monocyte chemotactic protein-1; MIP, macrophages inflammatory protein; MMSE, Mini-Mental State Examination; MRI, magnetic resonance imaging; no, numbers; OTU, operational taxonomic unit; PCoA, principal coordinate analysis; PiCRUSt, phylogenetic investigation of bacterial communities by reconstruction of unobserved states; QIIME, Quantitative Insights Into Microbial Ecology; RDP, Ribosomal Database Project; SCFAs, short chain fatty acids; SD, standard deviation; SparCC, sparse compositional correlation; STAMP, Statistical Analysis of Metagenomic Profiles software package; TNF-α, tumor necrosis factor-alpha; WAIS, Wechsler Adult Intelligence Scale.

METHODS

Subjects' Enrollment

A total of 100 well-controlled Chinese AD patients, who were diagnosed based on the criteria of the National Institute of Neurological and Communicative Diseases and Stroke/AD and Related Disorders Association, were recruited from Lishui, Zhejiang province (China) from February 2019 to November 2019, with 71 cognitively normal subjects as control. The

cognitive and functional status were scored using the Mini-Mental State Examination (MMSE, Chinese version), the current version in the Wechsler Adult Intelligence Scale series (WAIS-IV, published in 2008), and instrumental Barthel activities of daily living. Each participant was scanned on magnetic resonance imaging (MRI), with AD patients diagnosed as brain atrophy. The detailed demographic data and medical history (such as hypertension, diabetes mellitus, hypercholesterolemia, coronary heart disease, diarrhea, and constipation) were collected using a set of questionnaire. The exclusion criteria included: family history of dementia; any kind of other neurodegenerative disease such as Parkinson's disease; confirmed mental illness such as schizophrenia; any kind of tumor; antibiotic, prebiotic, probiotic, or synbiotic administration in the previous month; known active infections such as viral, bacterial, or fungal infections; other diseases such as inflammatory bowel disease, irritable bowel syndrome, or other autoimmune diseases. These protocols for the study were approved by the Ethics Committee of Lishui Second People's Hospital (Zhejiang, China) and written informed consent was obtained from each of the subject or their guardian before enrollment.

Fecal Sample Collection and DNA Extraction

Approximately 2 g of a fresh fecal sample was collected in a sterile plastic cup, and stored at −80°C after preparation within 15 min until use. Bacterial genomic DNA was extracted from 300 mg of homogenized feces using a QIAamp® DNA Stool Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions, with additional glass-bead beating steps on a Mini-beadbeater (FastPrep; Thermo Electron Corporation, Boston, MA, USA). The amount of DNA was determined using a NanoDrop ND-1000 spectrophotometer (Thermo Electron Corporation); the integrity and size were checked by 1.0% agarose gel electrophoresis containing 0.5 mg/ml ethidium bromide. All DNA was stored at −20°C before further analysis.

Amplicon Library Construction and Sequencing

Amplicon libraries were constructed with Illumina sequencing-compatible and barcode-indexed bacterial PCR primers 341F (5′-CCTACGGGNGGCWGCAG-3′)/785R (5′-ACTACHVGGGTATCTAATCC-3′), which target the V3–V4 regions of the 16S rRNA gene (Fadrosh et al., 2014). All PCR reactions were performed with KAPA HiFi HotStart ReadyMix using the manufacturer's protocol (KAPA Biosystems) and ∼50 ng of extracted DNA per reaction. Thermocycling conditions were set at 95°C for 1 min, 55°C for 1 min, then 72°C for 1 min for 30 cycles, followed by a final extension at 72°C for 5 min. All PCR reactions were performed in 50 μl triplicates and combined after PCR. The amplicon library was prepared using a TruSeq™ DNA sample preparation kit (Illumina Inc, San Diego, CA, USA). Prior to sequencing, the PCR products were extracted with the MiniElute® Gel Extraction Kit (QIAGEN) and quantified on a NanoDrop ND-1000 spectrophotometer (Thermo Electron Corporation) and Qubit 2.0 Fluorometer (Invitrogen). The purified amplicons were then pooled in equimolar concentrations and the final concentration of the library was determined by Qubit (Invitrogen). Negative DNA extraction controls (lysis buffer and kit reagents only) were amplified and sequenced as contamination controls. Sequencing was performed on a MiSeq instrument (Illumina) using a 300 × 2 V3 kit together with PhiX Control V3 (Illumina) (Ling et al., 2019; Liu X. et al., 2019). MiSeq sequencing and library construction were performed by technical staff at Hangzhou KaiTai Bio-lab.

Bioinformatic Analysis

The 16S rRNA gene sequence data set generated from the MiSeq run were first merged and demultiplexed into per samples using the QIIME version 1.9.0 with default parameters (Caporaso et al., 2010). Chimera sequences were detected and removed using the USEARCH software based on the UCHIME algorithm (Edgar et al., 2011). Open-reference operational taxonomic unit (OTU) pick was then performed with USEARCH V7 referenced against Greengenes database version 13.8 at 97% sequence similarity (Edgar, 2010; Mcdonald et al., 2012). OTUs with a number of sequences <0.005% of the total number of sequences were discarded as recommended (Navas-Molina et al., 2013). The result was an OTU table, which was used for subsequent downstream analysis.

For taxonomic assignment, the most abundant sequences were chosen as the representative sequences of corresponding OTUs. Taxonomic assignment of individual datasets were classified against the Greengenes database version 13.8 using both RDP classifier and UCLUST version 1.2.22 methods implemented in QIIME (Wang et al., 2007; Edgar, 2010). Any sequences that were identified as members of Eukarya, Archaea, Mitochondria, Chloroplasts, and Cyanobacteria lineages, were removed. Alpha diversity was calculated with QIIME software with Python scripts base on the sequence similarity at 97% level, including index of observed OTUs, abundance-based coverage estimator (ACE), Chao1 estimator, Shannon, Simpson, Evenness, and PD whole tree. Sequence coverage was assessed in mothur by rarefaction curves and Good's coverage (Good, 1953; Schloss et al., 2009). Beta diversity was measured by jaccard, bray-curtis, unweighted UniFrac, and weighted UniFrac distance calculated with 10 times of subsampling by QIIME. These distances were visualized by principal coordinate analysis (PCoA) (Lozupone and Knight, 2005). Hierarchical clustering was performed and heatmap was generated using a Spearman's rank correlation coefficient as a distance measure and a customized script developed in the R statistical package. The output file was further analyzed using Statistical Analysis of Metagenomic Profiles software package (STAMP) version 2.1.3 (Parks et al., 2014).

For the predictive functional analyses, PiCRUSt software package version 1.0.0 was used to identify predicted gene families and associated pathways from inferred metagenomes of taxa of interest identified from the compositional analyses, which was based on the fact that phylogeny and function are closely linked (Langille et al., 2013). Predicted functional genes were categorized into Clusters of Orthologous Groups (COG) and into Kyoto Encyclopedia of Genes and Genome

(KEGG) orthology (KO), and compared across patient groups using STAMP. Pathways and enzymes were assigned using KEGG database options built into the pipeline. The pathways that were non-prokaryotic, had fewer than 2 sequences in each cohort, or had a difference in mean proportions <0.1% were excluded from analysis. The characterization of microorganismal features differentiating the gastric microbiota was performed using the linear discriminant analysis (LDA) effect size (LEfSe) method (http://huttenhower.sph.harvard.edu/galaxy/) for biomarker discovery, which emphasizes both statistical significance and biological relevance (Segata et al., 2011). With a normalized relative abundance matrix, LEfSe uses the Kruskal-Wallis rank sum test to detect features with significantly different abundances between assigned taxa and performs LDA to estimate the effect size of each feature. A significant alpha at 0.05 and an effect size threshold of 3 were used for all biomarkers discussed in this study.

Correlation analysis was performed using sparse compositional correlation (SparCC) algorithm on the complete OTU table collapsed to the genus level, which was introduced by Friedman and Alm and was known for its robustness to the compositional effects that are influenced by the diversity and sparsity of correlation in human microbiome data sets (Friedman and Alm, 2012). SparCC was employed to represent co-abundance and co-exclusion networks between OTUs. For SparCC, 1000 bootstrap replicates were used to calculate significance values, and considered correlation coefficients greater or <0.2 and −0.2, respectively, and $p < 0.05$. This set of iterative procedures were applied separately to normal, peritumor and tumor data sets to infer the basis correlation values within and/or between paired sampling sites. Visualization of the network was achieved using Cytoscape version 3.4.1.

Systemic Inflammatory Cytokines Analysis

Serum samples from these participants were obtained using their fasting blood in the early morning. Using a 27-plex magnetic bead based immunoassay kit (Bio-Rad, CA, USA), the following cytokines were quantified: interleukin-1β (IL-1β), IL-1 receptor antagonist (IL-1ra), IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12(p70), IL-13, IL-15, IL-17, Eotaxin, Fibroblast growth factor-basic (FGF-basic), granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophages colony-stimulating factor (GM-CSF), interferon gamma (IFN-γ), interferon gamma-inducible protein 10 (IP-10), monocyte chemotactic protein-1 (MCP-1), macrophages inflammatory protein-1α (MIP-1α), platelet-derived growth factor (PDGF-bb), MIP-1β, regulated upon activation normal T-cell expressed and secreted (RANTES), tumor necrosis factor-alpha (TNF-α), and vascular endothelial growth factor (VEGF). The Bio-Plex 200 system was utilized for the analysis of Bio-Rad 27-plex human group I cytokines and the Bio-Plex assay (Bio-Rad) was performed according to the manufacturer's directions. The results expressed as picogram per milliliter (pg/mL) using standard curves integrated into the assay and Bio-Plex Manager v5.0 software with reproducible intra- and inter-assay CV values of 5–8%.

Statistical Analysis

White's non-parametric *t*-test, independent *t*-test, or Mann-Whitney *U*-test were applied for continuous variables. Pearson chi-square or Fisher's exact test were used for categorical variables between groups. Spearman's rank correlation test was utilized for correlation analyses. Statistical analysis was performed using the SPSS v19.0 (SPSS Inc., Chicago, IL) and STAMP v2.1.3 (Parks et al., 2014). R packaged and GraphPad Prism v6.0 were used for preparation of graphs. All tests of significance were two sided, and $p < 0.05$ or corrected $p < 0.05$ was considered statistically significant.

Accession Number

The sequence data from this study are deposited in the GenBank Sequence Read Archive with the accession number SRP262626.

RESULTS

Subject Characteristics

Table 1 shows the characteristics of the Chinese AD patients as well as the age- and gender-matched cognitively normal, healthy controls. There were no significant differences in terms of gender, body mass index, smoking, drinking and comorbidities of hypertension, hypercholesterolemia, diabetes mellitus, and coronary heart disease between the healthy controls and the AD patients ($p > 0.05$), while the MMSE, WAIS, and Barthel scores were clearly lower in Chinese AD patients than in the healthy controls ($p < 0.05$).

TABLE 1 | Characteristics of the participants.

Parameters	AD patients ($n = 100$)	Healthy controls ($n = 71$)	*p*
Age (y)	74.14 ± 9.21	73.11 ± 7.75	0.105
Gender (male/female)	43/57	35/36	0.415
BMI (Mean ± SD)	22.12 ± 3.45	23.45 ± 3.32	0.164
Smoking, no.	4	3	0.942
Drinking, no.	2	1	0.772
Antibiotics use, no.	0	0	
Complications, no.			
Hypertension	37	25	0.445
Diabetes mellitus	17	11	0.793
Hypercholesterolemia	18	10	0.495
Coronary heart disease	15	8	0.481
Diarrhea	2	3	0.395
Constipation	7	5	0.991
Cognitive and functional status			
MMSE score	4.27 ± 6.06	27.21 ± 2.04	<0.01
WAIS score	35.31 ± 15.35	90.14 ± 10.04	<0.01
Barthel score	23.22 ± 23.15	76.75 ± 7.79	<0.01

BMI, body mass index; SD, standard deviation; no, numbers; MMSE, Mini-Mental State Examination; WAIS, Wechsler Adult Intelligence Scale.

Altered Overall Structure of the Fecal Microbiota in AD

In total, 5,760,348 high-quality reads (2,421,229 reads of the controls and 3,339,119 of AD patients), with an average of 33,686 reads per sample, were obtained for the subsequent analysis of the microbiota. The value of Good's coverage was 99.24%, indicating that a majority of bacterial phylotypes (2,366 OTUs) in the fecal microbiota had been identified. Interestingly, the alpha-diversity indices, including Shannon's and Simpson's indices, were significantly different between the controls and the AD patients, indicating decreased bacterial diversity in AD-associated microbiota (**Figures 1A,B**). Richness indices, including the observed OTUs, ACE, and Chao1, were also significantly higher in the controls than in the AD patients (**Figures 1C–E**). Despite significant inter-individual variations, the PCoA based on the Jaccard, Bray–Curtis, unweighted UniFrac, and weighted UniFrac algorithms also divided the two groups into different clusters (Adonis test: $p < 0.01$; **Figures 1F–I**). Thus, the alpha- and beta-diversity analyses demonstrated that the overall structure of the AD-associated fecal microbiota had changed significantly compared with that of the controls.

Composition of Changed Fecal Microbiota in AD Patients

The compositions of the fecal microbiota in the AD patients and the controls were assessed at different taxonomic levels. Using the RDP classifier, the sequences were classified as 10 phyla, 76 families, and 203 genera. The distribution of the phyla and the genera are shown in **Supplementary Figures 1, 2**, respectively, and suggested significant inter-personal variations. By using the LEfSe, our discriminant analyses showed that many key taxa were clearly different between the AD and the control group (LDA score >3, $p < 0.05$, **Figure 2**). Only bacterial phylotypes with an average relative abundance of more than 0.01% were selected here for the LEfSe. The representative cladogram demonstrated the dysbiosis of AD-associated fecal microbiota in the Chinese AD patients. Of these differential functional bacterial taxa, we found that Actinobacteria and Verrucomicrobia had clearly increased in the AD patients, while Firmicutes had significantly decreased at the phylum level. At the family level, 13 key functional bacterial families including *Bifidobacteriaceae*, *Verrucomicrobiaceae*, *Coriobacteriaceae*, *Erysipelotrichaceae*, *Enterococcaceae*, and *Corynebacteriaceae* had significantly increased in AD patients while three families—*Ruminococcaceae*, *Lachnospiraceae*, and *Clostridiaceae* 1—had drastically decreased. At the genus level, 24 key functional bacterial genera had changed significantly between the groups while only eight genera—*Faecalibacterium*, *Roseburia*, *Clostridium sensu stricto*, *Gemmiger*, *Dialister*, *Romboutsia*, *Coprococcus,* and *Butyricicoccus*—had decreased in AD patients. **Supplementary Figure 3** shows the heatmap of the bacterial genera in the AD patients and the controls. It shows the relative percentages of most genera identified in each sample. Intriguingly, traditionally beneficial genera, such as *Bifidobacterium* and *Akkermansia,* had drastically increased in the Chinese AD patients.

In addition, the structure of the fecal microbiota was determined by dynamic interactions between these community members. Our SparCC algorithm with FDR adjustments was used to generate correlation-based networks of microbial interaction based on the relative abundance of OTUs between the groups (**Figure 3**). We found a more complex network of interactions in healthy controls than that in the AD patients. More positive and negative correlations among the bacteria were found in the healthy controls than in the AD patients. Our data indicate the structural dysbiosis of the AD-associated fecal microbiota in the AD patients.

Fecal Microbiota-Based Signature Discriminated Healthy Controls From AD Patients

We identified several differential taxa in the AD-associated fecal microbiota. We then evaluated the value of using six abundant genera as biomarkers: *Bifidobacterium*, *Faecalibacterium*, *Roseburia*, *Akkermansia*, *Lactobacillus,* and *Enterococcus*. The differential features of these genera are shown in **Figures 4A–F**, which show significant inter-personal variations. We first used only one of the differential bacteria as predictor to generate the area under the receiving operating characteristic curves to obtain the area under the curve (AUC) ranging from 0.304 to 0.797 (**Figure 4G**). **Figure 4** shows that enriched *Faecalibacterium* was the best discriminant predictor for the healthy controls (AUC: 0.797), with a best cut-off value of 3.2149%. Further, multivariable stepwise logistic regression analysis was applied to the list of AD-associated genera to determine the taxa that best distinguished the controls from the AD patients. We found that using all six abundant genera significantly improved predictive performance (AUC: 0.836). We also assessed the predictive value of the ratio of *Faecalibacterium/Bifidobacterium* (F/B ranged from 0.0001 to 2876.2660, **Figure 4H**). We found that the ratio of F/B could help discriminate between healthy controls and AD patients with an AUC of 0.788. Interestingly, the best cut-off value of the ratio of F/B was one. Therefore, these key differential genera can be used as potential biomarkers for discriminating between healthy controls and AD patients.

Microbial Functional Dysbiosis in AD

To identify the metabolic and functional changes in the fecal microbiota between the AD patients and the controls, PiCRUSt was used to analyze the functional potential of the microbiota based on closed-reference OTU picking. We compared 64 KEGG pathways at level 2 and identified seven KEGG categories with clearly differential abundances between the AD patients and the controls. We found that carbohydrate metabolism, xenobiotics' biodegradation and metabolism, and transport and catabolism significantly increased in the AD patients, while transcription, immune system, environmental adaptation, and cell motility significantly decreased ($p < 0.05$; **Figure 5**). Specifically, 15 pathways in level 3, including the metabolism of fatty acids and lipoic acid, and folate biosynthesis, increased significantly,

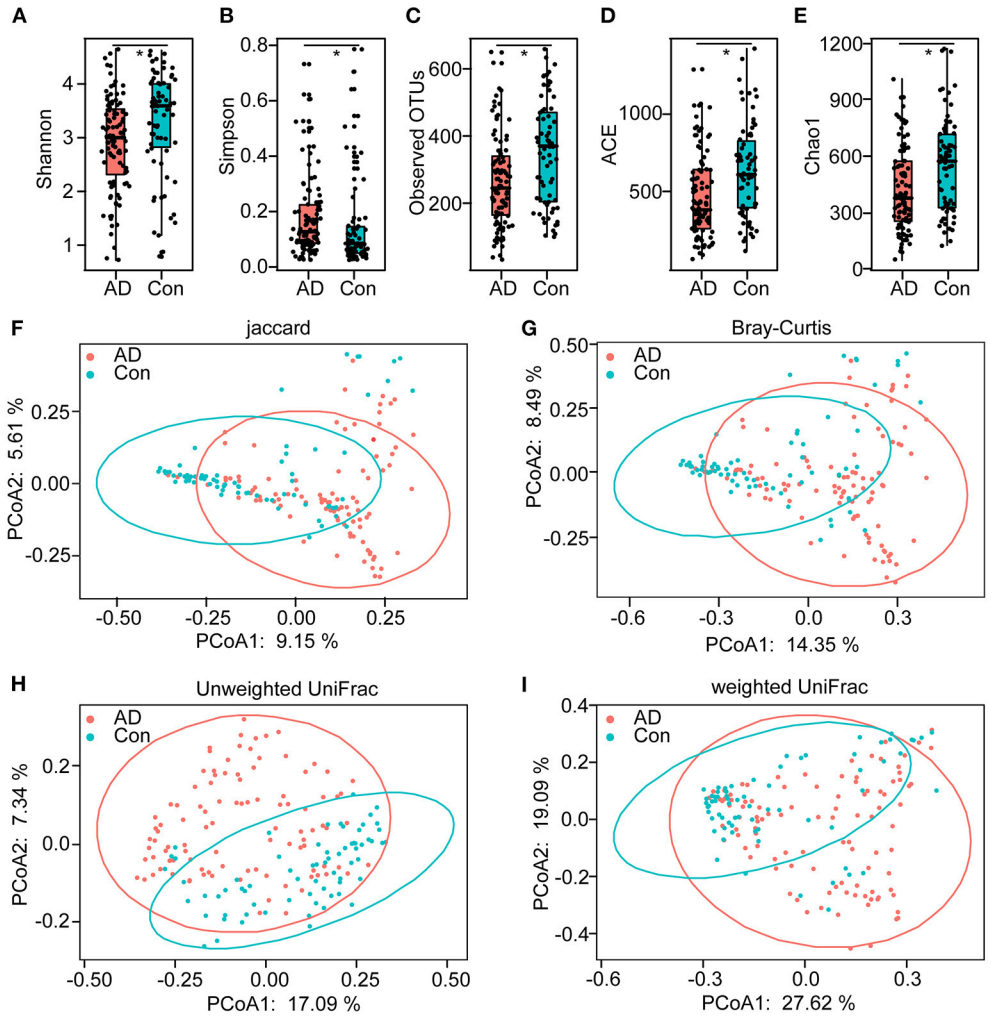

FIGURE 1 | The altered bacterial diversity and richness of the fecal microbiota in Chinese AD patients. The diversity indices of Shannon **(A)** and Simpson **(B)**, and the richness indices of the observed OTUs **(C)**, ACE **(D)**, and Chao1 **(E)** were used to evaluate the overall structure of the fecal microbiota in the AD patients and the healthy controls. The data are presented as mean ± standard deviation. Unpaired *t*-tests (two tailed) were used to analyze the variation between the groups. Principal coordinate analysis (PCoA) plots of individual fecal microbiota based on Jaccard **(F)**, Bray–Curtis **(G)**, and unweighted **(H)** and weighted **(I)** UniFrac distances in the Chinese AD patients and the healthy controls. Each symbol represents a sample. $^{*}p < 0.05$.

while 15 other pathways, including bacterial chemotaxis and the biosynthesis of fatty acid, decreased prominently in the AD-associated microbiota. Together, the functional dysbiosis of the fecal microbiota may participate in the pathogenesis and development of AD.

Correlations Between Differential Genera, and Clinical Indicators and Host Immunity

We found that the clinical indicators—the MMSE, WAIS, and Barthel scores—were significantly lower in AD patients ($p < 0.01$). By using the Bio-Plex Pro™ human cytokine group I panel 27-plex analysis, we observed that anti-inflammatory cytokines, such as IFN-γ, had significantly decreased, such pro-inflammatory cytokines as TNF-α had markedly increased, and several chemokines, such as IL-8, MCP-1, and MIP-1a, had clearly decreased. IP-10 had also decreased in the Chinese AD patients (**Supplementary Figure 4**; $p < 0.05$). To determine the associations between the deferential genera of the AD patients, and the clinical indicators and altered cytokines, we performed a correlation analysis using Spearman's rank correlation (**Figure 6**). Notably, such lactate producers as *Bifidobacterium* and propionate producers such as *Akkermansia* had the strongest negative correlations with clinical indicators such as MMSE, WAIS, and the Barthel scores, whereas butyrate-producing genera, such as *Faecalibacterium*, *Roseburia*, *Gemmiger*, *Coprococcus,* and *Butyricicoccus,* had positive correlations with the clinical indicators ($p < 0.05$). *Bifidobacterium* was negatively associated with IL-8, *Akkermansia* was negatively correlated with IFN-γ but positively correlated with IP-10, *Enterococcus* and *Corynebacterium*

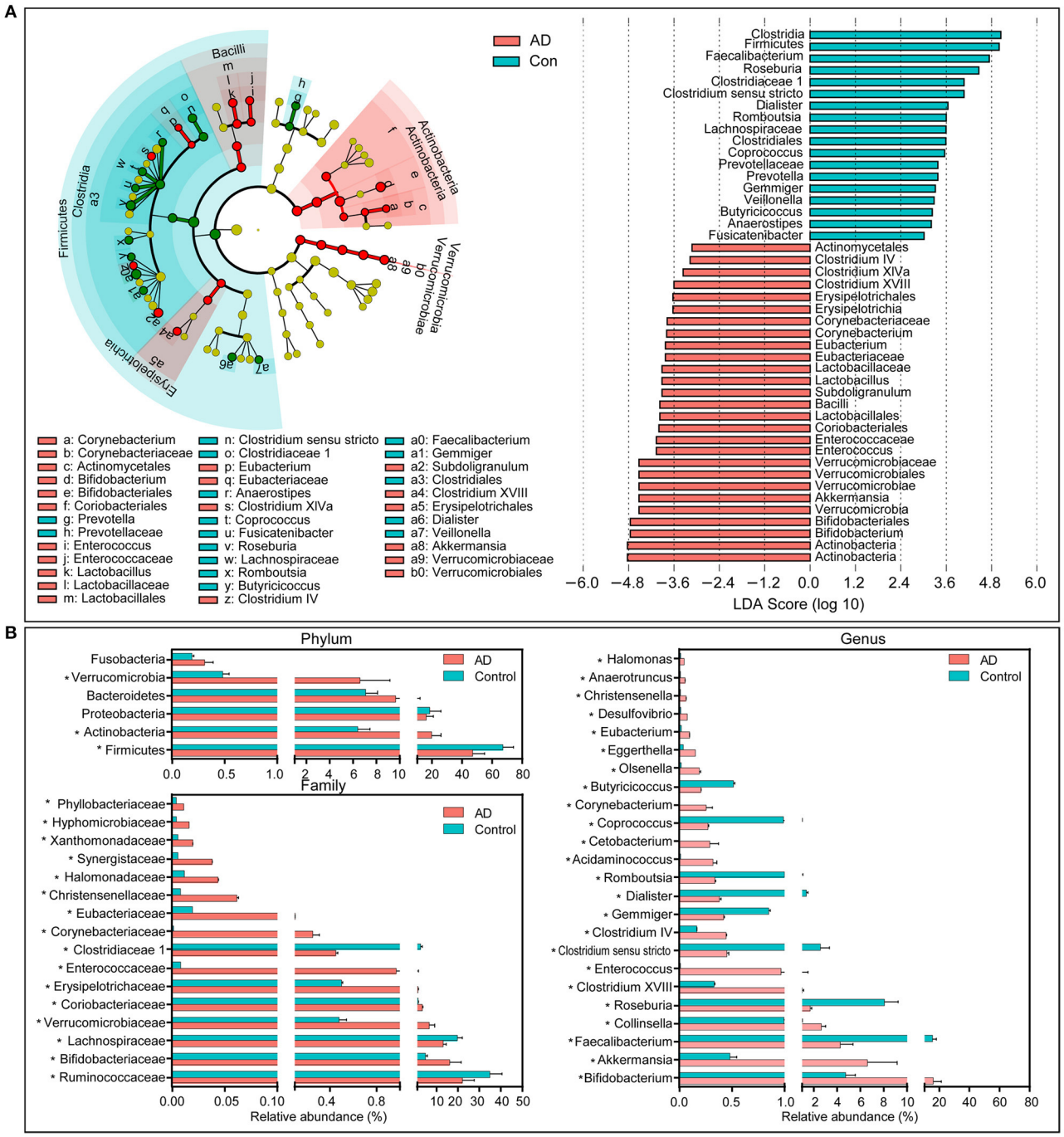

FIGURE 2 | Differential bacterial taxa between the Chinese AD patients and the healthy controls. The LEfSe identified the taxa with the greatest differences in abundance between the Chinese AD patients and the healthy controls. Only the taxa meeting a significant LDA threshold value of >3 are shown **(A)**. Comparisons of the relative abundance of the abundant bacterial taxa at the level of bacterial phylum, family, and genus **(B)**. The data are presented as the mean ± standard deviation. Mann–Whitney *U*-tests were used to analyze variation between the Chinese AD patients and the healthy controls. *$p < 0.05$ compared with the control group.

were positively correlated with the pro-inflammatory cytokine TNF-α, while *Faecalibacterium*, *Roseburia*, *Gemmiger*, and *Coprococcus* were negatively correlated with TNF-α and IP-10 ($p < 0.05$). Taken together, the enriched lactate-producing genera and the decreased butyrate-producing genera in the fecal microbiota of AD patients performed distinct roles in the progression of AD and differently modulated the immune response of the host. The altered fecal microbial profiles and

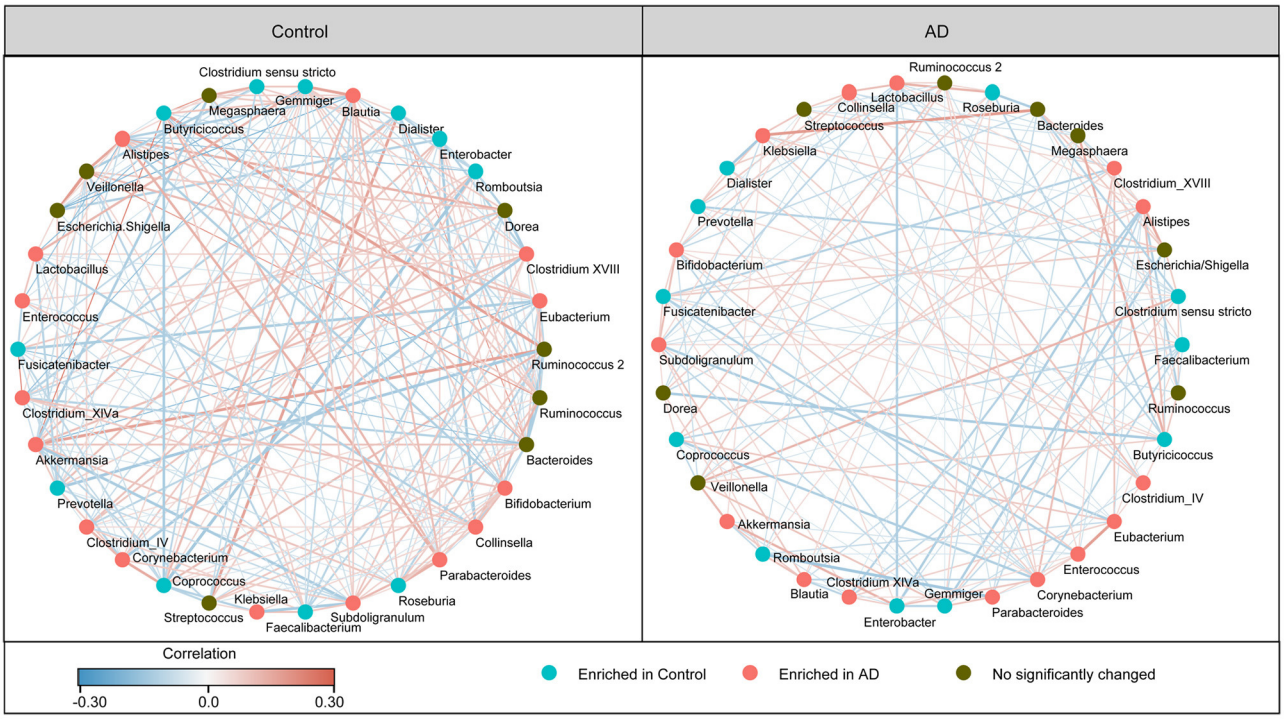

FIGURE 3 | Strengths of the correlation between abundant fecal microbiota in the Chinese AD patients and the healthy controls. Correlation network of the abundant fecal microbiota in the healthy controls and the AD patients. The correlation coefficients were calculated with the Sparse Correlations for Compositional (SparCC) data algorithm. Cytoscape version 3.4.0 was used for network construction. The red and blue lines represent positive and negative correlations, respectively. The correlation network became simpler in AD patients.

their related host responses might be the key pathophysiology of AD.

DISCUSSION

In recent years, multi-omics techniques have revealed that the gut microbiota play a crucial role in promoting human health, as a result of which they are often referred to as the "forgotten organ." Accumulating evidence indicates that the gut microbiota constitute a key factor in maintaining gut homeostasis by various complex mechanisms. Not only have the gut microbiota been invoked as a contributor to every gastrointestinal ailment, but the analyses of their influence have also been extended to other organs, such as the central nervous system (CNS). Exploring the roles and mechanisms of the gut microbiota in neurodegenerative diseases is an emerging field of research. Recently, several lines of research have suggested that changes in the composition and function of gut microbiota significantly affect neuronal function and, consequently, the host's behavior (Wang T. et al., 2015). The gut–brain axis of the microbiota has a proven role in regulating multiple neuro-chemical pathways. Microbiota–gut–brain axis signaling has uncovered a new era in psychiatry that is expected to provide novel targets for the diagnosis and treatment of psychiatric disorders and decipher their pathogeneses.

Aging is associated with an overstimulation of both innate and adaptive immune systems, resulting in a low-grade, chronic state of inflammation defined as inflammaging (Franceschi et al., 2000; Franceschi, 2007). This can increase gut permeability ("leaky gut") and bacterial translocation (Ulluwishewa et al., 2011; Tran and Greenwood-Van Meerveld, 2013). As a major age-related neurodegenerative disorder, the onset of AD has been closely correlated with alterations in the gut microbiota (Vogt et al., 2017; Zhuang et al., 2018; Li et al., 2019; Liu P. et al., 2019; Wang et al., 2019). Most of these previous "AD microbiome" studies have been mainly conducted on a small scale of patients. In this study, 100 well-controlled Chinese AD patients and 71 age- and gender-matched normal controls were enrolled for AD microbiome analysis. In total, the deeper sequencing and higher coverage allowed us to identify low-abundance taxa in AD-associated fecal microbiota. In the results, structural changes in the fecal microbiota were evident in Chinese AD patients, with decreased alpha-diversity indices and altered beta-diversity ones. Inconsistent with previous clinical studies on AD patients, our study also indicated that Shannon significantly decreased in the AD patients (Liu P. et al., 2019), and such richness indices as the observed OTUs, ACE, and Chao1 were also significantly reduced (Vogt et al., 2017). Liu P. et al. (2019) have also shown significant compositional differences between AD patients and controls in PCoA plots based on Bray–Curtis dissimilarity, which were consistent with our study. Taken together, both alpha-diversity and beta-diversity indices provide powerful evidence of structurally dysbiotic AD microbiota in the AD patients.

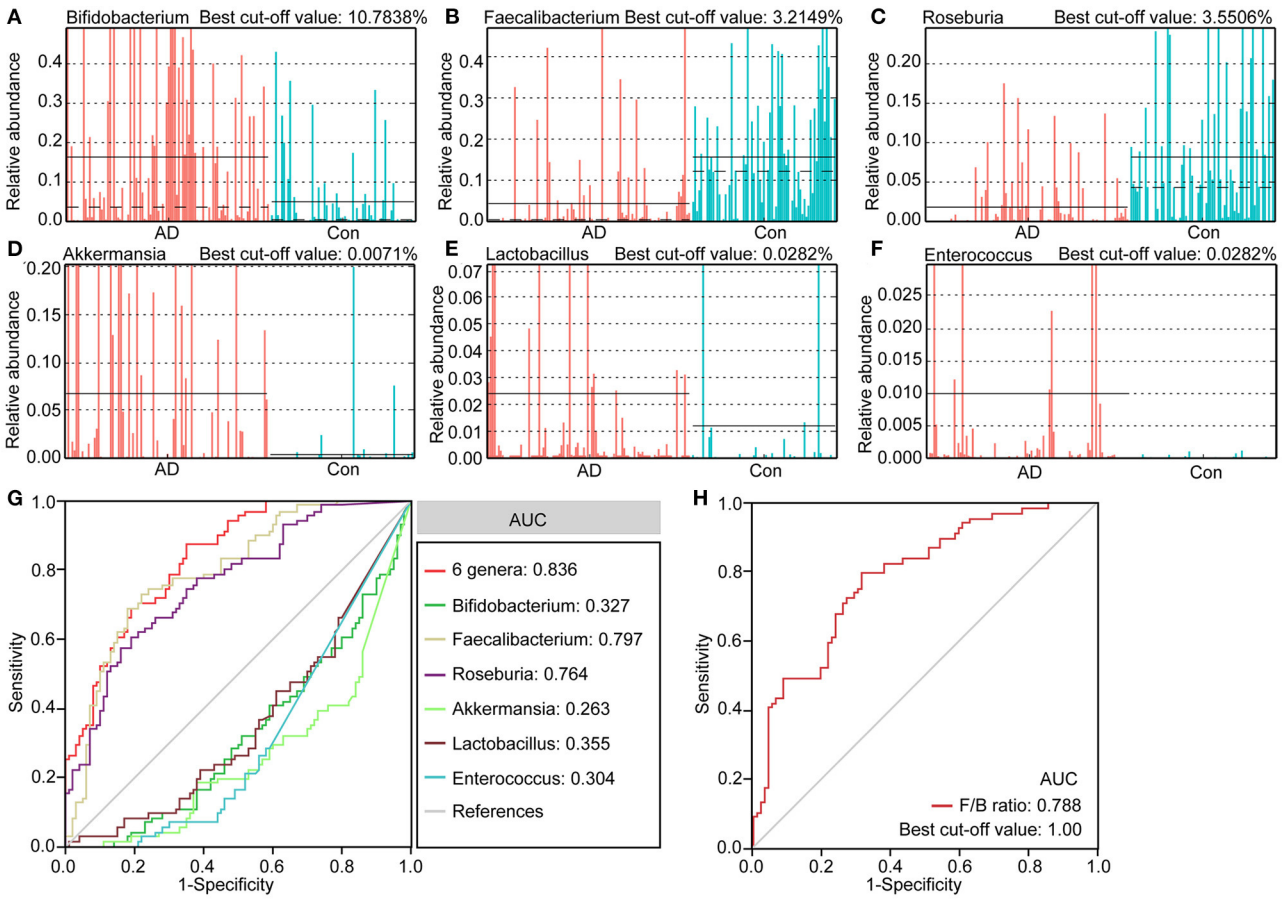

FIGURE 4 | The differential genera as AD diagnostic markers. The relative abundance and best cut-off values of the differential genera such as Bifidobacterium **(A)**, Faecalibacterium **(B)**, Roseburia **(C)**, Akkermansia **(D)**, Lactobacillus **(E)**, Enterococcus **(F)** in each sample. Receiver operating characteristic (ROC) curves for the differential genera alone or in combination **(G)**, and Faecalibacterium/Bifidobacterium **(H)**, used to discriminate healthy controls from AD patients. AUC, the area under the receiver operating characteristic curve.

In parallel with other similar studies, the structural dysbiosis observed in ours shows that the compositions of the AD-associated fecal microbiota also changed significantly. The distributions of bacterial taxa in the AD patients, at the levels of the phylum, family, and genus, were significantly different from those in healthy controls. An enrichment of the phyla Actinobacteria and Verrucomicrobia, and a decrease in the phylum Firmicutes were observed in AD-associated microbiota, while the amounts of such abundant phyla as Proteobacteria and Bacteroidetes did not change significantly, which is not consistent with previous studies (Zhuang et al., 2018; Liu P. et al., 2019). One aging indicator, the decreased ratios of Firmicutes/Bacteroidetes (Mariat et al., 2009), also did not change significantly in AD-associated microbiota. However, the ratios of Firmicutes/Actinobacteria were found to have decreased significantly ($p < 0.05$), which could reveal the bacterial dysbiosis in AD-associated microbiota. Several bacterial families—such abundant families as the *Ruminococcaceae, Lachnospiraceae*, and *Clostridiaceae* 1—decreased significantly in AD patients, while *Bifidobacteriaceae*, *Verrucomicrobiaceae*, *Coriobacteriaceae*, *Erysipelotrichaceae*, and *Enterococcaceae* increased significantly. *Ruminococcaceae* and *Lachnospiraceae* can produce different types of short-chain fatty acids (SCFAs). Among the SCFAs, butyrate has received particular attention in research owing to its beneficial effects on maintaining health. Butyrate can influence gastrointestinal physiology, the peripheral immunity of the liver metabolism, and the integrity of the blood–brain barrier, which can indirectly contribute to the functions of the brain (Fung et al., 2017). In addition, it can drive the maturation of the microglia, and is needed for the maintenance of mature microglia (Erny et al., 2015). However, the amounts of *Bifidobacteriaceae*, *Verrucomicrobiaceae*, *Coriobacteriaceae*, and *Enterococcaceae*, mainly lactate producers, increased in AD patients. Inconsistent with this study, Liu P. et al. (2019) found that the family *Enterobacteriaceae* is correlated with the presence and progression of AD, which can help distinguish between AD patients and healthy controls (AUC: 0.698). The changed fecal bacteria at the levels of phylum and family represent the dysbiosis of AD-associated fecal microbiota, but this is not suitable for

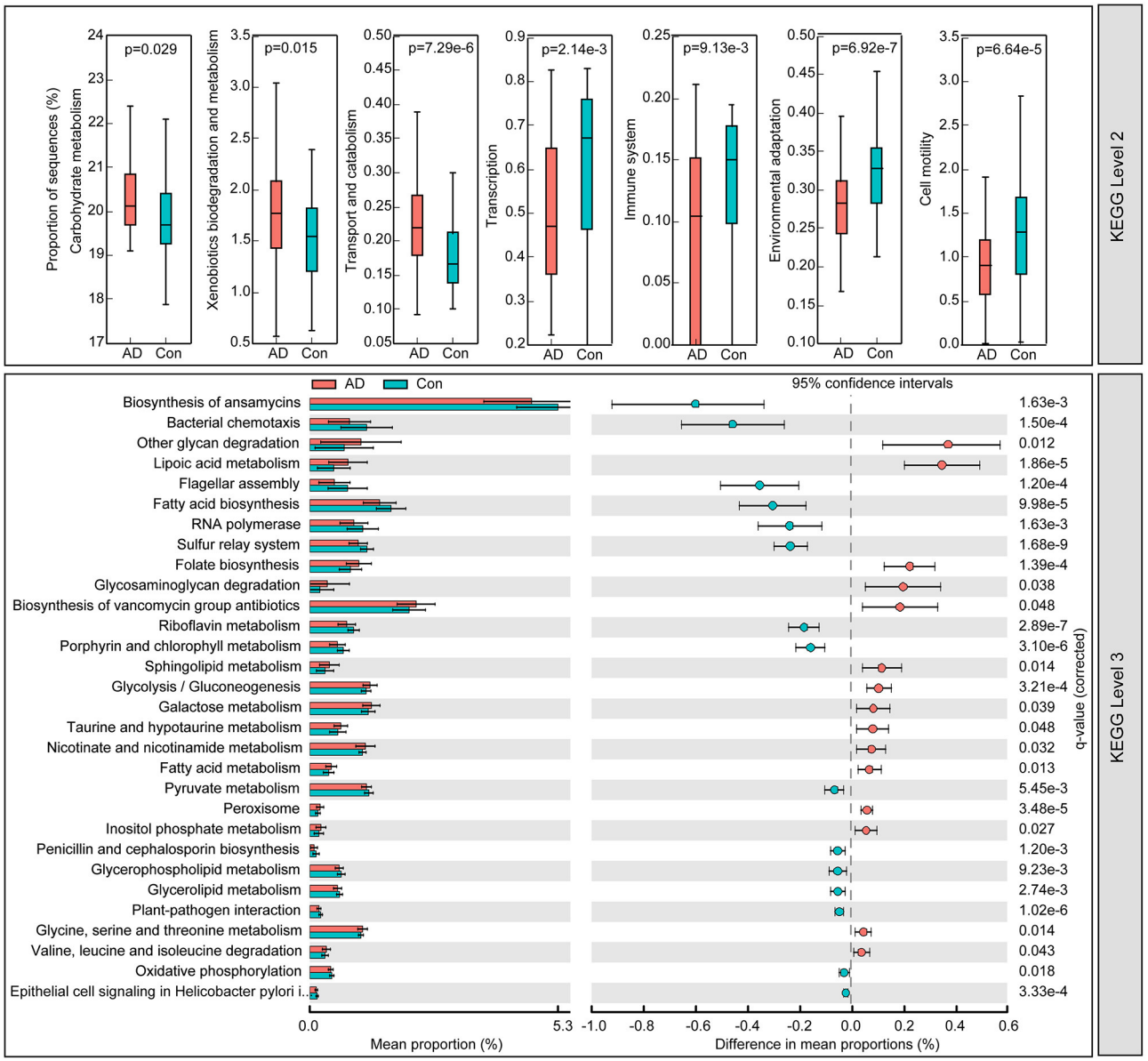

FIGURE 5 | PiCRUSt-based examination of the fecal microbiome of the Chinese AD patients and the healthy controls. The different bacterial functions were evaluated between them based on two-sided Welch's *t*-test. Comparisons between the groups for each KEGG functional category (levels 2 and 3) are shown by percentage. The Benjamini–Hochberg method was used for multiple testing correction based on the false discovery rate (FDR) through STAMP.

a non-invasive diagnosis of AD using fecal bacteria at higher taxonomic levels.

In agreement with the altered bacteria at the family level, many such genera as the *Bifidobacterium*, *Akkermansia*, *Faecalibacterium*, *Collinsella*, and *Roseburia* and changed significantly in content the AD-associated fecal microbiota. Interestingly, traditionally beneficial bacteria, such as the *Bifidobacterium* and *Akkermansia,* increase in these AD patients while *Faecalibacterium* and *Roseburia* decrease significantly. Our ROC curves also show that these differential genera can be used as biomarkers to discriminate the controls from the AD patients, alone or together, which provides novel targets for a non-invasive diagnosis of AD. *Bifidobacterium*, mainly a lactate producer, is highly beneficial to humans, and has been used as a food supplement in dairy products (Camfield et al., 2011). An open-label, single-arm, preliminary clinical study conducted by Kobayashi et al. (2017, 2019) found that oral supplementation using *Bifidobacterium breve* A1 can improve the cognitive function and maintain the quality of life of the elderly by suppressing the gene expression of inflammation and immune-reactive genes. Different from previous studies on animals and clinical studies (Vogt et al., 2017), this traditionally

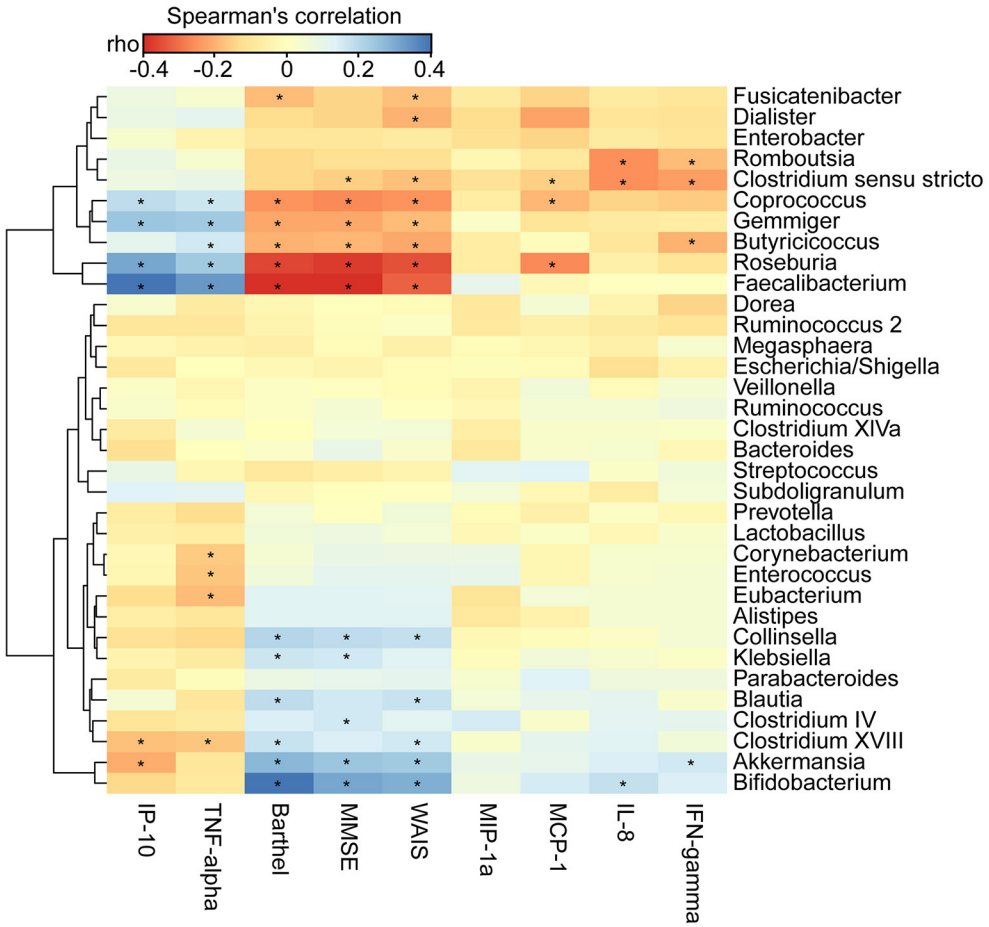

FIGURE 6 | Correlation between fecal microbiota, and pro- and anti-inflammatory cytokines and chemokines and clinical indicators. The heatmap shows partial Spearman's correlation coefficients between 34 genera, and clinical indicators and host immunity in AD patients. Spearman's rank correlation (r) and probability (p) were used to evaluate statistical importance. $^{*}p < 0.05$.

beneficial genus was among the most abundant genera in the AD-associated fecal microbiota, which suggests that the *Bifidobacterium* genus may play a crucial role in the pathogenesis and development of AD. It is challenging to link species to 16S metagenomic data, but different species of *Bifidobacterium* may have different effects that can explain why *Bifidobacterium* spp. are commonly associated with healthy and diverse microbiota but sometimes also isolated in other conditions (Pineiro and Stanton, 2007). Thus, we needed to re-examine the therapeutic potential of *Bifidobacterium* in terms of maintaining cognitive function and treating dementia. Our data also showed that *Bifidobacterium* was significantly negatively correlated with the MMSE, WAIS, Barthel, and IL-8, which also shows that *Bifidobacterium* was not a beneficial genus in our clinical study. *Akkermansia*, a specialized mucin-degrading genus, can utilize mucin-derived sugars like fucose to produce propionate through the propanediol pathway (Ottman et al., 2017). Previous work has shown that *Akkermansia muciniphila* (typical strain) is associated with protection against obesity, enhancement of wound healing, augmented antitumor responses, and induced intestinal adaptive immune responses during homeostasis (Everard et al., 2013; Greer et al., 2016; Routy et al., 2018; Ansaldo et al., 2019). Combinations of *Akkermansia*, two strains of *Clostridium*, one strain of Eubacterium, one strain of Bifidobacterium, and inulin have recently been used as special synbiotics to treat type-2 diabetes mellitus. The discovery of *Akkermansia muciniphila* has opened new avenues for the use of this abundant intestinal symbiont in next-generation therapeutic products, and they can be used to target the dynamics of microbiota. Surprisingly, our data indicate that *Akkermansia* was among the most abundant genera in the AD-associated fecal microbiota. Similarly to *Bifidobacterium*, *Akkermansia* was negatively correlated with clinical indicators of AD, such as MMSE, WAIS, and Barthel, and anti-inflammatory cytokine such as IFN-γ. Based on our present observations, *Akkermansia* cannot always be considered a potentially beneficial bacterium, it might be harmful for the gut–brain axis in the context of the AD development in the elderly. Of these AD-enriched genera, *Clostridium* IV, *Desulfovibrio,* and *Corynebacterium* have been reported to be involved in the pathologic development of AD

and other CNS diseases (Zhou et al., 2019), which is consistent with our findings. *Clostridium* IV was closely associated with type-2 diabetes and obesity in mice as well as the risk factors in AD development (Haan, 2006; Yamaguchi et al., 2016), which in turn is correlated negatively with the MMSE. Sawin et al. (2015) have also shown that *Desulfovibrio* can induce decreased levels of SCFAs that can influence pathologic conditions of CNS disease (Sampson et al., 2016). Previous studies have also found a decreased amount of *Corynebacterium* in patients suffering from depression and autism spectrum disorder (Strati et al., 2017; Yu et al., 2017), while *Corynebacterium* has been positively correlated with pro-inflammatory TNF-α. Different from Vogt's and Liu's studies, we found that such non-abundant genera as *Collinsella*, *Enterococcus*, *Olsenella*, *Eubacterium*, *Christensenella*, *Anaerotruncus,* and *Halomonas* were also enriched in AD-associated fecal microbiota (Vogt et al., 2017; Liu P. et al., 2019). *Collinsella*, one of the most abundant genera in the phylum Actinobacteria, was found to have increased prominently in the AD patients, but decreased in cases of relapsing–remitting multiple sclerosis (Chen et al., 2016), which was negatively correlated with MMSE, WAIS, and Barthel. *Enterococcus* (typical strain *E. faecalis*) can generate early Alzheimer-like neurofibrillary epitopes in primary rat cortical neurons (Underly et al., 2016), which can serve as harmful bacteria in AD etiopathology. Similarly to our findings, *Christensenella* was found to be increased in amounts in patients with Parkinson's disease in comparison with healthy controls (Petrov et al., 2017). These enriched AD-associated fecal genera, mainly lactate and propionate producers, may play a crucial role in the pathogenesis and development of AD.

However, reductions in the levels of *Faecalibacterium*, *Roseburia*, *Clostridium sensu stricto*, *Gemmiger*, *Dialister*, *Romboutsia*, *Coprococcus,* and *Butyricicoccus* were observed in AD-associated fecal microbiota. In particular, Biagi et al. (2016) and Wang F. et al. (2015) have shown that such butyrate producers as *Faecalibacterium*, *Roseburia*, and *Coprococcus* are negatively correlated with age. Our study found that these bacteria were positively correlated with AD clinical indicators, such as the MMSE, WAIS, and Barthel, and were negatively correlated with inflammatory cytokines, such as TNF-α and chemokines, such as IP-10. *Faecalibacterium* (typical strain *F. prausnitzii*), a major member of the Firmicutes phylum, is considered to be among the most important bacterial indicators of a healthy gut, and can modulate the inflammation of the level of the gut epithelium (Sokol et al., 2008). Beneficial *Faecalibacterium* has been found to be reduced in case of many intestinal disorders. Van Tongeren et al. (2005) observed a decreased relative abundance of *Faecalibacterium* in frail and elderly patients. In line with these findings, the decreased proportion of *Faecalibacterium* and increased *Bifidobacterium* have been found in elderly patients with Parkinson's disease (Scheperjans et al., 2015; Unger et al., 2016). All these changes may lead to a pro-inflammatory gut environment that may altogether lead to the chronic low-grade inflammation found in elderly persons with declining health. Previous studies have found that *Faecalibacterium* has anti-inflammatory properties due to its capability to produce butyrate and induce a tolerogenic cytokine profile (Sokol et al., 2008; Qiu et al., 2013), which can help extenuate these alterations in elderly AD patients. Liu J. et al. (2020) found that high-altitude Tibetan fermented milk can increase microbial diversity, and can elevate the levels of *Bacteroides* and *Faecalibacterium* in AD mice model, which are associated with cognitive improvements in mice afflicted with AD. The clinical comparative analyses and studies on animal mechanics confirm the beneficial roles of Faecalibacterium on mental health, which has prompted interest in considering this bacterium as a new-generation probiotic or psychobiotic. Gut *Roseburia* is part of commensal bacteria-producing SCFAs, especially butyrate, that affect immunity maintenance, colonic motility, and anti-inflammatory properties. The concomitant decreases in the well-known butyrate-producing bacterial genus *Roseburia* in many intestinal disorders (including type-2 diabetes, obesity, irritable bowel syndrome, nervous system conditions, and allergies), which suggests the potential of these bacteria as indicators of intestinal health (Tamanai-Shacoori et al., 2017). Consistently with our data, Keshavarzian et al. (2015) demonstrated the anti-inflammatory properties of *Roseburia*, and found that its levels are more abundant in feces of controls than in those of patients with Parkinson's disease. Neyrinck et al. (2011) found that the amount of *Roseburia* was inversely correlated with important markers of the metabolism and obesity of the host lipid. The role of *Roseburia* in protecting the nervous system from diseases has lately been highlighted, and has been shown to reduce neuroinflammation by regulating the gut–brain axis through its metabolite butyrate. *Coprococcus*, a less abundant bacterium in the large intestine, produces butyrate from fructose and propionate from lactate (via the acrylate pathway) (Reichardt et al., 2018). Together with Faecalibacterium, the butyrate-producing *Coprococcus* has been consistently associated with higher quality-of-life (QoL) indicators, which have been positively associated with several QoL scores (Valles-Colomer et al., 2019). Our previous study showed that *Coprococcus* is depleted in patients of depression (Jiang et al., 2015), even after correcting for the confounding effects of antidepressants (Valles-Colomer et al., 2019). Parashar and Udayabanu (2017) also found a reduction in fecal bacteria in the genus *Coprococcus* in patients with Parkinson's disease. Inconsistent with our microbiome study on AD patients here, Nagpal et al. (2019) found increased levels of *Coprococcus* in mild cognitive impairment participants in comparison with the controls. On the contrary, our study demonstrated that *Coprococcus* is positively correlated with clinical indicators of AD. *Butyricicoccus*, a butyrate-producing *Clostridium* cluster IV genus, was reduced in the feces of the AD patients. *Butyricicoccus* was found to be positively associated with the clinical indicators MMSE, WAIS, and Barthel, and anti-inflammatory cytokine IFN-γ, and negatively correlated with the pro-inflammatory cytokine TNF-α. Devriese et al. (2017) also found that reduced mucosa-associated *Butyricicoccus* activity in patients with ulcerative colitis was correlated with aberrant expressions of claudin-1, supporting its use as a pharmabiotic that preserves epithelial tight junction integrity. Zhang et al. (2017) also showed that the abundance of *Butyricicoccus* clearly decreases in a mouse model of AD in comparison with age-matched controls. Shen

et al. (2019) observed that the regulation of gut microbiota by using silibinin and silymarin, especially with an increase in *Butyricicoccus*, might prohibit AD. Another *Clostridium* cluster I genus, *Clostridium sensu stricto*, was found to have decreased in AD patients (Vogt et al., 2017). *Clostridium sensu stricto* was positively associated with HDL and negatively associated with VLDL particles (Vojinovic et al., 2019), which are associated with a decreased risk of cardiovascular disease and stroke (Holmes et al., 2018). *Gemmiger*, also an SCFAs-producing genus, was positively related with the Montreal Cognitive Assessment scale score in patients with post-stroke cognitive impairment (Liu Y. et al., 2020). Our study also found that decreased levels of *Gemmiger* were positively associated with the MMSE, WAIS, and Barthel, and negatively correlated with inflammatory cytokines such as TNF-α and chemokines such as IP-10. Consistent with our study, Vogt et al. (2017) found that the genus *Dialister* (belonging to *Veillonellaceae*) was less abundant in AD participants, and exhibited the strongest correlations with such AD biomarkers of cerebrospinal fluid as Aβ42/Aβ40, p-tau, and p-tau/Aβ42. Therefore, all these decreased levels of AD-associated fecal genera, interacting with the AD-enriched genera, where this contributed to shifts in the SCFAs, might have participated in the pathogenesis and development of AD.

The level of endogenous SCFAs is influenced by many factors, of which gut bacterial metabolism is the most important. The dysbiosis of microbiota in patients of AD can change the balanced levels of SCFAs in the human body, while abnormal levels of SCFAs may negatively affect human health. Zhao et al. (2018) have claimed that the SCFAs serve as the bridge within this associations among diet, intestinal microbiota, and health. We observed that several metabolic pathways, such as those for carbohydrate metabolism, xenobiotics biodegradation and metabolism, and the immune system, changed significantly in AD-associated fecal microbiota. The characteristics of the AD microbial profiles changed from butyrate producers, such as Faecalibacterium into lactate producers, such as *Bifidobacterium*. These alterations contributed to shifts in metabolic pathways from butyrate to lactate, which might have participated in the pathogenesis of AD. However, the specific roles of the AD-associated signatures and their functions should be explored in further studies.

Our study is limited in some ways. First, it used the 16S rRNA amplicon rather than metagenomic sequencing, which limited us to the finding of specific bacteria related to AD at the species level. Second, our cross-sectional study investigated only healthy controls and confirmed AD participants. To decipher the dynamic interplay between microbiota and AD, a longitudinal follow-up case-control study should include different stages of AD, such as the preclinical stage and the mild cognitive impairment stage, that signify the transition from health to AD. Third, the fecal microbial signatures and the corresponding metabolites as well as the non-invasive diagnostic model associated with AD still need a larger sample size of clinical studies to be validated. Fourth, culturomics should be used to obtain the AD-associated bacteria, and animal experiments can help determine the cause–effect relationship between these bacteria and the pathogenesis of AD.

In summary, we found altered bacterial composition and decreased bacterial diversity of the fecal microbiota in AD patients compared with healthy elderly subjects. The structural dysbiosis of the fecal microbiota of the AD patients was characterized by reductions in butyrate-producing bacteria, such as *Faecalibacterium,* and increases in lactate-producing ones, such as *Bifidobacterium*, which were both significantly correlated with host pro- and anti-inflammatory cytokines as well as clinical indicators of AD in the host. These changes in key functional bacteria, such as the *Faecalibacterium* and *Bifidobacterium*, can be used as non-invasive biomarkers to discriminate between healthy elderly subjects and AD patients. Transformations of the gut microbiota from lactate producers into butyrate producers through personalized diet or intervention from beneficial microbiota may be useful for patient-tailored early intervention in cases of AD. In addition, the functional dysbiosis in AD-associated fecal microbiota also suggests that the changed fecal microbiota is associated with changed functions and metabolic activities of the patients, which might play vital roles in the pathogenesis and development of AD. Therefore, our investigation of fecal microbiota using a large and confirmed AD cohort provides novel insights into disease pathogenesis, which can provide new avenues for the scientific trajectory of managing neurodevelopmental disorders by modulating the gut microbiome.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Lishui Second People's Hospital (Zhejiang, China). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

ZL, MZ, XY, and SW conceived and designed the experiments. ZL, MZ, XY, YC, LS, XL, and RJ performed the experiments. ZL, MZ, XY, and LS analyzed the data. ZL, MZ, LS, and SW wrote the paper and edited the manuscript. The final manuscript was read and approved by all authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.634069/full#supplementary-material

Supplementary Figure 1 | Variations in the compositions of fecal microbiota in the Chinese AD patients and the healthy controls. Relative proportions of bacterial phyla in AD patients ($n = 100$) and healthy controls ($n = 71$).

Supplementary Figure 2 | Variations in the composition of fecal microbiota in the Chinese AD patients and the healthy controls. Relative proportions of bacterial genera in AD patients ($n = 100$) and healthy controls ($n = 71$).

Supplementary Figure 3 | Heatmap of the genus-level taxa in the fecal microbiota of the Chinese AD patients and the healthy controls. The color of the spots in the panel represents the relative abundance (normalized and $\log_{10}$ transformed) of the genus in each sample. The relative abundance of the bacteria in each genus is indicated by a gradient of colors from blue (low abundance) to red (high abundance). The taxonomic classifications of the family are shown on the right. The corresponding Shannon's index in each sample is shown under the heatmap.

Supplementary Figure 4 | Mean concentrations (pg/ml) of pro- and anti-inflammatory cytokines and chemokines in patients with AD and in healthy controls determined using Bio-Plex immunoassays. The concentrations of TNF-α **(A)** and IP-10 **(D)** increased significantly in patients with AD, while those of IL-8 **(B)**, MCP-1 **(E)**, MIP-1a **(F)**, and IFN-γ **(C)** decreased significantly. $^*p < 0.05$.

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REVIEW
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Roles of Macrophages in the Development and Treatment of Gut Inflammation

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Macrophages, which are functional plasticity cells, have the ability to phagocytize and digest foreign substances and acquire pro-(M1-like) or anti-inflammatory (M2-like) phenotypes according to their microenvironment. The large number of macrophages in the intestinal tract, play a significant role in maintaining the homeostasis of microorganisms on the surface of the intestinal mucosa and in the continuous renewal of intestinal epithelial cells. They are not only responsible for innate immunity, but also participate in the development of intestinal inflammation. A clear understanding of the function of macrophages, as well as their role in pathogens and inflammatory response, will delineate the next steps in the treatment of intestinal inflammatory diseases. In this review, we discuss the origin and development of macrophages and their role in the intestinal inflammatory response or infection. In addition, the effects of macrophages in the occurrence and development of inflammatory bowel disease (IBD), and their role in inducing fibrosis, activating T cells, reducing colitis, and treating intestinal inflammation were also reviewed in this paper.

Keywords: macrophages, inflammation, homeostasis, intestinal, treatment

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INTRODUCTION

The intestinal tract is the largest independent immune system in the body. It is continuously exposed to foreign antigens and the distinction between harmful and harmless antigens is necessary for the intestine to ensure an appropriate response to every antigen (Weiner, 2000; Ma et al., 2020a). The gut needs to produce a strong protective immune response to resist the invasion of pathogenic antigens, while similar reactions to harmless antigens such as dietary proteins or symbiotic microorganisms, may lead to chronic inflammatory diseases. Macrophages are phagocytes found in tissues and maintain tissue homeostasis, regulate inflammation, and play a significant role in host protection. There are many microorganisms colonized in the human intestine, and more than 1000 bacterial species in the intestinal ecosystem of a single individual. Among them, Actinobacteria, Bacteroidetes, Firmicutes, Proteobacteria, and Tenericutes are the predominant bacterial phyla, while the abundances of Fusobacteria, Saccharibacteria, Spirochaetes, Synergistetes and Verrucomicrobia are lower (Fassarella et al., 2020).

The production of phagocytic cytotoxic substances by activated macrophages is a key process in the control of intracellular pathogens (Piacenza et al., 2019). The pattern recognition receptors on the surface of macrophages recognize and bind to the corresponding pathogen associated molecular pattern (PAMP)—a specific molecular structure shared by some pathogens—on pathogens, and

nonspecifically phagocytize and remove pathogenic microorganisms (Ley et al., 2016). Different kinds of microorganisms express different PAMPs, including mainly lipopolysaccharides (LPSs), phosphoteichoic acid, peptidoglycan, and other structures that usually do not exist in the host. Then, the pathogens are phagocytized and digested by macrophages, and the lymphocytes or other immune cells are activated to kill these pathogens (Jain et al., 2019). On the other hand, phagosomes are formed when the pathogen is engulfed by macrophages and fuse with lysosomes to release enzymes and toxic substances, resulting in killing or having cytotoxic effects on bacteria and tumor cells. The intestinal mucosa is the first line of defense for organisms against intestinal pathogens. The lamina propria of the small intestine is the main site of the intestinal immune system, which contains a large number of macrophages, CD4 T cells, and dendritic cells. These cells play a key role in early resistance to intestinal pathogens. Macrophages play a significant role in many processes, such as the human immune function, parasite infection, and tissue remodeling by secreting cytokines and producing reactive oxygen and nitrogen intermediates. In a broad sense, intestinal macrophages are divided into two categories: resident and inflammatory (Mills et al., 2000). The former maintains intestinal health, while the latter plays an important role in the occurrence of inflammatory reactions. Multiple studies have shown that macrophages are associated with the development of intestinal inflammation and secrete a large number of cytokines and bioactive substances that participate in the inflammatory response (Cummings et al., 2016; Joeris et al., 2017). Herein, we review the origin and development of macrophages and their role in intestinal inflammation and treatment.

INTESTINAL INFLAMMATION

The healthy gut can control inflammation through its powerful mechanisms, but inflammatory bowel disease (IBD) can occur if the inflammation is not resolved (Murray and Smale, 2012). IBD, which includes Crohn's disease and ulcerative colitis, is a kind of chronic gastrointestinal inflammatory disease with unknown etiology and recurrent attacks (Torres et al., 2017; Ungaro et al., 2017). The pathogenesis of IBD is unknown, but it is believed that the uncontrolled immune response of genetically predisposed individuals to environmental factors and intestinal microorganisms is the cause (Khor et al., 2011; Kostic et al., 2014; de Souza and Fiocchi, 2016; Liu and Stappenbeck, 2016; Ni et al., 2017; Ananthakrishnan et al., 2018). In other words, the combined effects of genetic, microbial, immune, and environmental factors lead to an abnormal and excessive immune response of the commensal microbiota (Wallace et al., 2014). When the intestine is invaded by pathogens, which can cross the damaged intestinal epithelial cell barrier, the intrinsic defense cells in the epithelium, especially the macrophages, will produce pro-inflammatory cytokines after being stimulated, and then release interleukin-1 (IL-1), IL-6, IL-18, transforming growth factor-β (TGF-β), and tumor necrosis factor-α (TNF-α). These cytokines directly or indirectly affect the intestinal epithelial cells, leading to the injury or necrosis of these cells, which promotes the occurrence and development of IBD (**Figure 1**). An over-secretion of cytokines and chronic inflammation are the typical features of IBD, with clinical symptoms of diarrhea, abdominal pain, fever, intestinal obstruction, and disability symptoms of blood or mucus or both (Baumgart and Carding, 2007; Geremia et al., 2014; Geremia and Arancibia-Cárcamo, 2017; Hidalgo-Garcia et al., 2018; Ding et al., 2019b). IBD occurs exclusively in the colon in ulcerative colitis and almost anywhere along the gastrointestinal tract in chronic diarrhea (Jones et al., 2018). In addition, IBD also has the characteristics of intestinal microbiota dysbiosis. Compared with the gut of healthy people, the quantity and diversity of intestinal bacteria is lower in IBD patients (Frank et al., 2007; Lepage et al., 2011; Kostic et al., 2014). In some sufferers, the inflammation of the mucosa is associated with these changes and bacterial factors (Gevers et al., 2014; Franzosa et al., 2019; Lloyd-Price et al., 2019). Some scholars have analyzed the pro-inflammatory and anti-inflammatory pathways of IBD patients, and the results show that the imbalance of immune responses is caused by the change of balance among inflammatory, regulatory and anti-inflammatory cytokines (Bouma and Strober, 2003). When IBD occurs, monocyte infiltration will increase and produce many pro-inflammatory mediators, including TNFα, IL-1, IL-23, and nitric oxide (Ogino et al., 2013; Bain and Mowat, 2014; Sanders et al., 2014; Magnusson et al., 2016; Joeris et al., 2017). Many types of mucosal immune cells are related to the pathogenesis of IBD: intestinal epithelial cells, innate arm dendritic cells, innate lymphoid cells, neutrophils, macrophages, $Foxp3^+$ regulatory T (Treg) cells of the adaptive arm, interferon-γ-producing type 1 helper T cells (Th1), interferon-γ helper T cells (Th17), and secretory mediators of the adaptive arm of the mucosal immune system—cytokines, chemokines, eicosanoic acid, reactive oxygen species and nitrogen species (Xavier and Podolsky, 2007; Wu et al., 2015). A study has found that, compared with quiescent IBD or the healthy intestine, IBD in active humans was related to the increase of colonic mRNA expression of TNF, IL-1β and IL-6, and of the $HLA\text{-}DR^{Int}$:$HLA\text{-}DR^{Hi}$ and $CD14^{Hi}$:$CD14^{Lo}$ cell ratios (Jones et al., 2018).

Molecular cues are also responsible for the contribution of intestinal macrophages in the development of IBD. Toll-like receptors (TLRs) play a key role in maintaining intestinal homeostasis. After recognizing PAMPs, TCLs are activated to regulate both innate and adaptive immunity. Innate immunity is regulated by mediating the phosphorylation of IκB, thereby activating NF-κB. Moreover, the proliferation and differentiation of Th1 and Th2 from T cells is regulated by TCLs to regulate adaptive immunity. When these regulations are disturbed, the expression of TLRs increases and the downstream signaling cascade is over activated, resulting in the over production of inflammatory cytokines and IBD (Lu et al., 2018). When IBD occurs, it is often accompanied by the death of intestinal epithelial cells (Abraham and Cho, 2009). Epithelial injury and inflammation in IBD patients are usually dependent on TNF (Zeissig et al., 2004). When the production of TNF

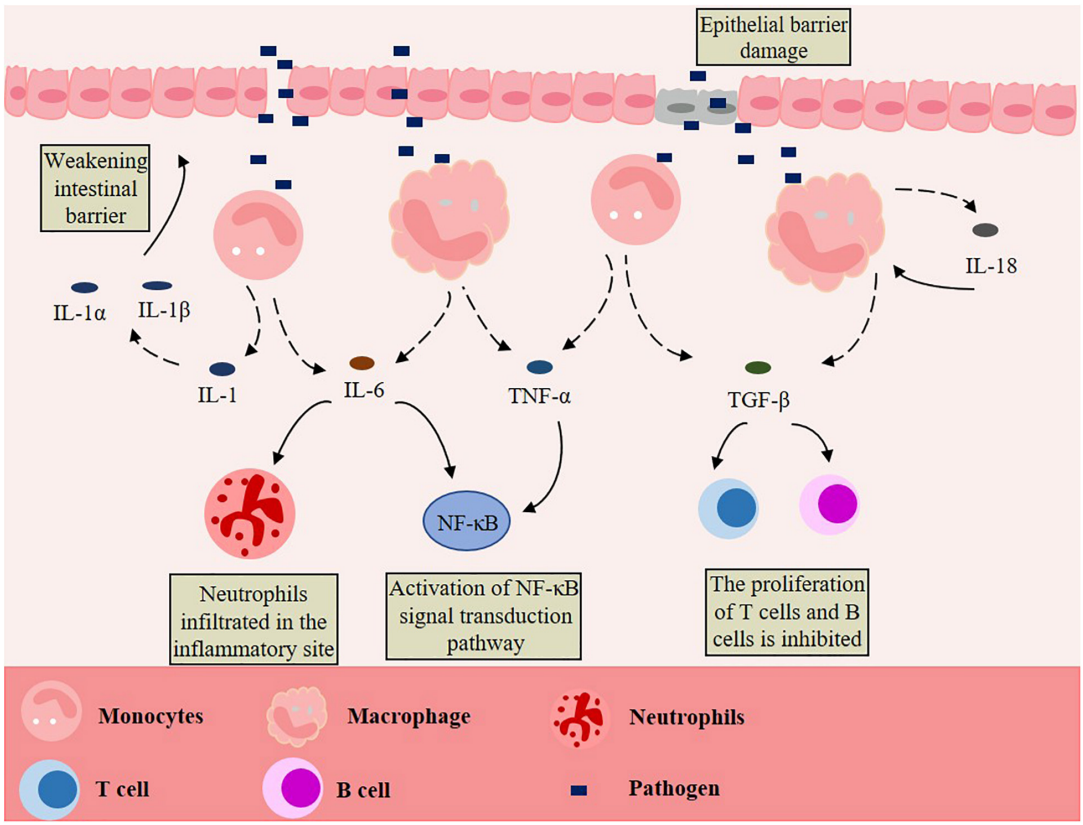

FIGURE 1 | Macrophages promote the development of IBD. Pathogens cross the damaged intestinal epithelial cell barrier and stimulate macrophages to produce pro-inflammatory cytokines, such as interleukin-1 (IL-1), IL-6, IL-18, transforming growth factor-α (TGF-α) and tumor necrosis factor-β (TNF-β) are released. These act on intestinal epithelial cells directly or indirectly, leading to the injury or necrosis of these cells, thus promoting the occurrence and development of IBD.

increased in IBD, the expression of the *TNFAIP3* gene, which encodes A20, also increases. The A20 protein is the negative feedback regulator of NF-κB. In the intestinal epithelium of IBD patients, A20 is expressed by an intestinal epithelial cell specific promoter and is highly sensitive to intestinal epithelial cell death, intestinal injury, and shock induced by TNF (Garcia-Carbonell et al., 2018).

Generally speaking, IBD often occurs in young individuals, and most patients with IBD are expected live to a normal life due to the progress of medical treatment. Despite the low mortality rate of IBD, the incidence rate is still a serious problem. Moreover, IBD is incurable and increases the risk of lymphoma, cholangiocarcinoma, and colorectal cancer (Samadder et al., 2019; Scharl et al., 2019). Many patients with IBD have to undergo surgery multiple times to relieve symptoms, which may lead to postoperative complications and infections, adversely affecting their quality of life (Torres et al., 2017; Ungaro et al., 2017; Liang et al., 2018). There have been some—although relative few—experiments using immunomodulators for IBD treatment, but the effect of the treatment declines with time (Torres et al., 2017; Ungaro et al., 2017; Friedrich et al., 2019). Tissue reparative programs may also contribute to restoring the barrier, but improper regulation may lead to fibrosis and intestinal structuring due to the dysregulation of intestinal, which is a possible complication of IBD (Rieder and Fiocchi, 2009; Rieder et al., 2017).

ONTOGENY, LOCATION, AND CHARACTERIZATION OF MACROPHAGES

Origin and Differentiation of Macrophages

Macrophages are white blood cells located in tissues. In general, it is believed that macrophages are derived from monocytes, and monocytes are derived from precursor cells in bone marrow, which is also known as the granulocyte-macrophage colony-forming unit (GM-CFUc) (**Figure 2**; van Furth and Cohn, 1968; van Furth et al., 1972). However, whether monocytes differentiate into tissue-specific macrophages in the blood is still controversial. Some scholars believe that monocytes continue to develop and mature in the blood, where they can migrate to different tissues to form cell groups with different functions and structures. According to their function during the migration from blood to tissue, they can be divided into "inflammatory" and "resident" monocytes (Geissmann et al., 2003). The resident

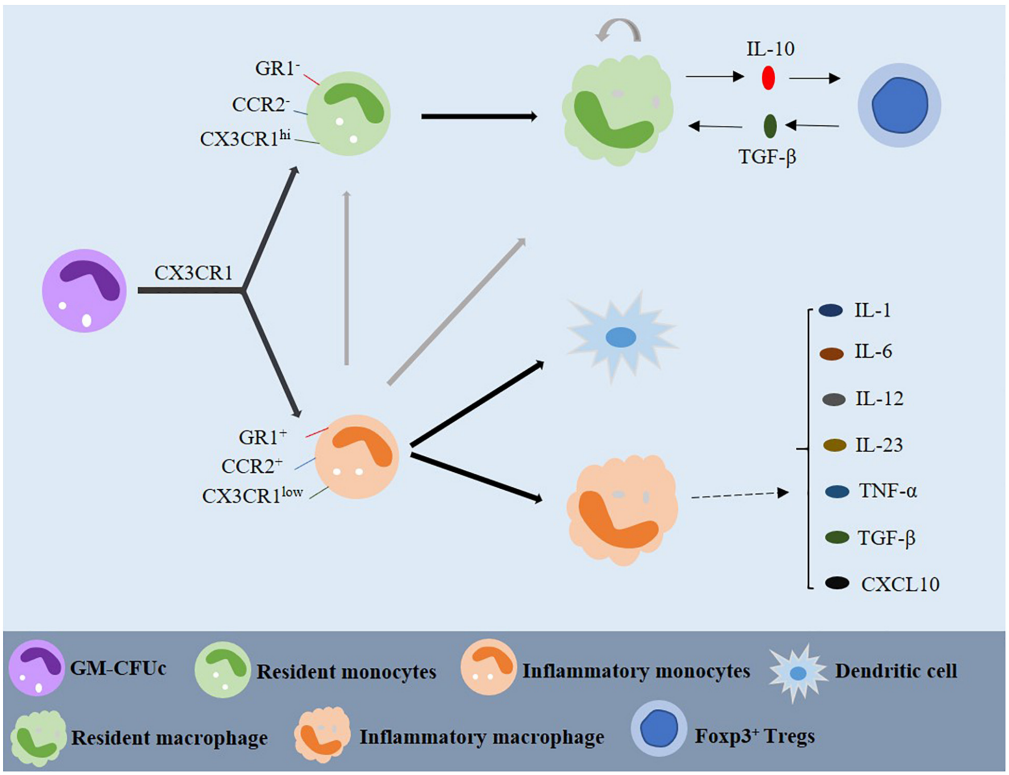

FIGURE 2 | Origin and differentiation of macrophages. GM-CFUc can be divided into resident and inflammatory monocytes by CX3CR1. Inflammatory monocytes may also be one of the sources of resident monocytes. Resident macrophages are usually produced by resident monocytes or, sometimes, by inflammatory monocytes. Resident macrophages and Foxp3$^+$ T cells play a significant role in maintaining intestinal homeostasis through IL-10 and TGF-β dependent mechanisms. When there is inflammation in the intestine, inflammatory monocytes migrate to the intestine and differentiate into dendritic cells and inflammatory macrophages, which can produce a variety of cytokines involved in the inflammatory reaction. GM-CFUc: granulocyte macrophage colony forming unit; IL: interleukin; TNF-α: tumor necrosis factor-α; TGF-β: transforming growth factor-β.

monocytes are defined as CCR2$^-$, CX3CR1^{hi}, and GR1$^-$, they exist in the non-inflammatory tissues and have a long half-life. The precursor inflammatory monocytes are CCR2$^+$, CX3CR1^{low}, and GR1$^+$ are found in the inflammatory tissues, having a short survival time. The two monocyte populations can be distinguished by the expression of CX3CR1, a cell surface marker (**Figure 2**; Geissmann et al., 2003; Strauss-Ayali et al., 2007). One study has shown that when blood vessels are damaged and infected, the colonized monocytes rapidly invade the tissues, and then initiate the innate immune response and differentiate into macrophages (**Figure 2**; Geissmann et al., 2003). By contrast, inflammatory monocytes reach the site of inflammatory response and differentiate into inflammatory dendritic cells after infection. It has been demonstrated that inflammatory monocytes can differentiate into inflammatory macrophages, and the migration of resident monocytes may depend on chemical signals from damaged tissues or endothelial cells (**Figure 2**; Mowat and Bain, 2011).

In the human bone marrow, monocytes can be divided into Ly6C^{lo} monocytes and Ly6C^{hi} monocytes through the expression of Ly6C/GR1, CCR2, and CX3CR1. Because Ly6C^{hi} monocytes tend to perform functions that traditionally belong to monocytes, they are now called "classical" monocytes. On the other hand, some scholars have proposed that Ly6C^{lo} monocytes are the precursors of tissue resident macrophages because they do not enter inflammatory tissue (Geissmann et al., 2003). In addition, a study has shown that the "non-classical" Ly6C^{lo} monocytes are not used as circulating intermediates, but their main function is to patrol the vascular system and remove necrotic endothelial cells (Carlin et al., 2013). Therefore, Ly6C^{lo} monocytes can be considered the macrophages of the circulatory system in some ways. In the normal colon, monocytes gradually differentiate into resident macrophages. When there is inflammation in the gut, resident macrophages still originate from monocytes in blood circulation but change from anti-inflammatory to inflammatory macrophages with high expression of TLRs. However, studies have shown that Ly6C^{hi} monocytes can also be converted to Ly6C^{lo} monocytes and returned to the bone marrow to replenish the resident macrophages (**Figure 2**; Gren and Grip, 2016). Some scholars proposed that resident macrophages also have the characteristics of self-renewal (**Figure 2**; Bain and Mowat, 2011).

Resident macrophages produce anti-inflammatory cytokines, such as IL-10 and TGF-β. Studies have reported that IL-10 produced by macrophages has the effect of regulating the expression of Foxp3$^+$ Tregs, and macrophages highly express the TGF-β receptor and participate in the signal transduction of

activated TGF-β (**Figure 2**; Mowat and Bain, 2011; Weinhage et al., 2015). TGF-β can combine with the Foxp3 expressed by Tregs to form $CD4^+$ $Foxp3^+$ Tregs, which can reduce the activation of macrophages and translocation of NF-κB in the mucosa (Li et al., 2010).

Some studies have shown that many tissue macrophages do not originate from blood mononuclear cells but exist independently of conventional hematopoiesis and originate from embryonic precursors produced by the yolk sac or fetal liver (Schulz et al., 2012; Hashimoto et al., 2013; Yona et al., 2013; Epelman et al., 2014; Sheng et al., 2015). As we know, the differentiation continuum of monocyte to macrophage exists in intestinal lipoprotein, which has been called the monocyte "waterfall" (Tamoutounour et al., 2012; Bain et al., 2013). $Ly6C^{hi}$ $CX3CR1^{int}$ $MHCII^-$ monocytes exist at one end of the "waterfall", and their phenotype and morphology are similar to those of their counterparts in the blood. In fact, the expression of molecules of monocytes in the mucosa, including CCR2, VLA-1, CD62L, Ly6C and LFA-1, is still preserved; these molecules are related to the chemotaxis and extravasation of circulation (Schridde et al., 2017). First, these monocytes show MHCII expression; Then, the molecules associated with extravasation, including LFA-1, CCR2, and CD62L, are downregulated; Finally, CX3CR1 is upregulated to obtain fully mature macrophages (Tamoutounour et al., 2012; Bain et al., 2013; Schridde et al., 2017). At the same time, it has been proved that the human intestinal mucosa presents a similar "waterfall", with the classic $CD14^{hi}CCR2^+CD11C^{hi}$ monocytes and mature $CD14^{lo}CCR2^-CD11C^{lo}$ macrophages at the two ends (Bain et al., 2013; Bernardo et al., 2018; Bujko et al., 2018).

Inflammation includes the detection of tissue injury or infection, the subsequent inflammatory response and the final resolution. Monocytes are equipped with a large number of scavengers and pattern recognition receptors, which can react to local danger signals quickly. Their high plasticity enables them to adapt to molecular changes in response to the production of effector molecules that drive inflammation. Although we now have a deeper understanding of the function of Ly6C, more research is needed to explain the molecular mechanism of monocytes acting in a restorative rather than pathological manner.

Distribution of Intestinal Macrophages

Macrophages play a significant role in regulating intestinal peristalsis. They are distributed throughout the gastrointestinal mucosa, with a large proportion of them being located in the natural layer (LP) near the epithelium and a small part of them appear in the smooth muscle layer of the intestinal wall (Tajima et al., 2012; Gabanyi et al., 2016). In different parts of the gastrointestinal tract, the number of macrophages varies in the intestinal mucosa. Both in humans and rodents, the number of macrophages in the colon and lamina propria was found to be more than that in the small intestine (Nagashima et al., 1996; Denning et al., 2011). However, the number of macrophages follows a continuous gradient trend between the proximal and distal intestines of mice, while the number of macrophages in different parts of the colon was similar in mice and humans (Nagashima et al., 1996; Grainger et al., 2017).

Functional Plasticity of Macrophages

Generally speaking, macrophages are phagocytes in tissues and play an important role in homeostasis of adipose and tissue, regulation of inflammatory response and defense protection of host. Macrophages have the property of plasticity and can change their physiology, being able to produce different cell populations with different functions, according to environmental cues (Mosser and Edwards, 2008). The activation state of macrophages was initially divided into classically activated M1 macrophages and alternatively activated M2 macrophages. Inflammatory macrophages are usually activated as the M1 phenotype, while resident macrophages usually belong to the activated M2 phenotype. M1 and M2 macrophages are induced by interferon-γ (IFN-γ) and IL-4, respectively, and participate in the anti-microbial response and the reaction of wound healing and tissue remodeling, respectively (Stein et al., 1992; Mills et al., 2000). It is difficult to distinguish M1 and M2 *in vivo* due to the mixing of activated M1/M2 macrophages caused by the multitude of stimulations, although the polarization state of prototypes M1/M2 has been established *in vitro* (Martinez and Gordon, 2014). Some studies have shown that macrophages become a continuum of activation states when they are stimulated by certain cytokines or complexes, such as TNF-α, LPS, TGF-β, IL-10, IL-13, Glucocorticoid or the immune complex, and macrophage activation with similar but different transcriptional and functional is subsequently produced along the M1/M2 axis (Martinez and Gordon, 2014; Murray et al., 2014; Xue et al., 2014; Murray, 2017). Moreover, some studies have found that macrophages are activated outside the M1-M2 continuum when they are stimulated by high-density lipoproteins, free fatty acids, or chronic-inflammation-related stimulants (Popov et al., 2008; Xue et al., 2014).

The activation and function of macrophages are complex, but the activated states can be identified and distinguished by the abundance of transcription factors, cytokines, and surface molecules (**Table 1**). For example, M1 macrophages usually produce high levels of pro-inflammatory cytokines, such as TNF-α, IL-6 and IL-12, and promote the induction of nitric oxide synthase (iNOS) and the expression of indoleamine 2,3-dioxygenase in mice and humans, respectively, while M2 macrophages are generally distinguished by stimuli-specific molecules and more general M2 markers (Murray et al., 2014; Xue et al., 2014). CD206 is one such surface marker induced by IL-4/IL-13 and IL-10 in mice and humans, respectively (Stein et al., 1992; Mantovani et al., 2004; Murray et al., 2014). The expression and activity of arginase I also constitute a marker of M2-polarized macrophages in mice, but not in human (Thomas and Mattila, 2014). The expression of IL-10 in several polarization states of M2 macrophages (except for those induced by IL-4/IL-13) is higher than for M1 macrophages, making it a frequently used marker of M2 macrophages.

In addition, macrophages can also differentiate into Mregs and TAM, which have different stimulating factors, surface markers, cytokines and functions (**Table 1**) (Mosser and Edwards, 2008).

However, it is not clear what causes the change of activation status of macrophages, the reasons may be the recruitment of monocytes and their response to local changes, the repolarization between M1 and M2 macrophages, or a combination of the two (Italiani and Boraschi, 2014). The traditional macrophage polarization model is not sufficient to describe the full range of macrophage activity. Due to the increased heterogeneity of macrophages in the gut, further work is needed to analyze the role of macrophage subsets in health and disease. Many technologies have been used to study the heterogeneity of macrophages. For example, single-cell RNA sequencing has been used for the transcriptomic profiling of haematopoietic cells in humans, and macrophage heterogeneity across multiple anatomical sites was mapped, with diverse subsets being identified (Bian et al., 2020). A rapid three-dimensional (3D) printing method was also used in the research of cell heterogeneity. Tang et al. reported a controllable, repeatable, and quantifiable 3D bioprinting model of the glioblastoma microenvironment, simulating the high cell heterogeneity and cell interaction in the tumor microenvironment (Tang et al., 2020). In addition, macrophages have highly specialized functions in different tissues, and their receptors are also different. They may cooperate or compete for ligand recognition, which will affect their function.

MACROPHAGES IN THE INTESTINAL MUCOSA DURING INFLAMMATION

Intestinal Homeostasis and Its Disruption During Inflammation

The gut, which is exposed to pathogens, commensal microbiota, and food antigens, is one of the main interfaces for contact with the outside ambient. The balance between immune responses to pathogens and tolerance is necessary for this bodily niche in order to maintain intestinal homeostasis and body health (Hill and Artis, 2010; Pabst and Mowat, 2012; Belkaid and Hand, 2014). The intestinal epithelium, which is mainly made up of a single layer of intestinal cells, is tightly connected with adjacent cells to form a critical continuous physical barrier, which regulates the selective permeability of luminal content (Odenwald and Turner, 2017; Chelakkot et al., 2018). Except for those in physical barriers, several other types of epithelial cells produced by stem cells, which are located at the base of the intestinal crypt, also play a role in the homeostasis of the body (Clevers and Bevins, 2013; Peterson and Artis, 2014; Johansson and Hansson, 2016; Martens et al., 2018). There is only one mucus layer in the small intestine, while both an internal and outer layer can be found in the colon, making it a habitat for many microbes (Johansson and Hansson, 2016). After passing through the epithelial barrier, the luminal antigens come in contact with the immune cells in the second and third lymphoid organs in the lamina propria (Buettner and Lochner, 2016; Ahluwalia et al., 2017; Da Silva et al., 2017; Mowat, 2018; Tordesillas and Berin, 2018). After the internalization of mononuclear phagocytes, the treated antigens are presented to lymphocytes to induce oral tolerance and interact with the intestinal flora and dietary factors (Hadis et al., 2011; Pabst and Mowat, 2012; Muller et al., 2014; Chinthrajah et al., 2016; Esterházy et al., 2016; Loschko et al., 2016; Nutsch et al., 2016; Belkaid and Harrison, 2017; Kim et al., 2018; Mowat, 2018). Moreover, conventional dendritic cells can polarize naïve T cells by migration, while macrophages lack the characteristics of active migration, but help to amplify the T cell response of lymphocytes (Gaudino and Kumar, 2019). In addition, intestinal macrophages maintain T cell function by scavenging apoptotic/dead cells, secreting cytokines, and remodeling epithelial cells, thus maintaining tissue homeostasis (Zigmond et al., 2012; Ortega-Gómez et al., 2013; Cerovic et al., 2014; Zigmond et al., 2014; Schett and Neurath, 2018; Sugimoto et al., 2019). These processes of active regulation, and T cell deletion and anergy are associated with maintaining oral tolerance (Sun et al., 2015; Luu et al., 2017; Wawrzyniak et al., 2017; Mowat, 2018). In addition, as a response to microbial induction, conventional dendritic cells also support the conversion of immunoglobulin M and immunoglobulin G to immunoglobulin A on B cells, which

TABLE 1 | Phenotype of macrophages and its correlation with stimulating factors, surface markers, cytokines, and functions.

Phenotype	Origin	Stimuli	Surface makers	Secreted mediator	Functions
M1	Hematopoietic stem cells in bone marrow and progenitor cells in the embryonic yolk sac	IFN-γ, LPS, GM-CSF	MHC-II, CD68, CD80, CD86, CD197, SOCS3, B7	IL-1β,IL-6, IL-10^{low}, IL-12^{hi}, IL-18, IL-23, TNF, CXCL9, CXCL10, iNOS	Pro-inflammatory Th1 response, key mediator of several autoimmune diseases
M2		IL-4, IL-13, CSF-1, TGF-β, helminth	CD206, CD200R, CD163, CD86, Arg-1	IL-6, IL-10^{hi}, IL-12^{low}, VEGF, CCL17, CCL18, CCL22, IL-13α1, CH13L1	Anti-inflammatory, Th2 activation, wound healing
Mregs		IgG, PG, IL-10, apoptotic cells, GPCRs, adenosine	CD80$^{low/int}$, CD86^{+}, CD163^{low}, CD206^{low}	IL-10^{hi}, IL-12^{low}, TGF-β	The regulatory agencies controlling immune response, a bridge between innate immunity and adaptive immunity
TAMs		TME	CD81, CD163, CD206, VCAM-1, MHC-II	IL-6, IL-10, TGF-β, CCL2, CCL17, VEDF, CTSC	Associated with tumors

is essential for the homeostasis of the intestinal environment because immunoglobulin A inhibits the interaction between microorganisms and epithelial cells by transporting across the epithelial cell layer (Litinskiy et al., 2002; Macpherson et al., 2018; Castro-Dopico and Clatworthy, 2019).

In general, mononuclear phagocytes control the stability of the intestinal environment and the ability to trigger the immune response to pathogens by maintaining immune tolerance to commensal animals and diet (Hadis et al., 2011; Bain and Mowat, 2014; Cerovic et al., 2014; Kim et al., 2018; Leonardi et al., 2018). Ideally, these immune responses can promote inflammation remission and rapid homeostasis recovery in tissues. However, due to the repeated and abnormal activation of the immune system, the chronic inflammatory microenvironment of IBD will be produced in the body (Caër and Wick, 2020). Destruction of intestinal homeostasis, including an immune response to commensal bacteria, dysfunction of the epithelial barrier function, the reduction of nutrient absorption, and changes in tissue autophagy and oxygenation, can induce the recruitment of immune cells (Maloy and Powrie, 2011; Johansson et al., 2013; Peterson and Artis, 2014; Colgan et al., 2016; Ramakrishnan and Shah, 2016; Odenwald and Turner, 2017; Okumura and Takeda, 2017; Ahluwalia et al., 2018; Mowat, 2018; VanDussen et al., 2018). These intestinal defects are associated with IBD, and the gene expression related to the prognosis variation of Crohn's disease can be detected in mononuclear phagocytes. Thus, we can speculate that mononuclear phagocytes play a significant role in the cellular signaling pathway that regulates tolerance and chronic inflammation in the intestine (Lee et al., 2017).

The Role of Macrophages in Intestinal Inflammation

The largest macrophage population in the body exists in the gastrointestinal mucosa, which plays a key role in maintaining epithelial and immune homeostasis (Lee et al., 1985; Pull et al., 2005; Isidro and Appleyard, 2016; Guan et al., 2019). When intestinal homeostasis is disturbed, the composition of the intestinal macrophage pool will change greatly. The inflammatory macrophages will accumulate in the intestinal mucosa of patients with Crohn's disease and ulcerative colitis, for example. Compared with $CD14^{low}$, these inflammatory macrophages can be identified by the expression of $CD14^{hi}$, which produces multiple inflammatory mediators, such as TNF-α, IL-1, IL-6, ROS mediators, and nitric oxide, which makes them different from macrophages in healthy intestines (Thiesen et al., 2014).

$Ly6C^{hi}$ monocytes and their derivatives play a significant role in intestinal pathology (Bain and Schridde, 2018). When inflammation occurs in the gut, classical monocytes ($Ly6C^{hi}$) respond to the stimulation of Toll-like receptors in a highly pro-inflammatory manner, expressing reactive oxygen intermediates (**Figure 3**; Varol et al., 2009; Weber et al., 2011; Rivollier et al., 2012; Tamoutounour et al., 2012; Zigmond et al., 2012; Bain et al., 2013). $CD11c^{high}CCR2^{+}CX3CR1^{+}$ monocytes infiltrate in the colonic mucosa of IBD patients in a CCR2-dependent manner and cannot completely differentiate into macrophages and produce pro-inflammatory cytokines (Bernardo et al., 2018). Intestinal macrophages in IBD patients produce more pro-inflammatory cytokines, which promote or perpetuate the pathological environment (Kamada et al., 2008; Kamada et al., 2009; Lissner et al., 2015; Barman et al., 2016; Friedrich et al., 2019). In patients with Crohn's diseases, some factors, such as IFN-γ, induce the differentiation of inflammatory monocytes and the secretion of IL-23, thus creating a vicious circle of inflammation (Kamada et al., 2008). In addition, other mechanisms and disease-related changes in the function of macrophages may also promote the occurrence and development of IBD. For instance, $TREM\text{-}1^{+}$ macrophages, which are mainly immature macrophages, increase in frequency and number in patients with IBD, especially in the active lesion area (Schenk et al., 2007; Brynjolfsson et al., 2016). It has also been suggested that bacterial clearance of intestinal macrophages in patients with IBD is impaired, and patients with Crohn's disease phenotype mainly through dysfunctional autophagy (Smith et al., 2009; Schwerd et al., 2017). After the removal of infectious or inflammatory factors, the intestinal tract must be restored to balance so that a chronic inflammatory reaction will not follow. At the same time, the macrophage pool changes significantly. During colitis in mice, the expansion rate of $CX3CR1^{int}$ macrophages returned to normal (Zigmond et al., 2012). On the other hand, $Ly6C^{hi}$ monocytes supplement $CX3CR1^{hi}$ macrophages in intestinal homeostasis. Once the inflammatory response begins to subside, some of the induced $Ly6C^{hi}$ cells may be transformed into resident macrophages with anti-inflammatory effects and may play an active role in tissue injury. IL-1β is believed to be induced mainly by monocytes, and its susceptibility to chemically induced colitis is reduced due to its neutralization (Seo et al., 2015). Meanwhile, the selective ablation of *Tnfa* in $Ly6C^{hi}$ monocytes also reduces the development of colitis (Varol et al., 2009). Mice with defective recruitment of inflammatory mucosal monocytes, which is due to the neutralization or deletion of CCL2, CCR2 or β 7 integrins, are protected from colitis induced by DSS (Platt et al., 2010; Takada et al., 2010; Zigmond et al., 2012; Bain et al., 2013; Becker et al., 2016; Schippers et al., 2016).

There are other additional functions of Ly6C monocytes (**Figure 3**). Studies have shown that CCL2, CCL3 and CCL11 may come from $Ly6C^{hi}$ and play a role in recruiting innate immune effector cells in the gut (Waddell et al., 2011; Schulthess et al., 2012; Bain and Mowat, 2014). $Ly6C^{hi}$ monocytes can also prevent immunopathology by inhibiting the production of TNF-α and ROI by local neutrophils (Grainger et al., 2013). Furthermore, intestinal macrophages participate in tissue repair and fibrosis (**Figure 3**; Karin and Clevers, 2016; Vannella and Wynn, 2017). Some with Crohn's disease have symptoms of intestinal fibrostenosis, while others develop fibrosis complications several years later (Rieder et al., 2017). Another study has shown that in the colon of patients with Crohn's disease with stenosis, the number of $IL\text{-}36\alpha^{+}$ macrophages in the intestine is increased (Scheibe et al., 2019). The direct effect of IL-36 on human mesenchymal cells leads to profibrosis transcription, which indicates that intestinal fibrosis in patients with IBD can be induced by the increase of $IL\ 36\alpha^{+}$ macrophages

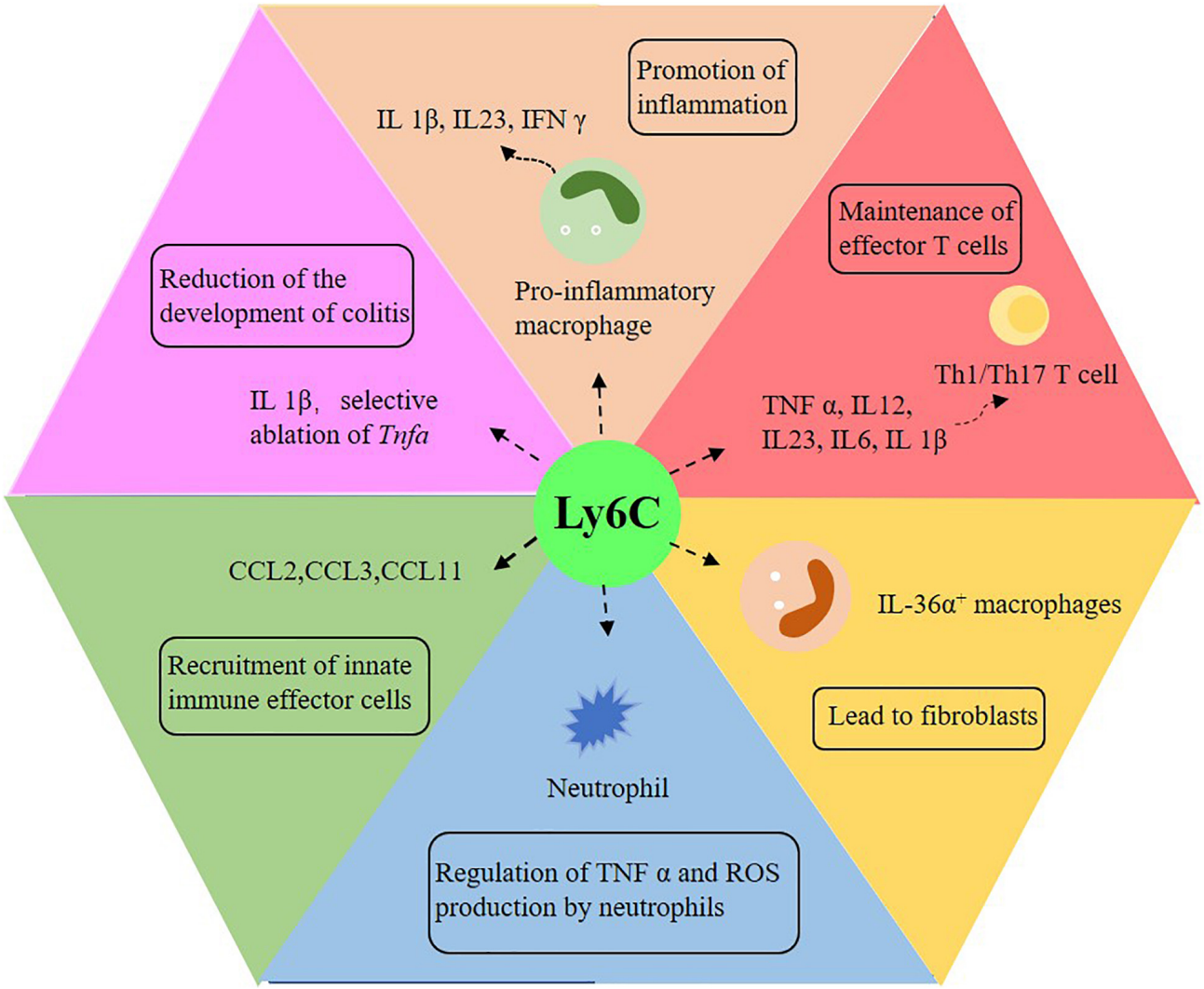

FIGURE 3 | The role of Ly6C monocytes in intestinal inflammation. Ly6C plays an important role in promoting intestinal inflammation, reducing colitis, activating T cells, promoting tissue fibrosis, regulating neutrophils and recruiting innate immune effector cells. IL: interleukin; TNF-α: tumor necrosis factor-α; IFN-γ: interferon-γ; Th1: interferon-γ-producing type 1 helper T cells; Th17: interferon-γ-producing helper T cells.

(Bettenworth and Rieder, 2017; Salvador et al., 2018; Scheibe et al., 2019). Other studies have reported that immature macrophages are always close to activated fibroblasts in the intestinal mucosa of patients with Crohn's disease, and immature macrophages, as well as conventional dendritic cells 2, activate fibroblasts to induce intestinal inflammation by oncostatin M/OSMR signaling (West et al., 2017; Martin et al., 2019; Smillie et al., 2019). Some genes expressed by intestinal macrophages also affect the development of intestinal inflammation. For example, the gene ablation of GPBAR1, a G protein–coupled receptor that is highly expressed in macrophages, enhances the recruitment of classically activated macrophages in the colonic lamina propria and aggravates the severity of inflammation (Biagioli et al., 2017).

Macrophages also play a role in the activation of T cells (**Figure 3**). Some studies have shown that, in patients with Crohn's disease, intestinal macrophages can induce the proliferation of naïve $CD4^+$ T cells and the expression of integrin β-7 and CCR9, while other works deem mature macrophages from patients with ulcerative colitis unable to inhibit the proliferation of T cells (Barman et al., 2016). In addition, $CD14^{hi}$ monocytes/macrophages in the IBD mucosa can produce IL-23 and express CD40 and CD80 to support the function of pathogenic T cells (Rugtveit et al., 1997; Carlsen et al., 2006; Kamada et al., 2008). Intestinal macrophages in patients with Crohn's disease induce the Th1 and Th17 polarization of naïve $CD4^+$ T cells, which seems to be caused by the accumulation of immature macrophages in the total macrophage population of patients with Crohn's disease (Kamada et al., 2009; Ogino et al., 2013). In fact, it has been proved by previous studies that immature macrophages from patients with IBD mainly produce IL-1β to induce Th17 cells and pathological IFN-γ^+

IL-17$^+$ T cells, which come from autologous colon CD4$^+$ T cells (Ramesh et al., 2014; Chapuy et al., 2019; Chapuy et al., 2020).

The Role of Intestinal Macrophages in the Treatment of Inflammation and Diseases

There are many methods to treat IBD, and regulating macrophage activation is one of them. In fact, it has been considered an attractive treatment for IBD to increase the phenotype of anti-inflammatory M2 (Hidalgo-Garcia et al., 2018). Endoplasmic reticulum stress, which is involved in the regulation of IEC inflammatory injury, is common in IBD patients (Woehlbier and Hetz, 2011; Hosomi et al., 2015). Grp78 is a marker of endoplasmic reticulum stress, and its expression is increased in inflammatory IEC. However, after increasing the expression of IL-10, the expression of Grp78 decreases, and endoplasmic reticulum stress is inhibited (Shkoda et al., 2007). IL-10 inhibits the NF-κB RelA phosphorylation induced by TNF by regulating Grp78, the expression of pro-inflammatory cytokines is subsequently down-regulated, and the IEC barrier function is maintained. According to a previous study, the neutralization of IL-10/TGF-β or alternatively activated macrophages did not show resistance to colitis induced by DSS in mice infected with schistosome (Smith et al., 2007). Parasites inhibited colitis induced by DSS through a new colonic infiltrating macrophage population—i.e., the schistosome infection stimulates a new macrophage population that preferentially migrates to the colonic LP, where it can inhibit colitis (Smith et al., 2007). This finding highlights a variety of immunomodulatory macrophage activation states. It is worth noting that infliximab, a monoclonal antibody of anti-TNF-α, has been successfully used in the treatment of human IBD, and the regulatory macrophages CD68$^+$CD206$^+$ were induced in patients with IBD responsive to treatment (Vos et al., 2012; Danese et al., 2015). Some studies have proved that macrophages are significant for the treatment of IBD. For example, alternative activated macrophages can activate the Wnt signaling pathway, which is related to ulcerative colitis, and promote mucosal repair in IBD, while Yes-associated protein (YAP), a Hippo pathway molecule, can aggravate the occurrence of IBD by regulating macrophage polarization and the imbalance of intestinal flora homeostasis (Cosín-Roger et al., 2016; Zhou et al., 2019).

Macrophages play an important role in in the treatment of colitis. For example, it has been found that intracolonic administration of chromofungin can induce macrophages to enter alternatively activated macrophages (AAM), which reduce the deposition of colonic collagen and maintain the homeostasis of intestinal epithelial cells, thus protecting colitis induced by DSS (Eissa et al., 2017; Ding et al., 2019a). MicroRNAs (miRNAs), which are noncoding RNAs, are essential for many biological processes in fine tuning. In macrophages, miR-155 acts as a pro-inflammatory regulator by promoting M2 polarization or affecting NF-κB signal transduction (Vigorito et al., 2013; Zhang et al., 2016). Li et al. found the central role of alternative M2 skewing of miR-155 in colitis and suggested that macrophages might be the main target of treatment (Li et al., 2018). The Grb2-associated binding protein 2 (Gab2), which plays a role in regulating the activation of macrophages and T cells, and Grb2-associated binding protein 3 (Gab3), which is highly expressed in some immune cell types, redundantly regulate the activation of macrophages and CD8$^+$ T cells to inhibit colitis (Uno et al., 2010; Bezman et al., 2012; Best et al., 2013; Festuccia et al., 2014; Kaneda et al., 2016; Wang et al., 2019; Ma et al., 2020b). Human catestatin (hCT), which has immunomodulatory properties, can reduce the severity of inflammatory recurrence by regulating M1 macrophages and releasing pro-inflammatory cytokines (Zhang et al., 2009; Rabbi et al., 2017). Triggering receptor expressed on myeloid cells-1 (TREM-1) is a pattern recognition receptor (PRR) of the surface immunoglobulin receptor superfamily and is expressed by activated macrophages. A study has found that when TREM-1 is deficient, the number of M1 macrophages, which produce IL-1β, in DSS-treated colons decreases, and the damage mediated by DSS can be alleviated by providing TREM-1 expressing macrophages to TREM-1 deficient mice (Yang et al., 2019). Other studies have found that vitamin D supplementation can also reduce the severity of Crohn's disease, and its active form, 1,25-dihydroxyvitamin D (1,25D), can inhibit the secretion of pro-inflammatory cytokines by macrophages (Dionne et al., 2017). Moreover, 1,25 D is also very important for the regulation of bone homeostasis and various immune responses (Hewison, 2012).

In addition to inhibiting intestinal inflammation, macrophages also play a significant role in other diseases. For example, REG3γ is a secretory antimicrobial lectin and REG3γ-associated *Lactobacillus* can enlarge the macrophage pools in the intestinal lamina propria, spleen and adipose tissue. The anti-inflammatory macrophages induced by REG3γ-associated *Lactobacillus* in the lamina propria may migrate to the adipose tissue and participate in the resistance to high-fat-diet-mediated obesity, and adipose tissue homeostasis (Huang et al., 2017). Since the gastrointestinal tract contains many HIV target cells, it has become the main site of HIV infection. Some studies have shown that Toll-like receptor 3 activation of macrophages can produce a variety of intracellular HIV limiting factors and effectively inhibit HIV infection (Wang et al., 2013; Zhou et al., 2013). The supernatant of activated intestinal epithelial cells can induce macrophages to express several key HIV limiting factors, thus inhibiting the replication of HIV (Guo et al., 2018). Whether in mice or human, the cross-talk between liver and intestine is vital in the development of metabolic diseases (Zhang et al., 2010; Qin et al., 2014). For example, non-alcoholic fatty liver disease is usually accompanied by changes in the intestinal microflora and bacterial overgrowth these are related to increased intestinal permeability and pathological bacterial translocation, in which macrophages may also be involved (Bain and Mowat, 2014; Hundertmark et al., 2018). Macrophage inducible C-type lectin expressed on macrophages may contribute to the integrity of the intestinal barrier, but in the advanced stages of chronic liver disease, once the intestinal barrier leaks, it seems to cause inflammation and fibrosis (Schierwagen et al., 2020). Receptor-interacting protein (RIP)-3, a member of the serine threonine kinase family, is the central mediator of necrosis and is associated with many human diseases (Ramachandran et al., 2013;

Roychowdhury et al., 2013; Linkermann and Green, 2014). It has been shown that the deficiency of RIP3 can inhibit macrophage accumulation and reduce inflammation in mice by inhibiting the TLR4–NF-kB pathway, and thus may be a potential therapeutic target for immune-mediated liver fibrosis (Wei et al., 2019).

Cytokine blockade has been used to suppress intestinal inflammation, but there are still some problems that should be considered, such as the prediction of the therapeutic effect and its prospect. Treating IBD by treating anti-tumor factors is an important breakthrough. However, many treatments have not achieved satisfactory results, and although some treatments are promising in animal models, they have not yet undergone rigorous clinical trials. Moreover, the deficiency of intestinal macrophages may increase the susceptibility to infection and inhibit the activity of tissue repair. Therefore, the potential risks associated with this immunotherapy require careful monitoring procedures. Other ways to improve intestinal homeostasis may consist of promoting the anti-inflammatory effects of macrophages. It is worth noting that, due to their high phagocytic capacity, intestinal macrophages can be promoted through "delivery systems" such as nanomaterials and biomaterials. Finally, the reprogramming of macrophages with metabolites may be a promising method to inhibit intestinal inflammation.

SUMMARY AND PROSPECT

This paper reviews the origin, development, and function of macrophages and their role in intestinal inflammation and treatment. In the past few years, we have made significant progress in understanding the ontogeny and differentiation of intestinal macrophages. Advancements have been made in the recognition and regulation of tissue-specific phenotypes and functional environmental signals as well. Macrophages not only have the function of phagocytizing pathogens, but can also secrete a variety of cytokines under certain conditions and combine with different immune cells to participate in the occurrence, development, and persistence of IBD in different ways. At the same time, macrophages play a role in treating IBD, inhibiting colitis, maintaining adipose tissue homeostasis, and inhibiting HIV infection. In conclusion, macrophages are vital in gut homeostasis and immune defense. However, many aspects of intestinal macrophages still need to be explored. For example, the understanding of heterogeneity in the septum of intestinal macrophages needs to be more complete. An important feature of IBD is pro-inflammatory monocyte/macrophage accumulation. Therefore, it is very important to elucidate the exact character of the molecular factors that control the differentiation of monocyte/macrophage, the changes of these factors in the course of disease, the local regulation, and long-term effects. In addition, the study of the interaction between macrophages and other cells, intestinal microorganisms and metabolites will also contribute to the treatment of intestinal inflammation.

AUTHOR CONTRIBUTIONS

XH did the writing. SD did the writing—review and editing. HJ did the supervision. GL did the funding acquisition. All authors contributed to the article and approved the submitted version.

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Gut Microbiota and Diarrhea: An Updated Review

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Diarrhea is a common problem to the whole world and the occurrence of diarrhea is highly associated with gut microbiota, such as bacteria, fungi, and viruses. Generally, diarrheal patients or animals are characterized by gut microbiota dysbiosis and pathogen infections may lead to diarrheal phenotypes. Of relevance, reprograming gut microbiota communities by dietary probiotics or fecal bacteria transplantation are widely introduced to treat or prevent diarrhea. In this review, we discussed the influence of the gut microbiota in the infection of diarrhea pathogens, and updated the research of reshaping the gut microbiota to prevent or treat diarrhea for the past few years. Together, gut microbiota manipulation is of great significance to the prevention and treatment of diarrhea, and further insight into the function of the gut microbiota will help to discover more anti-diarrhea probiotics.

Keywords: intestinal health, fecal microbiota transplantation, probiotics, gut microbiota, diarrhea

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INTRODUCTION

Diarrhea is a common health problem in the world, which induces 1.3 million deaths every year (Troeger et al., 2017), especially for infants and young children (Lamberti et al., 2016; Black et al., 2019). Generally, diarrhea is a clinical manifestation of intestinal ion transport channel proteins, channels, physical and chemical barriers being damaged, leading to disorders of water and electrolyte transport in the digestive tract (Chu et al., 2020). In addition, diarrhea may be a symptom of many diseases. Pathological bile acid absorption, bacterial and viral infections, carbohydrate malabsorption, disaccharidase insufficiency, and chronic inflammatory diseases are all related to diarrhea (Camilleri et al., 2017). Although the mortality rate associated with diarrhea has been significantly reduced over the years, it is still one of the common reasons for pediatric emergency department visits, especially in some low-income countries in Asia and Africa (Nataro, 2013).

The intestinal tract of mammals hosts a high and diverse number of different microorganisms, including bacteria, fungi, protozoa, and viruses (Ryan et al., 2020). The density and compositions of microorganisms change along the gastrointestinal tract and perform their functions in different parts. Homeostasis and symbiotic interactions promote peaceful coexistence between the microbiota and the host, which further inhibit the colonization of most introduced pathogens and participate in nutrient absorption and physiological functions (Kaiser et al., 2012; Hillman et al., 2017; Fan and Pedersen, 2020). For example, the healthy gut microbiome can protect against

epithelial cell injury and improve pathogen clearance from the gut lumen after non-typhoidal *Salmonella* diarrhea. In addition, the roles of microbial balance in the development and the maturation of the mucosal immune system and the integrity of the intestinal barrier have been also reported (Jandhyala et al., 2015). However, the compositions and diversity of gut microbiota are easily affected by various factors (i.e., diets, drugs, pathogens, and environmental factors), which further affect the health of humans and animals (Gao et al., 2013; Yin et al., 2020). A growing of evidence shows that imbalance of the gut microbiota increases the susceptibility to various pathogens and causes many diseases, including diarrhea, irritable bowel syndrome (IBS), allergies, cardiovascular disease, and obesity (Rajilić-Stojanović et al., 2015; Gresse et al., 2017; Torres-Fuentes et al., 2017).

Therefore, this review aims to summarize the microorganisms that mediate the occurrence of diarrhea and combining with the newly discovered probiotics that can treat and prevent diarrhea. At the same time, the application of fecal microbiota transplantation (FMT) in the treatment of diarrhea in recent years is discussed (**Figure 1**).

GUT MICROBIOTA-MEDIATED DIARRHEA AND ITS MECHANISM

Despite an improvement of living environment conditions and vaccination, diarrhea is still a common problem faced by the whole world, especially for young children (Stockmann et al., 2017). Dysbiosis (bacteria, fungi, and viruses disorders) characterized by pathogens domination is widely identified in diarrheal humans and animals, and one area of diarrhea currently receiving a large amount of attention is the interaction with gut microbiota. Firstly, invading pathogenic bacteria inhibits the growth of normal bacteria, resulting in a decrease in the number of beneficial bacteria in the gastrointestinal tract (Fan and Pedersen, 2020). Then, pathogen-produced toxic substances further cause abnormal gut function and immune responses, leading to the occurrence of diarrhea (Ward et al., 2016). In this review, bacteria, fungi, and viruses mediated-diarrhea are mainly discussed.

Bacteria and Diarrhea

Diarrhea caused by bacterial pathogens is a global health problem, especially in developing countries, and enteric bacterial pathogens are the main cause of infectious diarrhea. Currently, *Escherichia coli* (*E. coli*), *Shigella*, *Salmonella*, *Campylobacter*, *Clostridium difficile* (*C. difficile*), and *Aeromonas* are mainly considered to be the pathogens of diarrhea (Hodges and Gill, 2010; Kaakoush et al., 2015; Levine et al., 2020).

E. coli is a type of facultative anaerobic gram-negative bacteria to cause diarrhea. Different diarrheal *E. coli* strains exhibit different epidemiology and have been classified as enteropathogenic *E. coli* (EPEC, the main cause of infant diarrhea), enterohemorrhagic *E. coli* (EHEC/STEC, the cause of hemorrhagic colitis and hemolytic uremic disease), enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC, the main cause of travelers' diarrhea and infant

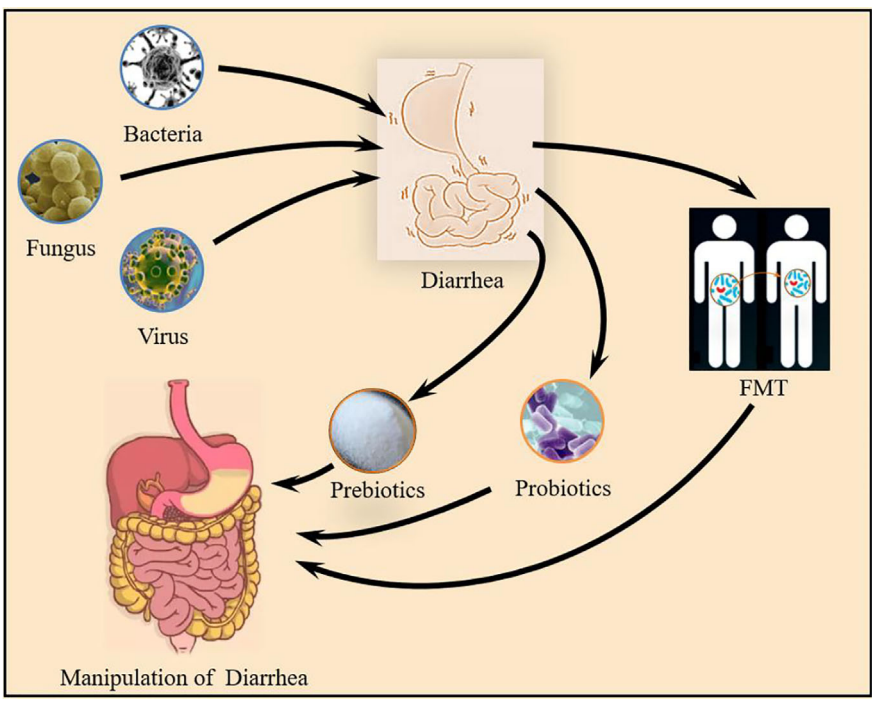

FIGURE 1 | Gut microbiota and diarrhea: Bacteria, Fungus and Virus all mediate the occurrence of diarrhea. microbial intervention by prebiotics, probiotics and FMT can regulate the composition of the intestinal flora to prevent and improve the occurrence of diarrhea.

diarrhea) and enteroinvasive *E. coli* (EIEC, the main cause of dysentery) according to the pathological types, colonization sites, virulence mechanisms, and clinical symptoms (Levine, 1987; Gomes et al., 2016). After infection, *E. coli* adheres to the intestinal epithelial cells through the adherent fimbriae, and then produces toxins and exerts pathogenic effects (Mirhoseini et al., 2018; Liu et al., 2020). Generally, diarrheal *E. coli* exhibit multi-drug resistance (Broujerdi et al., 2018), making it difficult to control the spread of pathogens.

Salmonella, a gram-negative and facultative anaerobic bacterium, is the third most common cause of diarrhea mortality (Troeger et al., 2017) and the infection is generally marked by gastroenteritis and diarrhea (De Jong et al., 2012). According to the clinical symptoms, *Salmonella* is divided into *Salmonella* typhi and nontyphoidal *Salmonella* (Crump et al., 2015). Typhoid *Salmonella* infections mainly occur in developing countries by causing 93.8 million food-borne cases and 155,000 deaths each year (Eng et al., 2015). While nontyphoid *Salmonella* has a wide host range (Crump et al., 2015). In addition to induce diarrhea, *Salmonella* can also cause fever and gastrointestinal complications such as pancreatitis and bleeding (Knodler and Elfenbein, 2019). Furthermore, inappropriate use of antibiotics has also led to an increase in antimicrobial resistance, and the development of multi-drug resistance in *Salmonella* serotypes has also led to an increase in the severity of *Salmonella* infections. For example, antibiotics have been found to damage the colonization resistance barrier, which further aggravates *Salmonella* infection that *Salmonella* typhimurium exceeds 10^8 CFU/g within 24 hours of infection and causes severe intestinal diseases (Ackermann et al., 2008). This epidemiological evidence underscores the relevance of these studies on mice to human diseases, that is, humans are more susceptible to *Salmonella* gastroenteritis after antibiotic treatment.

C. difficile is a gram-positive anaerobic bacterium with capable of forming a spore structure, which is widely present in the intestines of humans and animals. *C. difficile* infection has become the main cause of antibiotic-related diarrhea worldwide (Peng et al., 2018; Mileto et al., 2020). The clinical symptoms of *C. difficile* infection are ranging from asymptomatic carriers to various degrees of diarrhea even death. When the normal gut microbiota community is destroyed, *C. difficile* begins to colonize and dominate in the large intestine, releasing enterotoxin A and cytotoxin B (Androga et al., 2015). These toxins further damage the epithelial cells cytoskeleton, causing severe intestinal inflammation, diarrhea, and pseudomembranous colitis (Czepiel et al., 2019). Meanwhile, *C. difficile* may produce indole by manipulating the local microbial community, which has an effect on the abundance and diversity of bacterial communities in the colon, inhibiting the growth of beneficial indole-sensitive bacteria, such as *Bacteroides* and *Edwardsiella* (Darkoh et al., 2019). With the extensive use of broad-spectrum antibiotics, the resistance of strains has increased and highly virulent strains have emerged, which significantly increases the incidence of *C. difficile* infection. In mouse models, it was found that the isolated *C. difficile* variants from outbreaks of *C. difficile* – associated disease induced a strong inflammatory response (McDonald et al., 2005; Quesada-Gómez et al., 2015).

Shigella is a form of gram-negative bacterium to cause animal and human diarrhea (Liu et al., 2019). It is estimated that *Shigella* causes approximately 125 million diarrhea episodes and approximately 160,000 deaths every years, with a third are related to young children (Baker and The, 2018). *Shigella* produces *Shigella* enterotoxin and serotype toxin 1 into the enteric cavity, which further invade and destroy the epithelium of the large intestine, eventually leading to aggressive watery or mucus-like/bloody diarrhea (Nisa et al., 2020).

Vibrio cholerae is a gram-negative bacterium that causes watery diarrhea in the host. The cholera toxins, adnexa cholera toxin and closed band toxin produced by *V. cholerae* cause activation of anion secretion, inhibit absorption of electroneutral NaCl, destroy intestinal barrier function, and cause severe diarrhea (Ramamurthy et al., 2020). The serotypes O1 and O139 of *V. cholerae* strains with two main virulence genomes: cholera toxin (CT) and pathogenic toxin colonies cause an acute watery diarrhea (Ogura et al., 2020). A recent study showed that some non-01/O139 *V. cholerae* (NOVC) strains also cause diarrhea (Vezzulli et al., 2020). *V. cholerae* can also affect the virulence of pathogenic *Escherichia coli*, and the virulence of EPEC is enhanced with the elevated concentration of cholera autoinducer 1 (CAT-1) when grown in co-culture with *V. cholerae* (Gorelik et al., 2019).

Fungus and Diarrhea

Fungi are an important part of gut microorganisms and certain fungal communities have been confirmed to be highly associated with diarrhea (Sangster et al., 2016). *Candida* is generally considered to be a reliable cause of diarrhea, but its mechanism of inducing diarrhea is still unclear (Awoyeni et al., 2017). It is generally believed that *Candida* may selectively cause diarrhea in a clinical setting (Ponnuvel et al., 1996). *Candida albicans* (*C. albicans*), a conditional pathogen, is the most abundant fungus in the intestine of mammals (Nobile and Johnson, 2015). The role of C. albicans and diarrhea has been controversial for many years. While in mouse model, it was found that *C. albicans* could cause intestinal dysbiosis and enhance the severity of DSS-induced colitis (Panpetch et al., 2020). The mechanisms may be associated with dectin-1, which is a C-type lectin-like receptor that mediates the fungal immune response. Dectin-1 expression is positively correlated with (1,3)-β-Glucan and mediates fungal infections by recognizing the (1,3)-β-glucan structure on the fungal cell wall (Gantner et al., 2005). In addition, *Candida krusei*, *Candida tropicalis*, *Candida glabrata*, *Candida guilliermondii*, *Candida parapsilosis* are also the main pathogens that cause invasive candidiasis that may be implicated in diarrhea, but the detailed mechanisms need to be further studied (Awoyeni et al., 2017; Hirayama et al., 2020).

Virus and Diarrhea

The viral microbiome is a complex community composed of eukaryotic RNA viruses, DNA viruses, and bacteriophages, which plays an important role in maintaining human and animal health. Previous studies showed that some viruses also contribute to diarrhea in humans and animals and the clinical symptoms of viral diarrhea include diarrhea, fever, and vomiting (Goodgame, 2001). Rotavirus is a common diarrheal pathogen in

infants and young animals, causing more than 200,000 deaths every year (Ramig, 2004). Rotavirus, a double RNA virus without envelope, infects intestinal epithelial cells to stimulate intestinal secretion and activation of the enteric nervous system, causing the destruction of absorptive intestinal epithelial cells, thereby inducing diarrhea (Tafazoli et al., 2001; Crawford et al., 2017). Reduction of absorption function of the intestinal epithelium by rotavirus infection has been widely reported to cause damage and death of intestinal cells *in vivo* and vitro (Ball et al., 1996; Morris et al., 1999). In addition, viruses that cause diarrhea also include Norovirus, Astrovirus, Enterovirus, and Boca virus, while Rotavirus infection is usually more serious compared with other sources of infection (Crawford et al., 2017).

MANIPULATION OF DIARRHEA BY REPROGRAMING GUT MICROBIOTA

Gut microbiota plays a great significance to intestinal health, and healthy gut microbiota can resist the colonization of diarrhea pathogens. It was found in germ-free mice and antibiotic-treated mice that the gut microbiota has a protective effect on diarrhea infection (Kamada et al., 2012; Vogt and Finlay, 2017). Modulating the gut microbiota to improve human health also become more and more important. Diet is an important modulator of the gut microbiota, that can regulate the composition and function of the community of microbes in humans and other mammals to resist diseases (Sonnenburg and Bäckhed, 2016). In past studies, it was also found that microbial intervention can regulate the composition of the intestinal flora to prevent and improve the occurrence of diarrhea (Gallo et al., 2016).

Probiotics and Diarrhea

Probiotics are considered to be beneficial to the host's health and contain a sufficient amount of non-pathogenic specific live bacteria preparations, such as *Lactobacillus*, *Yeast*, *Bifidobacterium*, *Enterococcus*, and *Bacillus*. Probiotics have been widely reported to treat pathogens-caused diarrhea by maintaining or improving the balance of gut microbiota and the mechanisms may be associated with the inhibitory effect on the colonization of harmful bacteria by competing for nutrients and producing antibacterial compounds (Bron et al., 2017). Probiotics can reduce the severity of *Citrobacter rodentium*, *Listeria monocytogenes*, EHEC, and *Salmonella typhimurium* infections (Bayoumi and Griffiths, 2010; Emara et al., 2016; Wen et al., 2019). For example, *Bifidobacterium breve* and *Bifidobacterium pseudocatenulatum* DSM20439 inhibit the expression of intestinal Shiga toxin EHEC (Asahara et al., 2004). In clinic, the incidence of antibiotic-associated diarrhea and *C. difficile*-associated diarrhea in patients who received probiotic capsules per day was lower than that in the placebo group (Gao et al., 2010). Similarly, oral Lactobacillus LGG reduced the incidence of diarrhea in children and shorten the course of diarrhea (Li et al., 2019). Similarly, the administration of fortified milk containing probiotic Bifidobacterium lactis HN019 (1.9×10^7CFU) and prebiotic oligosaccharides administered three times a day for a year can reduce the incidence of dysentery in children (Sazawal et al., 2010). In addition, Szajewska and Mrukowicz reported that probiotics reduced the duration of Rotavirus diarrhea (Szajewska and Mrukowicz, 2001). Other studies further confirmed that probiotics such as *Bifidobacterium* and *Lactobacilli* played an important role in the treatment of Rotavirus infection (Rigo-Adrover et al., 2018; Azagra-Boronat et al., 2020). For example, *Lactobacillus rhamnosus* GG regulates the maturation and differentiation of dendritic cells (DCs) and the secretion of inflammatory factors, thereby preventing diarrhea caused by Rotavirus (Jiang et al., 2017). Together, the beneficial effects of probiotics on diarrhea are related to the strain and dosage and the selection and use of the best probiotics for the treatment of diarrhea need to be determined by more clinical trials.

The anti-diarrheal mechanisms of probiotics have not yet been fully elucidated, the currently considered mechanisms of action of probiotics mainly rely on the following pathways: (1) regulation of the balance of gut microbiota; (2) improvement immunity; (3) manipulation of intestinal defense barrier (Monteagudo-Mera et al., 2019); (4) metabolites. Firstly, probiotics have been widely considered to maintain gut microbial hemostasis and a healthy gut microbiota community predicate a low incidence of diarrhea. In addition, probiotics can produce organic acids in the metabolic process and lower the pH of the gut cavity, thereby inhibiting the growth of pathogens (Peters et al., 2019). Secondly, probiotic strains serve as a key activator for the gut innate and adaptive immune systems, thereby signaling antimicrobial and inflammatory responses and enhancing the diarrheal resistance (Kawashima et al., 2018). Thirdly, probiotics can stimulate the production and secretion of mucin, cathelicidins, and defensins from goblet cells and epithelial cells to form an immune barrier, preventing the invasion of pathogenic bacteria (Do Carmo et al., 2018). Meanwhile, probiotics have been reported to increase the expressions of tight junction proteins and reduce the damage to the gut tissues caused by pathogenic bacteria such as *E. coli* and Rotavirus (Zeng et al., 2017; Paparo et al., 2020). More interestingly, Hu Jun et al. transplanted fecal microbiota between CM piglets (a native Chinese breed, are more resistant to early-weaning stress-induced diarrhea) and commercial crossbred LY piglets and identified *Lactobacillus gasseri* LA39 (*L. gasseri* LA39) and *Lactobacillus frumenti* (*L. frumenti*) that could mediate diarrhea resistance. The mechanisms may be associated with microbiota-derived bacteriocin gasericin A, which further binds to Keratin 19 on the plasma membrane of intestinal epithelial cells to regulate fluid absorption and secretion (Hu et al., 2018). Recent studies have reported that adjusting the abundance and type of intestinal bacteria through *Debaryomyces hansenii* treatment can alleviate diarrhea caused by antibiotics. The reason may be that *Debaryomyces hansenii* changed the bacterial community structure, and promoted the growth of some key lactase-producing bacteria, such as *Enterobacter* sp. *638* and *Modestobacter* (He et al., 2017; Wu et al., 2020). Intestinal lactase is mainly produced by microorganisms such as *Lactobacillus* sp., *Bacillus* sp., *Escherichia coli*, *Bifidobacterium* sp., *Enterobacter aerogenes* and *Streptococcus thermophilus*, low activity and lactase deficiency can cause diarrhea (Long et al., 2017). Summarily, probiotics not only inhibit the overgrowth of pathogens, but also enhance the anti-pathogenic ability against microbiota associated with diarrhea.

Prebiotics and Diarrhea

Prebiotics are defined as "substrates that are selectively utilized by host microorganisms to confer health benefits" (Gibson et al., 2017). Consumption of prebiotics can improve the gut microbiota, which is beneficial to health. Recent study has shown that interventions prebiotic can increase the production of SCFA, play an important role in maintaining the intestinal barrier (Azad MAK et al., 2020; Snelson et al., 2021). The gut lymphoid tissue can induce secrete cytokines and anti-microbial peptides, such as β-defensins, to defense against the invasion of microorganisms (De Santis et al., 2015). Some prebiotics, such as fructo oligosaccharide, inulin, pectin oligosaccharides, etc., can resist the colonization of pathogen by acting as soluble decoy receptors that mimic the binding site of pathogens, thereby promoting the eliminating of pathogens from the intestine (Pujari and Banerjee, 2021). Previous studies have shown that prebiotics can shorten the duration of acute watery diarrhea and has a good therapeutic effect on diarrhea (Rigo-Adrover et al., 2017).

FMT and Diarrhea

FMT has become the focus of research in biomedicine and clinical medicine in recent years. FMT refers to the process of transplanting the functional flora in the feces of healthy people into the gastrointestinal tract of the patient to rebuild the gut flora with normal functions to treat intestinal and extra-intestinal diseases (De Groot et al., 2017). The clinical response of FMT to different diseases (i.e., Clostridium difficile infection, inflammatory bowel disease, diabetes, cancer, liver cirrhosis, and brain diseases) provides direct evidence for the interaction between the microbiota and the host (Aron-Wisnewsky et al., 2019; Tariq et al., 2020; Zhang et al., 2020). In a randomized double-blind trial, patients received anaerobic prepared donor FMT relieved ulcerative colitis (Costello et al., 2019). In another study, FMT significantly alleviated symptoms in IBS patients by double-blind randomized trials, but there have also been contradictory results (Halkjaer et al., 2018; Johnsen et al., 2018). In dextran sodium sulfate (DSS)-induced murine colitis model, fecal microorganisms from normal biological donors reduced colitis by regulating the expression of pro-inflammatory genes, antibacterial peptides and mucins (Burrello et al., 2019). In another two studies, the results showed that FMT increased the number of beneficial bacteria in the intestinal tract and reduced the number of harmful bacteria, and further research showed that FMT triggered the intestinal mucosal autophagy and reduce the damage of the intestinal barrier caused by *E. coli K88* (Cheng et al., 2018).

The therapeutic effect of FMT in gut diseases is related to reprogram gut microbiome, and the healthy flora reintroduced through FMT will compete with the ecological environment and prevent the colonization of pathogens. One way for the gut microbiota to directly inhibit the pathogen of diarrhea is to compete for nutrients to reject the pathogen (Vogt and Finlay, 2017). At the same time, the gut microbiota secrete antibacterial compounds that further inhibit the growth of harmful bacteria, such as bacteriocins or small molecular metabolites (Antunes et al., 2014; McKenney and Pamer, 2015). For example, some symbiotic bacteria can produce short-chain fatty acids (SFCAs), which can change the local pH to inhibit the growth of pathogens (Shin et al., 2002). However, the research on the mechanisms of FMT is limited, and it is not clear whether the changes of these microbiota have played a sufficiently important role. In recent studies, it has been proposed that the role of FMT in disease may not only be explained by simply restoring intestinal bacteria itself (Ott et al., 2017). Therefore, the application of FMT in diarrhea related diseases and the function of FMT in recipients need further studies.

CONCLUSION

Diarrhea is related to changes of gut microbiota and balanced gut microbiota is resistant to the colonization of diarrhea pathogens. Based on bacterial therapy, the gastrointestinal tract constitutes a healthy microbiota throughout life, which plays an important role in the prevention and treatment of diarrhea. Although the specific mechanism of gut microbiota affecting diarrhea remains to be studied in depth, it is effective to prevent and treat diarrhea by regulating the gut microbiota by dietary probiotics and FMT. Despite the progress made in the understanding gut microbiota and diarrhea, there are a number of prominent research avenues remain to be explored. For example, diarrhea is highly associated with gut microbiota alterations, thus microbial quorum sensing during the pathogen infection should be studied to unveil the pathogenesis of diarrhea; In addition, different probiotics exhibit different probiotic properties, the detailed mechanisms of probiotics-mediated diarrheal pathogen clearance are suggested; Nutritional regulation has been widely introduced to manipulation of gut microbiota compositions, personalized diets are designed to improve gut microbiota compositions against pathogen infections; Currently, FMT has been widely recommended to treat gut microbiota-related diseases, donor selection, ethics, operation method, and the follow-up should be standardized to guarantee the safe and effective of FMT in clinical practice. Further studies believe that the research on the function of microflora will help to discover some potential probiotics with anti-diarrhea function.

AUTHOR CONTRIBUTIONS

YL: Writing-Original draft preparation, revision and investigation. JY, SX and JM: Revision. XJ, CF and SG: Conceptualization. ZF: investigation. YY: Supervision. JY: Validation. All authors contributed to the article and approved the submitted version.

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The Impact of Gut Microbiota on Radiation-Induced Enteritis

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Radiotherapy is an important treatment for abdominal tumors. A critical side effect for this therapy is enteritis. In this review, we aim to summarize recent findings in radiation enteritis, in particular the role of gut microbiota dysbiosis in the development and therapy of the disease. Gut microbiota dysbiosis plays an important role in the occurrence of various diseases, such as radiation enteritis. Abdominal radiation results in changes in the composition of microbiota and reduces its diversity, which is mainly reflected in the decrease of *Lactobacillus* spp. and *Bifidobacterium* spp. and increase of *Escherichia coli* and *Staphylococcus* spp. Gut microbiota dysbiosis aggravates radiation enteritis, weakens intestinal epithelial barrier function, and promotes inflammatory factor expression. Pathogenic *Escherichia coli* induce the rearrangement and redistribution of claudin-1, occludin, and ZO-1 in tight junctions, a critical component in intestinal epithelial barrier. In view of the role that microbiome plays in radiation enteritis, we believe that intestinal flora could be a potential biomarker for the disease. Correction of microbiome by application of probiotics, fecal microbiota transplantation (FMT), and antibiotics could be an effective method for the prevention and treatment of radiation-induced enteritis.

Keywords: microbiota, radiation enteritis, intestinal epithelial barrier, inflammatory cytokines, probiotics

HIGHLIGHTS

In this article, we reviewed the alteration of intestinal flora and its functional impact on radiation enteritis. Specifically, we summarized changes in the composition and diversity of microbiota in radiation-induced enteritis. We further reviewed the mechanisms by which gut microbiota dysbiosis promotes the development of radiation enteritis. In addition, we summarized recent findings in the treatment of radiation enteritis by correction of microbiome with probiotics and FMT. Our goal is to consolidate the current literature to better delineate the relationship between microbiota and radiation enteritis.

INTRODUCTION

Emil Grubbe's treatment of breast cancer with radiotherapy back in 1896 (Hodges, 1964), pioneered modern cancer radiation therapy. As the number of patients receiving radiation increases steadily, the incidence of radiotherapy-associated complications has risen in parallel (Theis et al., 2010; Li et al., 2020b). While radiotherapy improves the survival of cancer patients (Hale, 2020),

the oxidative stress caused by radiotherapy can produce reactive oxide species (ROS), which result in broad DNA damage to normal tissues, e.g., the gastrointestinal mucosa (Kumagai et al., 2018). It was reported that 6% to 78% of the long-term post-radiation survivors suffer from complications, leading to a compromise in patient's quality of life (Andreyev, 2005). The most common side effect of this therapy is intestinal mucositis caused by injuries of normal intestinal epithelial cells. Intestinal mucosal atrophy and ulcer caused by radiotherapy hinder the renewal of basal epithelial cells (radiation-induced enteritis) (Sonis et al., 2004; Hauer-Jensen et al., 2014). The incidence of radiation-induced enteritis is largely dependent on radiation treatment scheme (Shaw and Taylor, 2012; Siegel et al., 2012). Patients with acute radiotherapy enteritis manifest symptoms, such as diarrhea, abdominal pain, constipation, hematochezia and loss of weight. Severe radiation-induced enteritis can lead to life-threatening systemic infection over a course of 3 months (Lalla et al., 2014; Marin et al., 2014; Lian et al., 2017). Therefore, prevention of adverse side effects associated with radiotherapy has become an urgent priority (Cui et al., 2019). It was reported that 90% of patients receiving abdominal radiotherapy develop gastrointestinal symptoms in a few weeks after the treatment (Andreyev, 2005). The intestinal microecology is disordered, and the infection of *Clostridium difficile* increases accordingly (Hautmann et al., 2011). In view of the key role of intestinal microecology in diseases, understanding the relationship between radiation enteritis and intestinal microecology and modulating the intestinal microecology to alleviate radiation enteritis may demonstrate a therapeutic benefit.

MICROBIOME FUNCTIONS IN HUMAN BODY

Intestinal mucosal barriers include those induced by mechanical, chemical, immunological and biological factors (Baumgart and Dignass, 2002). The biological factors refer to microbiome parasitizing in gastrointestinal tract of healthy people. The size of population for microbiome in an average adult intestine is around 100 trillion (Xu and Gordon, 2003). In healthy individuals, the intestinal flora maintains a stable and mutualism relationship with human host, and involves in almost all physiological aspects in nutrition, immune, and digestion. Gut microbiota is beneficial to host homeostasis and immune system development, regulating innate and adaptive immune response (Sommer and Backhed, 2013). The physical condition of host depends on the interaction between the microbiome and the immune system (Barroso-Batista et al., 2015). In addition, gut microbiota also regulates host metabolism. Dysbiosis is related to the occurrence of multiple metabolic diseases, such as type 2 diabetes (Amar et al., 2011) and obesity (Sonnenburg and Backhed, 2016), indicating that a stable gut microbiome is essential to human health.

Gut microbiome dysbiosis is also closely related to the occurrence and metastasis of dietary-associated cancers (Schulz et al., 2014; Feng et al., 2015). Intestinal flora may be used as biomarkers for the diagnosis of cancer. For example, the development of gastric cancer is associated with dysbacteriosis. The microbial dysbiosis index improves the sensitivity and specificity in detecting gastric cancer, indicating that changes in the overall flora rather than changes in individual flora lead to gastric cancer (Ferreira et al., 2018). In addition, *Fusobacterium* and related bacteria may also promote the growth and metastasis of colorectal cancer. Microbiota co-exists in primary and matched metastatic tumors, and is continuously associated with distant metastasis of primary human colorectal cancer (Bullman et al., 2017). Microbiome is also strongly linked to cancer treatment. Reports showed that the composition of gut microbiota can impact anti-cancer treatment by improving antitumor immunity. Targeting gut microbiota strengthens the efficacy of drugs and reduces adverse reactions (Gorjifard and Goldszmid, 2015; Roy and Trinchieri, 2017) (**Figure 1**).

Radiotherapy remains to be essential for most of cancers. The imbalance in the diversity of microbiome is associated with the occurrence and treatment failure of a variety of diseases, including radiation enteritis (Sokol and Adolph, 2018). It is necessary to clarify the interaction between intestinal flora and radiation-induced enteritis to provide optimal treatment for patients.

PATHOGENESIS OF RADIATION-INDUCED ENTERITIS: EMERGING ROLES OF GUT MICROBIOTA

The symptoms of radiation-associated enteritis, such as diarrhea, are correlated with the onset time and duration of radiotherapy (Hogan and Kellison, 2002; Keefe, 2007). It has been reported that radiotherapy-induced enteritis is a common reason for reduced dosage of irradiation in radiotherapy. Such reduction can severely compromise body functions and increase mortality among cancer patients (van Vliet et al., 2010; Tarricone et al., 2016). High doses of radiotherapy lead to intestinal villi atrophy, intestinal epithelial damage, increased apoptosis, and spike in inflammation. Moreover, radiotherapy can also result in the dysfunction of intestinal epithelial barrier, leading to increased intestinal permeability, diarrhea, and disruption of water and electrolyte processing. This may ultimately result in hypovolemic shock, hence endangering life (Barnett et al., 2006; Shen et al., 2018). Radiation enteritis includes five phases, (i) the initial phase with the formation of ROS that result in DNA damage, (ii) the primary damage response phase with inflammation and apoptosis, (iii) the signal amplification phase, in which more inflammation and apoptosis occurs, (iv) the phase of ulcer formation, with discontinuity of the epithelial barrier that promotes bacterial translocation, and (v) the healing phase, with cell proliferation once radiotherapy has ceased (Sonis, 2004). Although recent studies have begun to reveal the pathogenesis of radiation enteritis, our current knowledge is far from being able to provide an effective prevention or treatment for the disease.

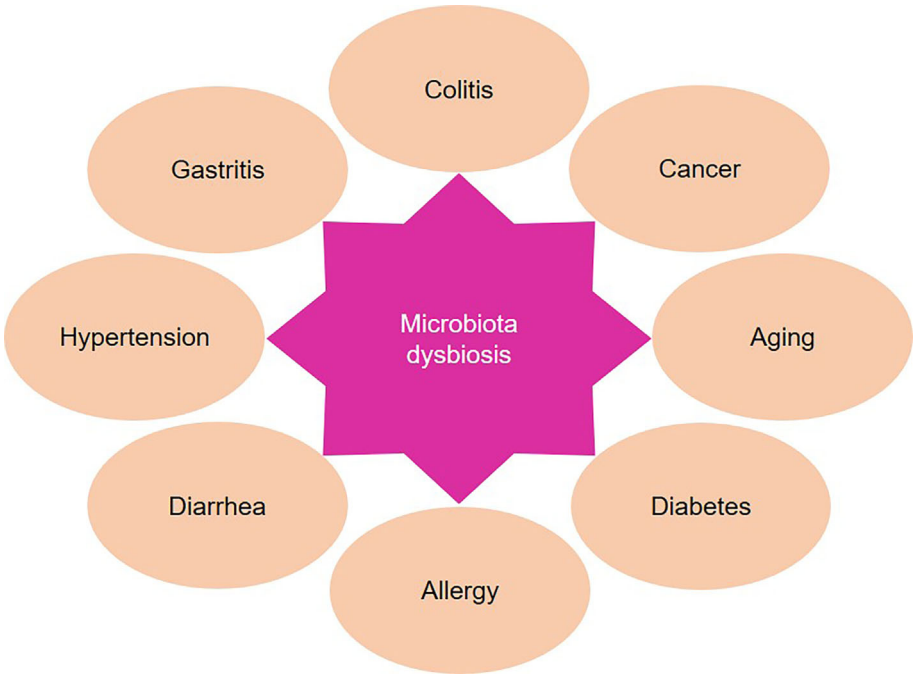

FIGURE 1 | Microbiota dysbiosis is involved in the development of multiple diseases. The intestinal flora affects immune, metabolism and other functions of the host. Microbiota dysbiosis leads to a series of diseases, such as diabetes and colitis.

There is evidence that the pathophysiology of intestinal injury caused by radiation is related to the dysbiosis of gut microbiota. To demonstrate the influence of gut microbiota on intestinal inflammation, García-Lafuente et al. evaluated the effect of colonic microbiota on 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced intestinal inflammation in rats. Animals whose colon segments were excluded from fecal transport were recolonized with pre-selected bacteria to test the effects of different species on intestinal inflammation and damage. Rats with excluded colon and sterile culture of intracavitary washings showed slight inflammation and low mucosal damage in response to TNBS. Rats colonized with anaerobic bacteria showed significantly higher release of eicosanoids than rats colonized with aerobic bacteria only. In addition, mucosal lesions were mainly observed in rats with anaerobic bacteria, which suggest that colonic anaerobic bacteria may play a role in intestinal inflammation (García-Lafuente et al., 1997). Therefore, it is necessary to clarify the relationship between radiation intestinal injury and intestinal flora. We will give an overview of the characteristics of alteration of intestinal microbiota in radiation enteritis and its possible mechanisms in radiotherapy-induced intestinal injury.

Although limitations exist in studies with animals, the majority of the intestinal flora in mice is unique, with high similarities to those in humans (e.g., phylum level of both mice and humans are mainly dominated by *Firmicutes* and *Bacteroidetes* (Ley et al., 2005). Animal models, if used within ethical bounds, are crucial in identifying the relationship between radiotherapy enteritis and gut microbiota (Jones et al., 2020). We will not only summarize the changes of microbiome in clinical patients with radiation enteritis, but will also discuss the relationship between radiation enteritis and intestinal flora in animal models.

RADIATION LEADS TO ALTERATIONS IN THE COMPOSITION AND DIVERSITY OF GUT MICROBIOTA

The development and application of 16S rRNA sequencing greatly promote the study of gut microbiota. 16S rRNA sequencing is a cost-effective method to study the microbiota diversity and composition in a large number of samples. However, this method is limited to the detection in flora composition. In order to obtain a comprehensive understanding of the microbiota, combined applications of 16S rRNA sequencing with metabolomics, transcriptomics, shotgun metagenomics, and other methods are recommended (Goudarzi et al., 2016).

Cui et al. determined the relationship between microbiome and the radiation sensitivity in the radiation enteritis through the 16S rRNA sequencing, and found that 6.5 Gy gamma ray with whole body radiation changed community composition of intestinal microbiota in C57BL/6 mice. The authors further confirmed that alterations in intestinal flora affect post-irradiation survival of mice mainly due to microbiome changes, which deregulate lncRNA expression of the host,

hence sensitizing the mice to radiation (Cui et al., 2017). In addition, Johnson et al. found that approximately two hours after radiation, *aerobic bacteria* and *Lactobacillus* counts in the intestine become markedly reduced (Johnson et al., 2004). Zhao et al. obtained a similar result, proving that abdominal radiation disrupted the intestinal flora balance and significantly reduced gut microbiota diversity in mice (Zhao et al., 2020). Together, current studies demonstrate that in animal models, abdominal radiation induces gut microbiota dysbiosis and reduces the survival of radioactive mice.

Clinically, the incidence of grade 2 or even worse diarrhea was as high as 17% in patients undergoing radiotherapy before surgery (Bosset et al., 2004). Excessive growth of gram-negative bacilli is detected in radiation-enteritis-induced diarrhea. Comparison of microbiota immediately before, immediately after, and two weeks after radiotherapy indicated that patients with diarrhea showed an increase in *Actinobacteria* phylum and a reduction in *Clostridium*. Impairment of intestinal motility after radiation is the main pathogenic factor for colonization of gram-negative bacilli in gastrointestinal tract. During bacterial overgrowth, absence of intestinal migrating motor complex (MMC) correlates with the existence of mass colonization of gram-negative bacilli (Bosset et al., 2004). Thus, abnormal intestinal motility and mass colonization of gram-negative bacilli are important factors for the development of severe late-stage radiation-induced enteropathy. In addition, the susceptibility and protection to diarrhea and other bacterial changes after radiotherapy may also be related to different initial microbiota colonization (Husebye et al., 1995; Manichanh et al., 2008).

Manichanh et al. analyzed the intestinal bacteria of patients receiving radiotherapy (Manichanh et al., 2008; Stringer et al., 2013). In radiation enteritis patients without diarrhea, *Actinobacteria* phylum was not detected, whereas in patients with diarrhea, *Bacilli class* showed a higher abundance. Radiation therapy reestablishes the intestinal flora, and acute diarrhea induced by radiation enteritis is associated with the reconstitution of colonized intestinal flora (Manichanh et al., 2008; Stringer et al., 2013). Differences in the level of gut microbiota diversity reveal an obvious variance in microbiota composition and structure between cancer patients and healthy individuals. Stool samples were collected from 18 patients with cervical cancer during radiotherapy. Wang et al. used the Illumina HiSeq platform to characterize the microbiota based on 16S rRNA sequencing. Microbiota dysbiosis was observed in radiation enteritis patients with a significant decrease in α diversity and an increase in β diversity. The abundance of *Proteobacteria*, *Gamma proteobacteria* and *Coprococcus* was increased, while the level of *Bacteroide*s was reduced (Wang et al., 2019).

Manichanh et al. demonstrated that there are different intestinal floras in abdominal cancer patients and in healthy individuals, because shifts in intestinal health status (such as chronic inflammation or abnormal function of epithelial cells) may directly affect microbiome (Manichanh et al., 2008). There is no direct causal relationship between gynecological cancer and intestinal flora, but Nam et al. found that there were significant differences in gut microbiota between gynecological cancer patients and healthy individuals. As compared with healthy individuals, gynecologic cancer patients receiving radiation therapy mainly possess a relative abundance of the phylum. *Actinobacteria* were 30 times higher than those in healthy individuals whereas *Bacteroidetes* and *Fusobacteria* were lower than those in healthy people. At the family level, the relative abundance of *Eubacteriaceae* was obviously higher, and *Prevotellaceae*, *Oscillospiraceae* and *Fusobacteriaceae* were lower than those in healthy group (Nam et al., 2013). Therefore, even if the location of disease development is far from or not related to the intestinal tract, the alteration in health status of the host will also affect the overall homeostasis of intestinal flora. Gut microbiota in different types of cancer patients may also be different as compared with healthy individuals.

Together, current studies suggest that gut microbiota in radiation-associated enteritis patients show marked changes in terms of composition and diversity. In radiation enteritis, the abundance of most bacteria belonging to the phylum *Actinobacteria* and *Proteobacteria* increases, and most of these bacteria are conditional pathogens, such as *Escherichia coli*. In contrast, microorganisms from *Firmicutes* and *Bacteroides* are reduced. Most of these bacteria are probiotics, such as *Lactobacillus*. When the abundance of probiotics decreases, conditional pathogens will reproduce and occupy the niche, which, in return, further inhibit the growth of probiotics and promote the release of endotoxin, and hence enhance intestinal inflammation. A vicious cycle may thus exacerbate radiation enteritis (**Table 1**).

MECHANISMS BY WHICH DYSBIOSIS OF GUT MICROBIOTA CONTRIBUTES TO RADIATION-INDUCED ENTERITIS

Radiation-induced enteritis refers to the intestinal mucosa damaged by the free radicals derived from ionization. Radiation enteritis is manifested by phenotypes such as impaired intestinal mucosal barrier, increased inflammatory factors, enhanced pathogen invasion and endotoxin release, and decreased immune barrier.

The Gut Microbiota Dysbiosis Weakens the Function of Intestinal Epithelial Barrier

The intestinal epithelial barrier is composed of mucus layer, epithelial glycoglobulin and epithelial cells, and plays a critical role in the prevention of pathogen invasion (Sánchez de Medina et al., 2014). As an important component of the intestinal epithelial barrier, intercellular junctions, which include tight junction, gap junction, adhesion junction and desmosome, form multiple functional complexes (Anderson and Van Itallie, 2009; Groschwitz and Hogan, 2009). Tight junction, as the most important intercellular junction, determines the intestinal epithelial permeability and maintains physiological functions of the intestinal barrier (Zihni et al., 2016). Zonula occludens-1

TABLE 1 | Radiotherapy affects the composition and diversity of intestinal microbiota.

Samples	Clinical setting	Techniques used	Disease	Alterations in the gut microbiota composition	References
Feces of male C57BL/6J mice	A single dose of 6.5 Gy gamma ray at a rate of 1.0 Gy/min	16S rRNA sequencing	–	*Bacteroides*↓	Cui et al., 2017
Irradiated ileals of male C57BL/6J mice	Ileal *in vitro* absorbed dose of 19 Gy and dose rate was 3.2 Gy/minute	37°C incubate	–	*Aerobic bacteria*↓ *Lactobacillus*↓	Johnson et al., 2004
Feces of male C57BL/6J mice	High-dose abdominal precision radiation with a single dose of 10 Gy	16S rRNA sequencing	–	*Proteobacteria*↑ *Bacteroidete*↓ *Firmicutes*↓ *Actinobacteria*↓ *Verrucomicrobia*↓	Zhao et al., 2020
Culture of gastric and duodenal samples	Abdominal radiotherapy	Glucose gas test and [14C] D-xylose breath test	Gynecologic cancer	*Gram-negative bacilli*↑	Husebye et al., 1995
Feces of patients	Abdominal radiotherapy	16S rRNA sequencing	Abdominal tumor	*Bacilli*↑ *Actinobacteria*↑ *Clostridia*↓	Manichanh et al., 2008
Feces of patients	Pelvic radiotherapy	16S rRNA sequencing	Cervical cancer	*Proteobacteria*↑ *Gammaprote-obacteria*↑ *Coprococcus*↑ *Bacteroides*↓	Wang et al., 2019
Feces of patients	Pelvic radiotherapy, five times a week during a 5 weeks period	454 pyrosequencing	Gynecologic cancer	*Gram-negative bacilli*↑ *Eubacteriaceae*↑ *Prevotellaceae*↓ *Oscillospiraceae*↓ *Fusobacteriaceae*↓	Nam et al., 2013

The meaning of a symbol in the table: ↑, increased; ↓, decreased.

(ZO-1), occludin, and claudins are three most important tight junction proteins, which play a vital role in maintaining cell polarity and intestinal epithelial barrier (Bazzoni et al., 2000). It was found that tight junction proteins are essential in repairing intestinal epithelial damages. Accumulating evidence shows that intestinal microbiome involves in the intestinal epithelial cell signaling and affects the intestinal barrier function (Ulluwishewa et al., 2011). Gut microbiota regulates intestinal barrier function by maintaining tight junction protein expression and distributions, which are important in intestinal barrier integrity (Ulluwishewa et al., 2011).

The intestinal epithelial mucus is a protective layer, which is regulated by bacteria such as *Lactobacilli*, *Bifidobacteria*, and *Streptococci* that play a positive role in strengthening the intestinal mucosal barrier. In the rat model, microbiota markedly promotes the expression of MUC2 gene and secretion of colonic MUC2 (Caballero-Franco et al., 2007). *Akkermansia muciniphila* is a type of gram-negative intestinal symbiotic bacteria that also promotes intestinal barrier function by enhancing mucous generation. Short-chain fatty acids (SCFAs) produced by *Akkermansia muciniphila* enter the intestinal epithelial cells through G protein-coupled receptor (GPCR) 41/43 to increase the expression of tight junction protein claudin-3 and occludin (Le Poul et al., 2003; Maslowski et al., 2009; Grander et al., 2018). Administration of *Lactobacillus* to healthy subjects significantly increases the scaffold protein ZO-1 and occludin in the vicinity of the tight junction structure, forming a cell side seal between the epithelial cells (Karczewski et al., 2010).

In radiation-associated enteritis patients, probiotics can also maintain micro-ecological stability and protect the intestinal barrier function by interrupting the pathogen's infection or inhibiting the growth of pathogens. After intestinal pathogenic *Escherichia coli* infection, changes in host cell cytoskeleton reduce the absorbing surface of intestinal epithelial cells, leading to pathogenic *Escherichia coli* infection-associated persistent diarrhea. However, *Lactobacillus plantarum* prevents pathogenic *Escherichia coli*-induced rearrangement and redistribution of claudin-1, occludin, ZO-1 and JAM-1 (Qin et al., 2009). *Lactobacillus* competes with pathogens to bind to receptors on the surface of intestinal epithelial cells, inhibits pathogen growth, and reduces the vitality and toxicity of pathogenic *Escherichia coli* (Sherman et al., 2005). In addition, certain harmful bacteria in *Enterobacteriaceae* can form a biofilm on the surface of the epithelium, altering and destroying the mucus layer (Hansson and Johansson, 2010). The increase of *Citrobacter rodentium* digests mucins with glycosidase, and participates in the degradation of mucus barrier (Bergstrom et al., 2010). Thus, the colonization of probiotics decreases, and the pathogenic bacteria multiply without competitors. The imbalance of intestinal flora homeostasis will weaken the intestinal epithelial barrier integrity, resulting in increased intestinal permeability.

Microbiome Dysbiosis Contributes to the Expression of Inflammatory Cytokines

Grander et al. found that recombination with *Akkermansia muciniphila* reduces expression of IL-1β and TNF-α and decrease the infiltration of MPO+ neutrophils in mice (Grander et al., 2018). Gut probiotics inhibit inflammation *via* suppressing the activation of NF-κB and TNF-α. This signaling pathway plays an important role in intestinal microbiota balance, which helps to regulate intestinal homeostasis, maintains intestinal barrier function and promotes repair and regeneration of tissues damaged (Stringer, 2013). *Ruminococcus*, *Coprococcus*, *Dorea*,

Lachnospira, *Roseburia*, *Bifidobacterium* and *Clostridium* can alleviate inflammation by inhibiting the TLR-NF-κB pathway (Lakhdari et al., 2011). *Bifidobacterium* inhibits intestinal epithelial cell inflammation by attenuating the inflammatory response induced by TNF-α and lipopolysaccharide (LPS) (Riedel et al., 2006). In addition, increased *Citrobacter* can activate NF-κB (Wang et al., 2006), and also involve in the degradation of the mucus barrier, thus increasing the inflammatory response. NF-κB and TNF-α are able to increase the production of myosin light-chain kinase, resulting in the disassembly of tight junction proteins. *Biffidobactrium* diminishes the formation of intestinal endotoxins and increases the production of tight junction proteins, thus decreasing intestinal permeability and bacterial translocation (Ewaschuk et al., 2008). Microbiome dysbiosis induced by radiation enteritis leads to severe side effects in immunodeficiency cancer patients because intestinal flora plays a vital role in innate and adaptive immune responses of hosts (Maynard et al., 2012). Interestingly, less apoptosis was observed in endothelial cells and lymphocytes in small intestine of germ free (GF) mice received a lethal dose of irradiation, demonstrating that GF mice may be resistance to lethal radioactive enteritis (Crawford and Gordon, 2005). Together, these studies suggest that microbiota dysbiosis may be detrimental to the maintenance of effective intestinal barrier function after radiation.

The mechanisms by which changes in the gut microbiota affect intestinal injury in radiation are expected to be extremely complicated. The dynamic changes of the microbial community interact with various components within the intestine rather than a single compound in the system. Current evidence supports and illustrates that intestinal microbiome plays a critical role in radiation-associated enteritis *via* up-regulating key regulatory factors. Gut microbiota and the body form a symbiotic and stable relationship in the protection of the intestinal epithelium, reduction of inflammatory responses, and maintenance of normal immune tolerance in order to sustain host's immune homeostasis, growth, and development (Sender et al., 2016). If there is an imbalance in intestinal flora, the relative abundance and diversity of the probiotics will be severely compromised, leading to limited intestinal barrier function, harmful bacteria overgrowth, accumulation of endotoxin in the blood, increased inflammatory factors, and eventually aggravating radiotherapy-induced enteritis. Thus, a stabilized intestinal microbiota is crucial for radiation enteritis (**Figure 2**).

THE ROLE OF INTESTINAL FLORA IN THE TREATMENT OF RADIOTHERAPY-INDUCED ENTERITIS

Probiotics Regulate the Intestinal Microbiota to Improve Radiation Enteritis

Recent studies have shown that probiotics are beneficial in strengthening the number and activity of host T cells, directly affecting the immune response mediated by T cells and improving immune functions (Redman et al., 2014). Furthermore, probiotics promote the diversity of gut microbiota, and increase the species of intestinal flora, ensuring the integrity of intestinal mucosal barrier so as to avoid bacterial translocation (Reiff and Kelly, 2010). The probiotic VSL# 3 is a mixture of 8 probiotics, which has been used broadly for many years with high safety. Clinically, it was proved that VSL#3 alleviates intestinal inflammation and enhances intestinal barrier functions by improving intestinal flora balance (Mimura et al., 2004; Sartor, 2006; Tankou et al., 2018). Treatment with VSL# 3 probiotics increases the diversity of bacterial community in patients, reduces fungal variety, and elevates the abundance of

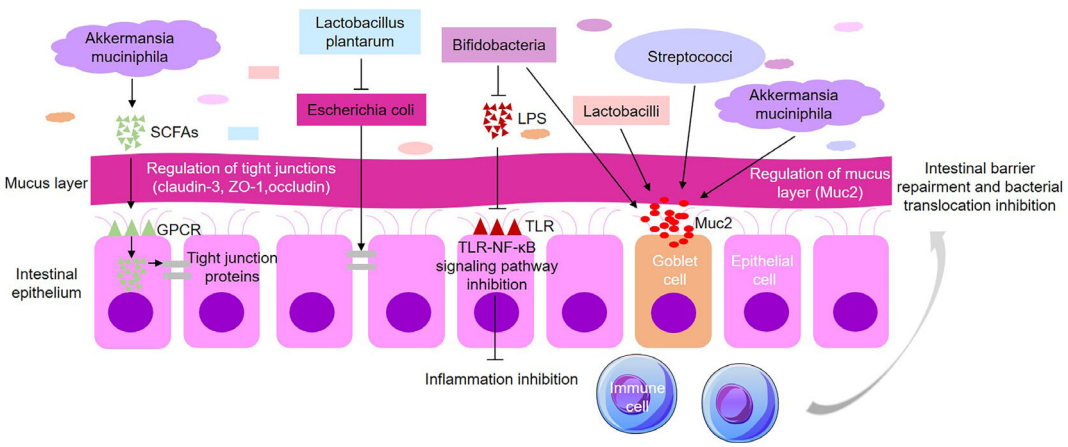

FIGURE 2 | Gut microbiota affects the function of intestinal epithelial barrier. Different types of microbiota act on various targets associated with intestinal epithelial barrier. *Biffidobactrium*, *Lactobacilli*, *Streptococci* and *Akkermansia muciniphila* stimulate the secretion of MUC2. SCFAs produced by *Akkermansia muciniphila* enter into the intestinal epithelial cells through GPCR to increase the expression of tight junction protein claudin-3 and occludin. *Lactobacillus plantarum* inhibits pathogenic *Escherichia coli* growth and increases the expression of ZO-1 and occludin. *Biffidobactrium* diminishes the formation of LPS and inhibits the TLR-NF-κB signaling to alleviate inflammation, hence decreasing intestinal permeability and bacterial translocation.

Lactobacillus and *Bifidobacterium*, and hence reduces the incidence and severity of radiotherapy enteritis-associated diarrhea (Kuhbacher et al., 2006; Chitapanarux et al., 2010).

A randomized double-blind controlled trial showed that a standard dose of *Lactobacillus acidophilus LAC-361* and *Bifidobacterium longum BB-536* decreased level 2, 3, and 4 diarrhea caused by radiation enteritis in patients with surgery. Demers et al. analyzed a total of 229 patients and found that for patients who had undergone surgery, the incidence of diarrhea was reduced after probiotic treatment. For patients who did not undergo surgery, there was no statistical difference (Demers et al., 2014). In fact, for patients without surgery, patients treated with standard doses of probiotics had fewer moderate to severe diarrhea than the placebo group at the end of radiotherapy. For patients with surgery before radiotherapy, administration of probiotics tends to reduce all degrees of diarrhea, especially the most severe grade 4 diarrhea. These results may be attributable to the individualized nutritional interventions during the treatment, and these interventions were adjusted as the treatment progressed. It is difficult to compare these results with other published studies due to different treatment options. In addition, compared to probiotic treatment group, the placebo group showed a significant destruction of gut microbiota after radiotherapy enteritis (e.g., through an increase in *Enterobacteriaceae* (Osterlund et al., 2007). The intragastric administration of *Lactobacillus rhamnosus* increased the crypts survival in radiation-induced enteritis by approximately two-fold and reduced epithelial cell apoptosis at the crypt basement, which depends on intact Toll-like receptor 2 (TLR2) and MyD88-dependent signals that involve in the relocation and expression of cyclooxygenase 2 (COX-2) in mesenchymal stem cells of crypt region (Ciorba et al., 2012; Uribe et al., 2018; Riehl et al., 2019). *Bifidobacterium infantis* produces indole-3-lactic acid (ILA) to protect intestinal epithelial cells in culture *via* activation of the aryl hydrogen receptor (AhR) and nuclear factor erythroid 2-related factor 2 (Nrf2) (Ehrlich et al., 2020). Thus, prebiotics and synbiotics can also promote the growth and reproduction of probiotics and hence reduce diarrhea in radiotherapy patients (Garcia-Peris et al., 2016).

Probiotics-mediated homeostasis of intestinal microbiota may also prevent other symptoms from radiation enteritis, but more studies are needed to clarify the observation, which is the key to maintaining the biological strategies of healthy intestine during radiotherapy of cancer (Bentzen, 2006; Fuccio and Guido, 2013; Florez et al., 2016).

Fecal Microbiota Transplantation Maintains the Balance of Gut Microbiota to Improve Radiotherapy-Induced Enteritis

FMT is a treatment method to reestablish the gut microbiota of patients by separating and transplanting the intestinal flora of healthy persons into patient's intestine tract (Xu et al., 2016). In 1958, Eiseman et al. applied FMT to treat Pseudomembranous enteritis caused by the treatment of antibiotics, leading to an improvement of patient's condition (Eiseman et al., 1958). With the analysis of the 16S rRNA sequencing, Cui et al. confirmed that FMT treatment increases the relative abundance of the intestinal microbiota, such as escalation (or stabilization) of *Bacteroidetes* (or *Lactobacillus*), augment of *Prevotella* at the genus level. In addition, FMT-improved gut microbiota can reduce the intestinal leakage and enhance the intestinal functions and epithelial integrity in radiotherapy-induced enteritis (Cui et al., 2017). Ding et al. proved that the intestinal flora diversity of all patients underwent radiotherapy increased after FMT treatment. Radiation-induced intestinal edema was strikingly alleviated after eight weeks of FMT, and the beneficial bacteria such as *Alistipes*, *Phascolarctobacterium*, *Streptococcus* and *Bacteroides* expanded, whereas the abundance of *Faecalibacterium* decreased (Ding et al., 2020). FMT of gut microbes from healthy donors may reduce the toxicity caused by radiotherapy (Cui et al., 2017). In addition, FMT could elevate the level of microbial-derived indole 3-propionic acid (IPA) in the feces of irradiated mice, so that the mice had a lower level of systemic inflammation, a reduced hematopoietic organ injury and catabatic myelosuppression, and improved gastrointestinal tract functions and epithelial integrity (Xiao et al., 2020). Li et al. proved that FMT increased the level of SCFAs in feces and valeric acid (VA) produced by microbiota exerted the most important radioprotective effect by increasing the expression of keratin 1 (KRT1). VA supplementation increased the survival of irradiated mice, protected their hematopoietic organs, and alleviated gastrointestinal injury of irradiated mice (Li et al., 2020a).

Fresh stool used for bacterial transplantation can effectively treat *Clostridium difficile* infection. For patients with severe *Clostridium difficile* infection, multiple stool transplantation may effectively relieve diarrhea (Hui et al., 2019). FMT capsules were also used to treat pediatric diarrhea and other diseases. It significantly improves both overall and gastrointestinal health after FMT (Youngster et al., 2014). FMT also has an obvious effect on the treatment of pediatric inflammatory bowel disease (IBD). Patients prior to FMT treatment exhibited reduced biodiversity and significantly differed in gut microbiota composition characterized by an increase in *Enterobacteriaceae*, *Enterococcus*, *Haemophilus*, and *Fusobacterium* compared to donors, and an augment in species diversity at 30 days post-FMT treatment (Goyal et al., 2018). Paramsothy et al. demonstrated that microbiota diversity increased with the persistence of FMT, which could alleviate the clinical symptoms of ulcerative colitis, and was mainly associated with the improvement of microbial diversity (Paramsothy et al., 2017). Ishikawa et al. also confirmed that FMT is a potential treatment for restoring normal intestinal flora in patients with ulcerative colitis (Ishikawa et al., 2017). Selection of healthy donors for FMT may avoid potential adverse effects transmitted from donor's feces in the therapy of radiation-induced enteritis. Standardization of this technology and humanization of FMT are two key factors in the usage of the therapy to meet the needs of patients (Ren et al., 2016).

Antibiotics Reconstruct the Intestinal Microbiota to Alleviate Radiation-Induced Enteritis

Abdominal radiation disrupts the intestinal microbial balance. It reduces microbiota diversity, and increases the relative abundance

of pathogenic bacteria, such as *Proteobacteria* in mice. Antibiotic cocktail (ABX) and metronidazole pretreatment are beneficial to the reconstruction of gut microbes in irradiated mice. It has been reported that Abx pretreatment effectively reduces the level of LPS in the ileum and inhibits the TLR4/MyD88/NF-κB signaling, thereby reducing intestinal inflammation. In addition, Abx pretreatment regulates macrophage polarization in the ileum and downregulates the expression of TGF-β1 and phosphorylated Smad-3 and α-SMA thereby preventing intestinal fibrosis and ultimately improving the survival of mice with radiation-induced intestinal injury (Cui et al., 2017; Zhao et al., 2020). These results indicate that antibiotic pretreatment can effectively alleviate gut microbial disorders and intestinal injury caused by abdominal radiation. In general, these findings have increased our understanding of the radiation enteritis pathogenesis.

CONCLUSIONS AND PROSPECTS

The intestinal flora is affected by multiple factors, as well as original diseases, such as cancers. Although the location of a disease is far from or not related to the intestinal tract, alterations in health status of the host may also affect the overall homeostasis of intestinal flora. Thus, gut microbiota in various cancer patients may be different as compared with healthy individuals. Studies with strictly selected healthy people for comparison may help to uncover bacterial genera changed in radiation enteritis. In terms of treatment, with intestinal flora in healthy people as references, those in patients with radiation-induced intestinal injury can be manipulated to a comparable level in healthy individuals.

In the current review, we emphasized the relationship between microbiota and radiation enteritis. Radiation reduces the diversity of intestinal flora and modifies gut microbiota composition. We summarized the types of bacteria, which have been changed in radiation enteritis. Alterations in the composition of the intestinal flora aggravate radiation enteritis, which may form a vicious circle and amplify the changes in microbiota. Radiation enteritis could weaken the intestinal epithelial barrier and increase the expression of inflammatory factors. Mounting evidence shows that intestinal microbiota is the initiator for the pathogenesis of radiation enteritis. Targeted treatment of gut microbiota with probiotics, fecal bacteria transplantation and antibiotics can alleviate radiation enteritis. In addition, intestinal flora could be a potential biomarker for radiation enteritis and may help to establish a personalized radiation treatment plan. Further studies regarding the role of intestinal flora in radiation enteritis are needed in order to set up a more reliable preventive strategy for the disease. In the future, on the basis of 16S rRNA sequencing, metabolomics, transcriptomics, shotgun metagenomics, and other state-of-art methods, functions of gut microbiota in radiation enteritis are expected to be explored more extensively. Reversing the changes in microbiome may eventually prove to be an effective therapeutic strategy for patients with life-threatening radiation enteritis.

AUTHOR CONTRIBUTIONS

YJ wrote the manuscript. Z-XX, YW, DZ, and ML contributed to critical revision of the paper. All authors contributed to the article and approved the submitted version.

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Plasma Metabolomic and Intestinal Microbial Analyses of Patients With Severe Aplastic Anemia

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Aplastic anemia results from bone marrow failure caused by an autoimmune abnormality, but the pathogenesis of severe aplastic anemia (SAA) is not well characterized. To identify potential metabolic markers of SAA and to further elucidate the pathogenetic mechanisms of SAA, we performed a metabolomic study of plasma samples and characterized the intestinal microbiota of patients with SAA and healthy controls. Patients with SAA had more Enterobacteriales and Lactobacillales, but fewer Bacteroidales, Clostridiales, and Erysipelotrichales than healthy controls. At the species level, the abundances of *Escherichia coli* and others including *Clostridium citroniae* were higher, whereas those of *Prevotella copri*, *Roseburia faecis*, and *Ruminococcus bromii* were lower. Eight metabolites showed significantly different plasma concentrations in the SAA and healthy control groups. Coumaric acid, L-phenylalanine, and sulfate were present at higher concentrations in the SAA group; whereas L-glutamic γ-semialdehyde, theobromine, 3a, 7a-dihydroxy-5b-cholestane, γ-δ-dioxovaleric acid, and (12Z)-9, 10-dihydroxyoctadec-12-enoic acid were present at lower concentrations. In conclusion, patients with SAA show abnormalities in both their plasma metabolomes and intestinal microbial compositions. These differences might reflect the molecular mechanisms involved in the defective immunity that characterizes SAA.

Keywords: aplastic anemia, metabolomics, microbiota, plasma, gut

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INTRODUCTION

Severe aplastic anemia (SAA) is a class of hematological diseases that is characterized by pancytopenia and bone marrow failure. Immunosuppressive therapy (IST) using antithymocyte globulin and cyclosporine A has been used as the first-line treatment for patients with SAA, and this improves their prognosis (Killick et al., 2016). SAAs are currently considered to be immune disorders of the bone marrow hematopoietic cells that involve damage resulting from hyperfunction of cytotoxic T lymphocytes (Fogarty et al., 2003; Maciejewski and Risitano, 2003). Patients with SAA also show an imbalance of T-helper (Th)1 and Th2 cells (Solomou et al., 2006), fewer regulatory T cells (Tregs) (Solomou et al., 2007), abnormally activated myeloid dendritic cells (mDCs) (Nakao, 2013; Ogawa, 2016), and abnormally high concentrations of Th1-type cytokines (Gidvani et al., 2007). However, the precise immunopathogenesis of SAA is unclear.

In recent studies, abnormal metabolism and abnormal composition of the intestinal microbiota have been shown to play important roles in autoimmune diseases. Abnormal composition of the gut microbiota is linked to a number of human diseases (Qin et al., 2012; Dodd et al., 2017). Furthermore, metabolomics studies have shown differences in concentrations of key metabolites

in hematological diseases (Chen et al., 2014). The immune-mediated defects in bone marrow hematopoiesis have been shown to be triggered by certain types of chronic inflammation or infection (Maciejewski et al., 2000; Chihara et al., 2018). Furthermore, alterations to the intestinal microbiota and chronic enteritis may provide persistent stimuli that induce and sustain the immune pathophysiology (Espinoza et al., 2016). A previous case report that described a 30-years-old male patient with refractory SAA revealed an inadvertently good hematological response to the treatment of intestinal inflammation, which supports a hypothetical but plausible pathogenic association (Zhao et al., 2020). However, although evidence for associations between the intestinal microbiota, diseases, and symptoms is accumulating, the design of novel therapies that are based on these links necessitates much fuller knowledge of the roles of these intestinal microorganisms.

In this study, we performed a metabolomic study of the plasma and characterized the intestinal microbiota of patients with SAA and healthy controls to identify potential metabolic markers and further elucidate the pathogenetic mechanisms of SAA.

MATERIALS AND METHODS

Study Participants

A total of 10 patients with SAA that was diagnosed at the Hematology Department of Tianjin Medical University were enrolled between January 2018 and January 2019 (six men and four women; median age = 56.5 years, range = 17–77 years). In addition, 14 healthy adults (3 men and 11 women; median age = 43.5 years, range = 26–63 years) were recruited as the control group.

A diagnosis of SAA was made according to the criteria of the International AA Study Group (Marsh et al., 2009). Bone marrow biopsy and aspiration for morphology and cytogenetics were performed before enrolment. All the patients were tested for paroxysmal nocturnal hemoglobinuria (PNH) using a flow cytometric assay, but no PNH clones were identified. None of the participants had taken antibiotics or probiotics within the 3 months prior to admission. The clinical data for all of the participants are shown in **Table 1**. There were no statistically significant differences in the clinical data between the two groups ($p > 0.05$), and the severity of the disease was similar in all the patients. The study was approved by the Ethics Committee of the Tianjin Medical University.

TABLE 1 | Clinical data for all the participants.

	SAA group	Control group
N	10	14
Females (%)	40.0	78.6
Median age	56.5	43.5
Age range	17–77	26–63
Hemoglobin (g/L)	68.30 ± 2.32	130.43 ± 1.68
Platelet (× 10^9/L)	12.60 ± 1.44	266.86 ± 14.05
Neutrophil (× 10^9/L)	0.61 ± 0.09	3.27 ± 0.24
Reticulocyte (× 10^9/L)	13.80 ± 0.95	55.71 ± 4.60

Intestinal Microbial Analysis

Sample Collection and Preservation

None of the participants had undergone antibiotic or IST within the 3 months preceding admission. Stool samples were collected within 2 h after the participants were consuming their standard diet. Samples from the middle and rear of the stool were collected using sterile cotton swabs. Half a milliliter of each sample of feces was collected into a 1.5-mL sterile Eppendorf tube, taking care to avoid contamination with urine or other substances. Two aliquots were collected from each participant, and these were stored at −80°C.

DNA Extraction and Quality Testing

Stool samples were thawed, and fecal microbial genomic DNA was extracted using a MagPure Stool DNA KF Kit B, according to the manufacturer's instructions. A Qubit® dsDNA BR Assay Kit was used to accurately quantify the DNA concentrations. One-percent agarose gel electrophoresis (150 V for ∼40 min) was used to check the quality of the DNA.

16s rDNAV3–V4 Segment Amplification

The primer sequences used for the polymerase chain reaction (PCR) reaction were 341F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). The 16S libraries were sequenced at the Analytical Genomics Core of Sanford Burnham Prebys Medical Discovery Institute (Lake Nona, FL, United States) and the Beijing Genomics Institute (Beijing, China). The original FASTQ files were processed using the novel 16S amplicon sequencing pipeline HiMap[1] (bioRxiv 565572), which generates Operational Strain Unit as its output. The read counts were then converted to relative abundances. Log10-transformed relative abundances were used for comparisons of samples from each group.

PCR Product Purification

The PCR products were purified using Agencourt AMPure XP magnetic beads, dissolved in Elution Buffer, labeled, and used for library construction.

Library Quality Inspection

An Agilent 2100 Bioanalyzer was used to characterize the sizes and concentrations of the fragments that constituted the libraries. Qualifying libraries were sequenced on the Illumina HiSeq platform according to the size of the inserted fragments and using the Illumina standard pipeline, generating read areas of 2 × 300 bp.

Bioinformatics Analysis

Data preprocessing

Off-machine data filtering was performed to remove low-quality, joint pollution, N, and low-complexity reads. The filtered reads were spliced using Fast Length Adjustment of Short reads (FLASH v1.2.11). The minimum matching length was 15 bp, and the allowable mismatch rate in the overlapping area was

[1]http://github.com/taolonglab/himap

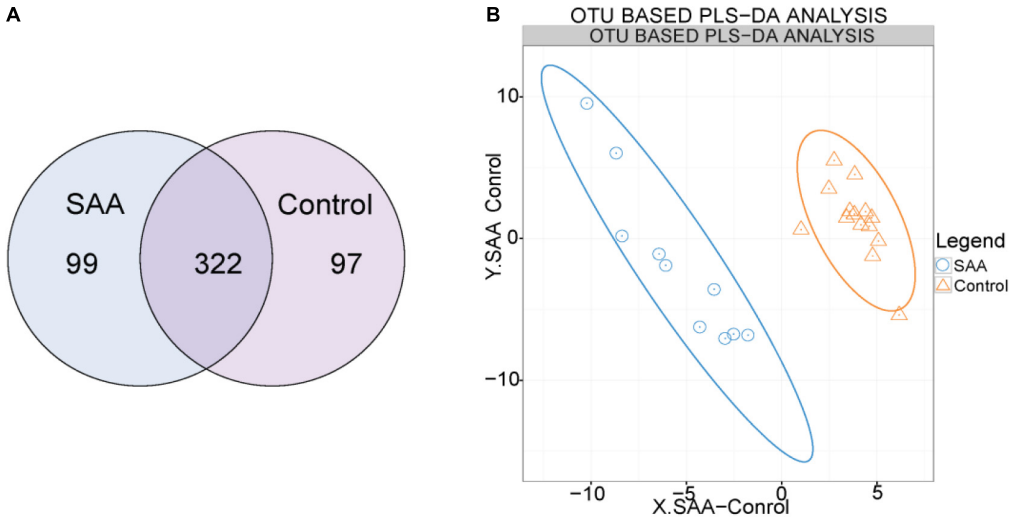

FIGURE 1 | (A) Venn diagram of the OTUs (A: SAA group, N: control group). **(B)** Out-based PLS-DA analysis. Orange triangles represent intestinal microbial samples from the control group, and blue circles represent samples from patients with SAA.

0.1. The reads were then spliced into tags using the overlaps between the reads.

Operational taxonomic unit cluster analysis

To facilitate analysis, certain taxonomic units were identified using unified marks, which are used in the study of phylogeny and population genetics, and were referred to as operational taxonomic units (OTUs). To study all the bacterial taxa that were sequenced in a sample, the sequences were classified into groups according to their similarity, and each group represented an OTU. In general, tags with a similarity of $>$ 97% were clustered into an OTU.

We used Venn diagrams to demonstrate the numbers of common and unique OTUs for a variety of samples and also visually displayed the OTU overlap between the samples graphically. Partial least-squares discrimination analysis (PLS-DA) is a method of multivariate statistical analysis and a method of supervision that is used for discriminant analysis, which reflects the differences between groups to the greatest extent. Various colors and shapes were used to represent the sample groups under various conditions.

Analysis of species composition

We used the RDP classifier Bayesian algorithm to perform taxonomic analysis of representative sequences of each OTU to obtain species classification information corresponding to each OTU. The community compositions of the SAA and healthy control groups were analyzed at the levels of phylum, class, order, family, genus, and species and are displayed in the form of abundance histograms. R software (v3.4.1) was used to analyze the relative abundance graphs for the two groups.

Alpha diversity analysis

Alpha diversity is a means of assessing the species diversity of an individual sample and is described in the form of the Chao index, observed species index, Ace index, Simpson index, Shannon index, and good-coverage index. The Chao, Ace, and observed species indices were used to describe the species richness of the samples, and the Simpson and Shannon indices were used to describe the species diversity of the samples, which comprises the species richness and species evenness. When the species richness is the same, the species evenness in the community is proportional to the species diversity. Therefore, the larger the Chao, Ace, observed species, and Shannon indices, and the smaller the Simpson index, the higher the species diversity of the sample. The good-coverage index reflects the coverage of the sample library, and its value is inversely proportional to the probability that the sequence was measured in the sample, such that the higher its value is, the more representative it is of the real composition of the sample.

Differential species analysis

The Wilcoxon rank-sum and Kruskal–Wallis tests were used to identify significant differences in the abundance of microbial species between the groups ($p < 0.05$).

Association analysis and model prediction

Species that were present at differing abundances according to the results of rank-sum tests were analyzed using a Spearman correlation heatmap of the dominant species, drawn using R software. Important patterns and relationships between the dominant species are identified by color: the darker the color is, the stronger the correlation is between the species.

Plasma Metabolomic Analysis

Fresh whole-blood samples (5 mL) were collected from the patient and control groups, placed into EDTA anticoagulation tubes, and centrifuged at 1,600 $\times$ *g* for 10 min at 4°C to separate the plasma. All the samples were analyzed using ultrahigh-performance liquid chromatography (UPLC) (Waters, United Kingdom), an Acquity UPLC BEH C18 column

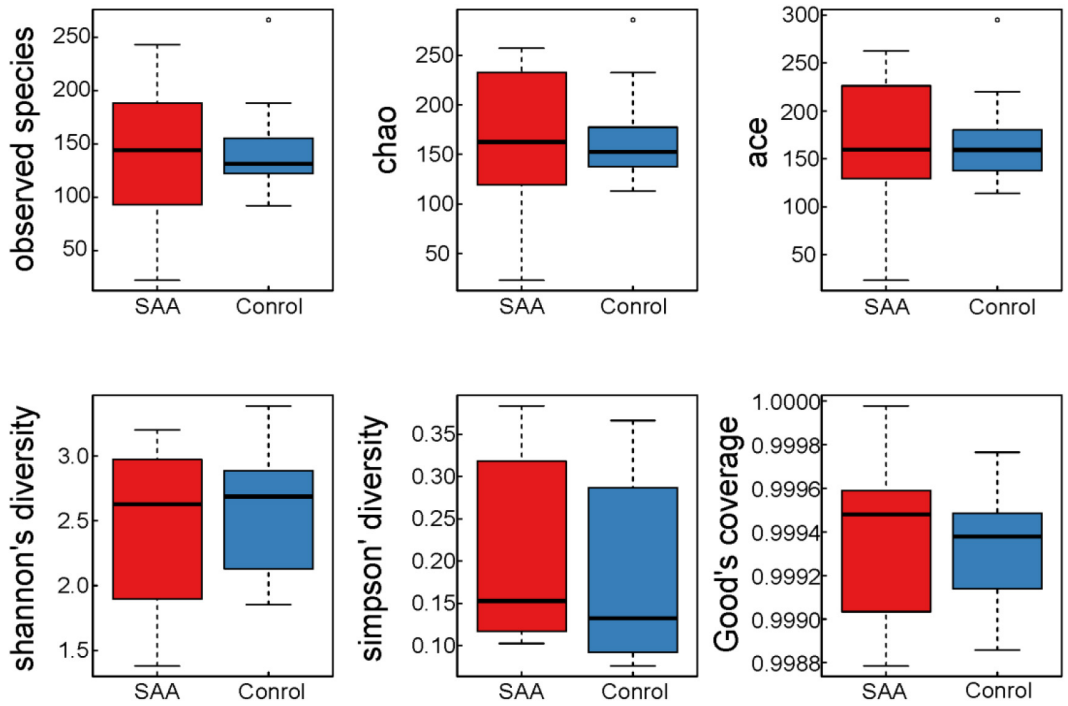

FIGURE 2 | Boxplots of indices of the alpha diversity of the intestinal microbiota in the SAA and control groups.

(100 mm × 2.1 mm, 1.7 μm, Waters, United Kingdom) for reverse-phase separation, and a high-resolution tandem mass spectrometer [Xevo G2 XS quadrupole-time of flight (Q-TOF), Waters, United Kingdom] to identify metabolites eluted from the column. The Q-TOF was operated in both positive and negative ion modes.

Statistical Analysis

Student's *t*-test, fold-change analysis, and PLS-DA were used to identify metabolites that were present at differing concentrations in the SAA and control groups. Differences were considered statistically significant when there was a fold difference ≥ 1.2 or ≤ 0.8333 and a $p < 0.05$.

RESULTS

Composition of the Intestinal Microbiota of Patients With SAA and Controls

OTU Analysis

OTU clustering was performed with a sequence similarity of 97%. The Venn diagram of the OTUs shows that the healthy control group contained a total of 419 OTUs, the SAA group contained 421 OTUs, and the two groups shared 322 OTUs (**Figure 1A**). Furthermore, we performed PLS-DA analysis in the R (v3.2.1) mixOmics package to analyze the OTU data, which showed that patients with SAA had a different intestinal microbiome to the healthy controls (**Figure 1B**).

Alpha Diversity Analysis

The mean good coverage values for the SAA and healthy control groups were > 99%, indicating that the sequences obtained for each sample covered almost all the bacterial sequences in the library. As shown in **Figure 2**, the values of the Chao, Observed species, Ace, and Shannon indices for the SAA group were slightly lower than those for the healthy control group, and the Simpson index was higher (**Figure 2** and **Table 2**).

Structures of the Microbiota of Patients With SAA and Controls

Intestinal Microbial Composition of the SAA and Control Groups at the Phylum Level

Composition analysis showed that the relative abundance of *Proteobacteria* in the intestinal microbiota was higher in the SAA group than in the control group, but that the relative abundance of *Bacteroidetes* was lower (**Figures 3A,B**). However, there were no significant differences in differential microbial phylum between the SAA group and control groups, according to the Wilcoxon rank-sum *t*-test (**Figure 3C**).

Intestinal Microbial Composition of the SAA and Control Groups at the Class Level

Composition analysis showed that the relative abundances of *Bacillus* and *Gammaproteobacteria* in the intestinal microbiota were higher in the SAA group than in the control group, but that the relative abundance of *Bacteroidia* was lower (**Figures 4A,B**). Further analysis of the top 10 most abundant species in the SAA and control groups showed that there were no significant

TABLE 2 | Results of the richness and diversity analysis of the intestinal microbiota.

Alpha diversity index	SAA (n = 10)	Normal control (n = 14)	p-value
Sobs	135.1	143.21429	0.52977
Chao	161.60401	163.85759	0.80945
Ace	164.78136	168.27365	0.83967
Shannon	2.39946	2.57708	0.18377
Simpson	0.20437	0.18683	0.23579
Coverage	0.99937	0.99934	0.95995

differences between the groups (Kruskal–Wallis and Wilcoxon rank-sum tests) (**Figures 4C,D**).

Intestinal Microbial Composition of the SAA and Control Groups at the Order Level

Composition analysis showed that the relative abundances of *Enterobacteriales* and *Lactobacillales* in the intestinal microbiota were higher in the SAA group than in the control group, but the relative abundances of *Bacteroidales*, *Clostridiales*, and *Erysipelotrichales* were lower (**Figures 5A,B**). Further analysis of the top 10 most abundant species in the SAA and control groups showed that the abundance of *Enterobacteriales* was higher in the SAA group than in the control group ($p < 0.05$), according to the Kruskal–Wallis test (**Figure 5C**). However, there were no other significant differences between the two groups (Wilcoxon rank-sum test) (**Figure 5D**).

Intestinal Microbial Composition of the SAA and Control Groups at the Family Level

Composition analysis showed that the relative abundances of *Ruminococcaceae* and *Paraprevotellaceae* in the intestinal microbiota were higher in the SAA group than in the control group (**Figures 6A,B**). Further analysis of the top most abundant 10 species in the two groups showed that the abundance of the *Enterobacteriaceae* was higher in the SAA group than in the control group ($p < 0.05$), according to the Kruskal–Wallis test (**Figure 6C**). However, there were no other significant differences between the two groups (Wilcoxon rank-sum test) (**Figure 6D**).

Microbial Composition of the SAA and Control Groups at the Genus Level

Composition analysis showed that the relative abundances of *Clostridium*, *Escherichia*, *Morganella*, and *Veillonella* in the intestinal microbiota were higher in the SAA group than in the control group, but the relative abundances of *Coprococcus* and *Roseburia* were lower (**Figures 7A,B**). Further analysis of the top 10 most abundant species in the two groups showed that the abundance of *Roseburia* was lower in the SAA group ($p < 0.05$; Wilcoxon rank-sum test) (**Figures 7C,D**).

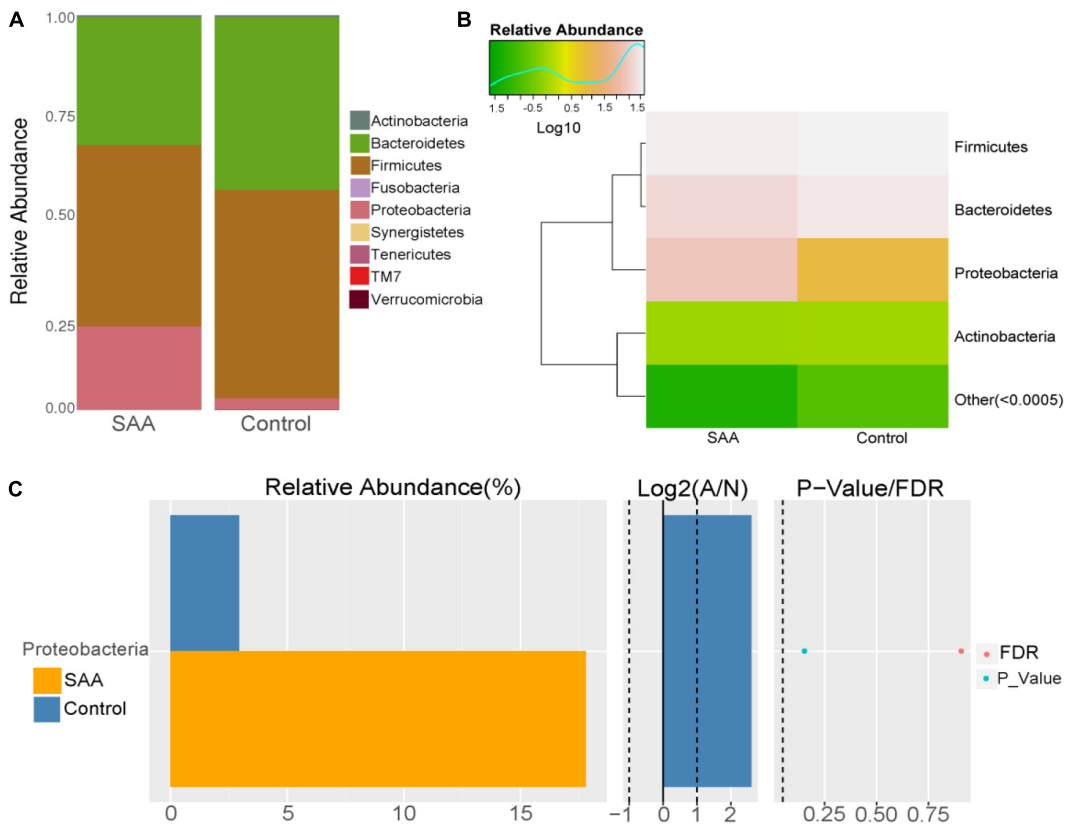

FIGURE 3 | Intestinal microbial compositions of the SAA and control groups at the phylum level. **(A)** Bar plot, **(B)** heatmap, **(C)** Wilcoxon rank-sum test.

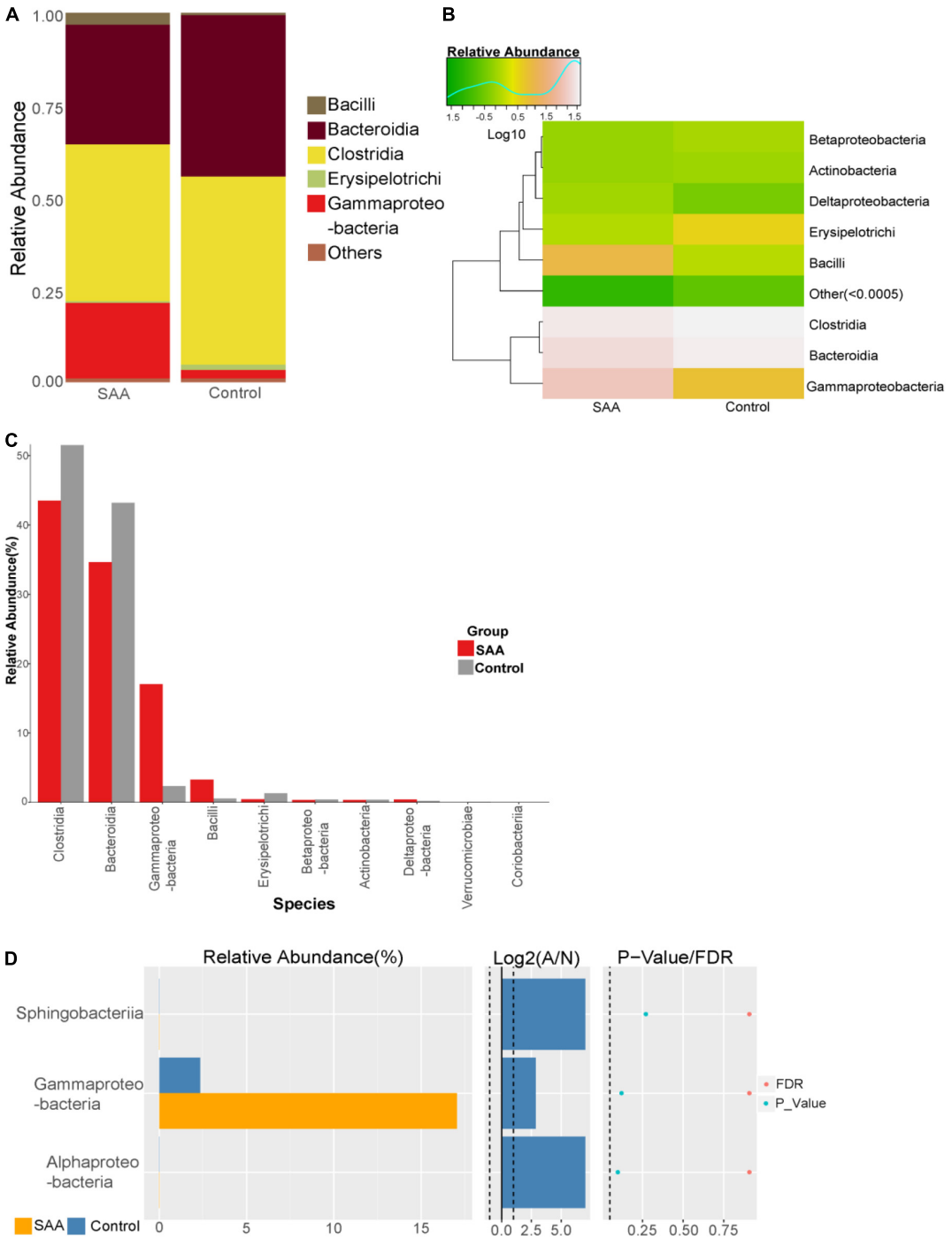

FIGURE 4 | Intestinal microbial compositions of the SAA and control groups at the class level. **(A)** Bar plot, **(B)** heatmap, **(C)** top 10 most abundant species, **(D)** Wilcoxon rank-sum test results.

Intestinal Microbial Composition of the SAA and Control Groups at the Species Level

Composition analysis showed that the relative abundances of *Gemmiger formicilis*, *Escherichia coli*, *Clostridium citroniae*, *Morganella morganii*, and *Veillonella dispar* in the intestinal microbiota were higher in the SAA group than in the control group, but the relative abundances of *Bacteroides coprophilus*, *Coprococcus eutactus*, *Prevotella copri*, *Roseburia faecis*, and *Ruminococcus bromii* were lower (**Figures 8A,B**). Further analysis of the two groups showed that the abundances of *C. citroniae* and *Coprococcus cateriicrmis* were higher, and those of *R. faecis*, *C. eutactus*, *Clostridium clostridioforme*, *Lactobacillus*

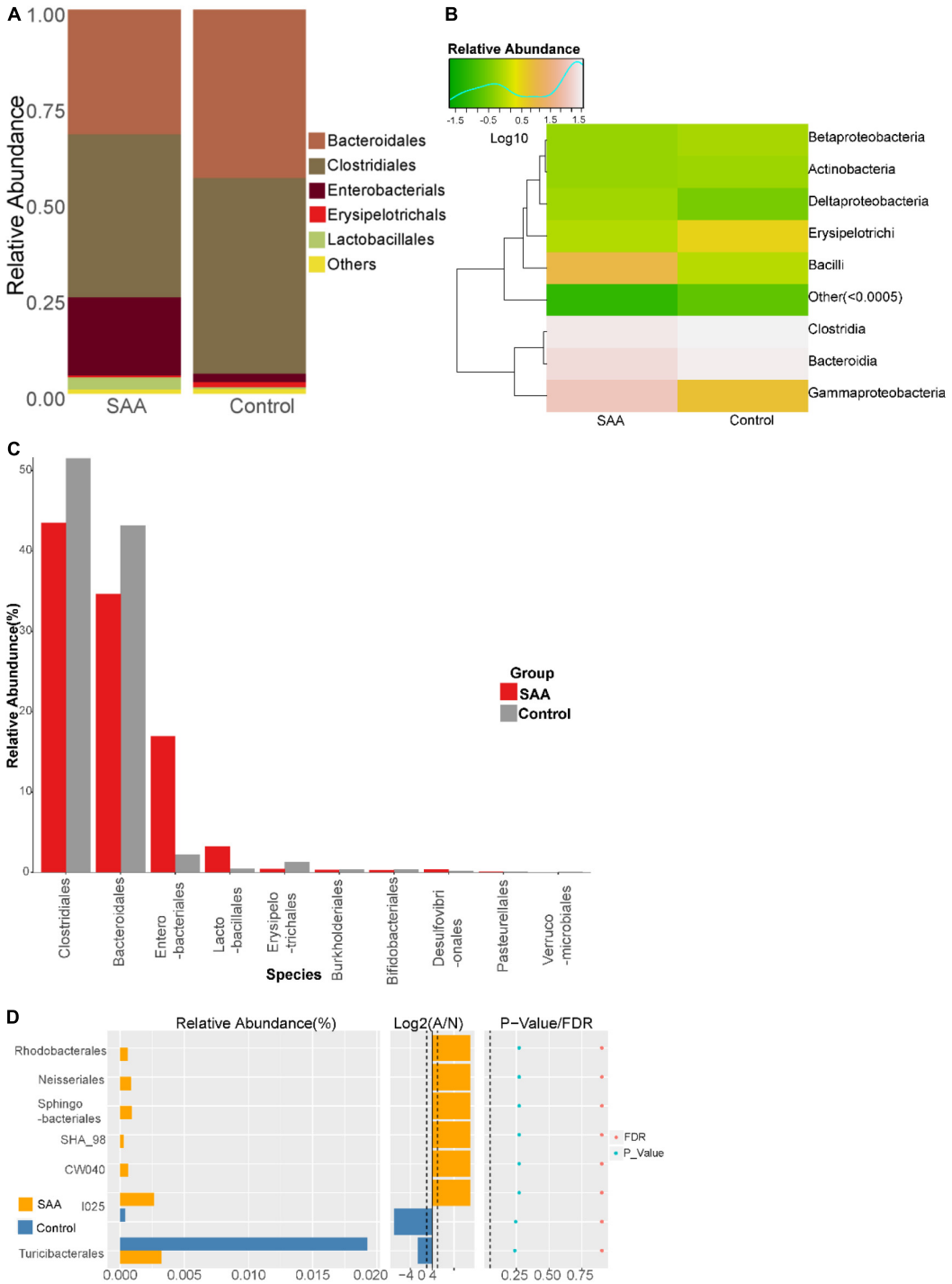

FIGURE 5 | Intestinal microbial compositions of the SAA and control groups at the order level. **(A)** Bar plot, **(B)** heatmap, **(C)** top 10 most abundant species, **(D)** Wilcoxon rank-sum test results.

ruminis, and *P. copri* were lower in the SAA group (all $p < 0.05$; Wilcoxon rank-sum test) (**Figure 8C**). In addition, the SAA group showed higher abundance of *C. citroniae* and lower abundance of *R. faecis* among the top 10 most abundant species (Kruskal–Wallis test) (**Figure 8D**).

Correlation Analysis and Model Prediction

The correlations between the associated dominant species in the SAA and control groups are shown in the form of a heatmap (**Figure 9**). This shows a strong competitive relationship between *P. copri* and *C. citroniae*.

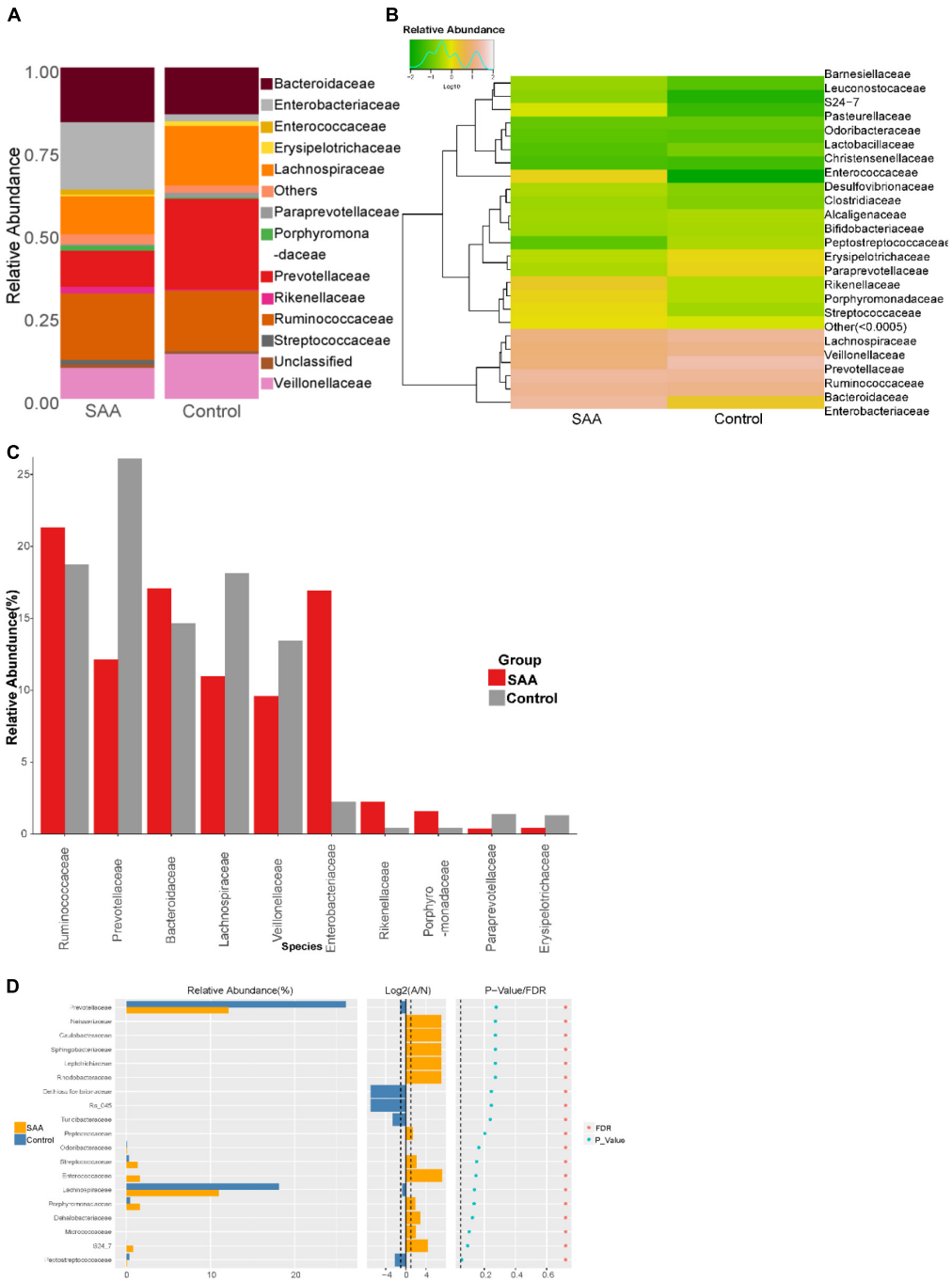

FIGURE 6 | Intestinal microbial compositions of the SAA and control groups at the family level. **(A)** Bar plot, **(B)** heatmap, **(C)** top 10 most abundant species, **(D)** Wilcoxon rank-sum test results.

Findings of the Metabolomic Analysis

The metabolomic analysis showed that patients with SAA had a different metabolite profile to that of healthy controls. The results are displayed using PLS-DA (**Figure 10A**), a visual volcanic map (**Figure 10B**), and a heatmap (**Figure 10C**). Differences were present in both the positive and negative mass spectrometric ion modes.

Eight metabolites were found to be present at significantly different plasma concentrations in the SAA and healthy control groups by the screening and identification of derived ions. Coumaric acid, L-phenylalanine, and sulfate were present at higher concentrations in the SAA group, whereas L-glutamic γ-semialdehyde, theobromine,3a, 7a-dihydroxy-5b-cholestane, γ-δ-dioxovaleric acid, and (12Z)-9,

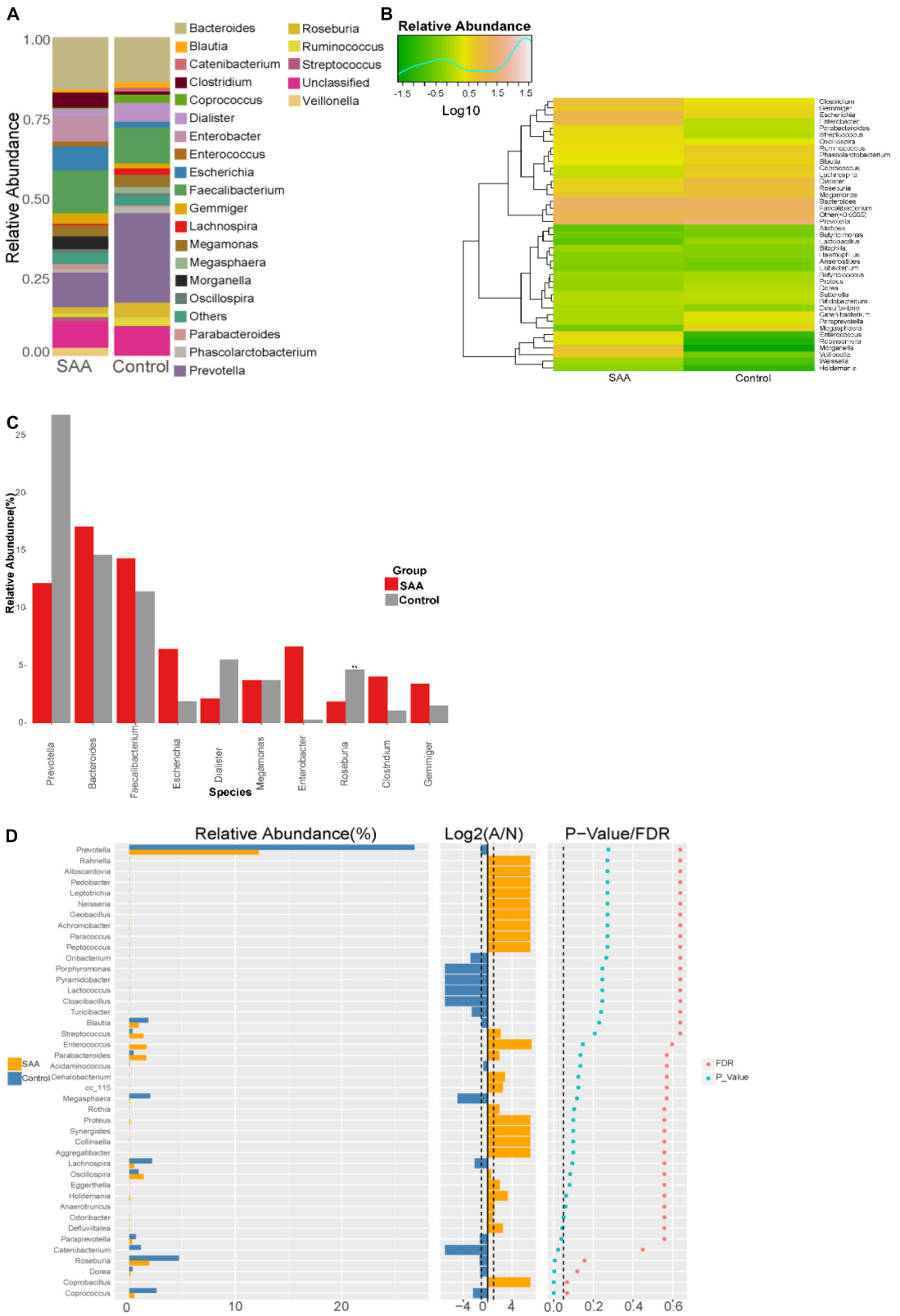

FIGURE 7 | Intestinal microbial compositions of the SAA and control groups at the genus level. **(A)** Bar plot, **(B)** heatmap, **(C)** top 10 most abundant species, **(D)** Wilcoxon rank-sum test results.

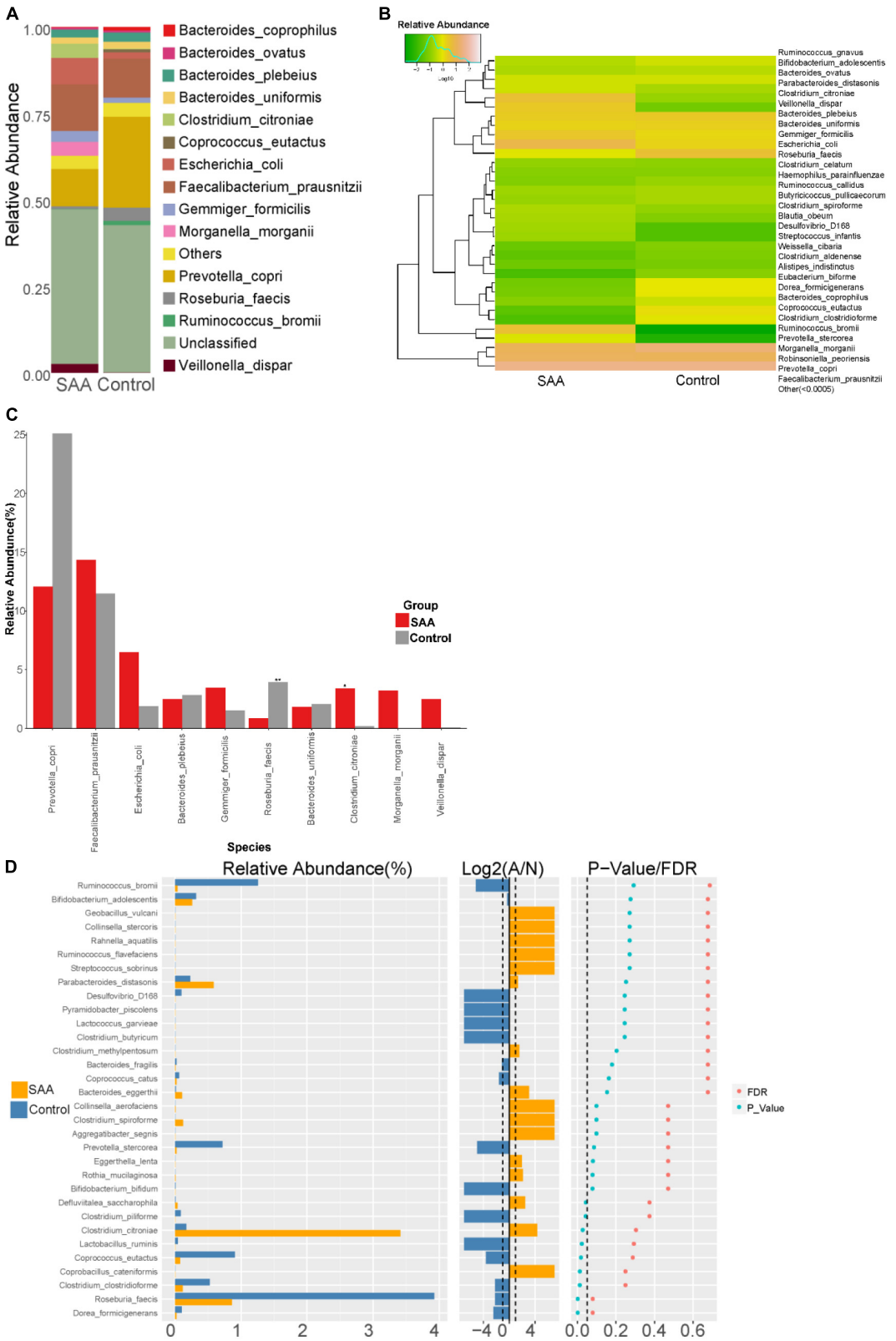

FIGURE 8 | Intestinal microbial compositions of the SAA and control groups at the species level. **(A)** Bar plot, **(B)** heatmap, **(C)** Wilcoxon rank-sum test results, **(D)** top 10 most abundant species.

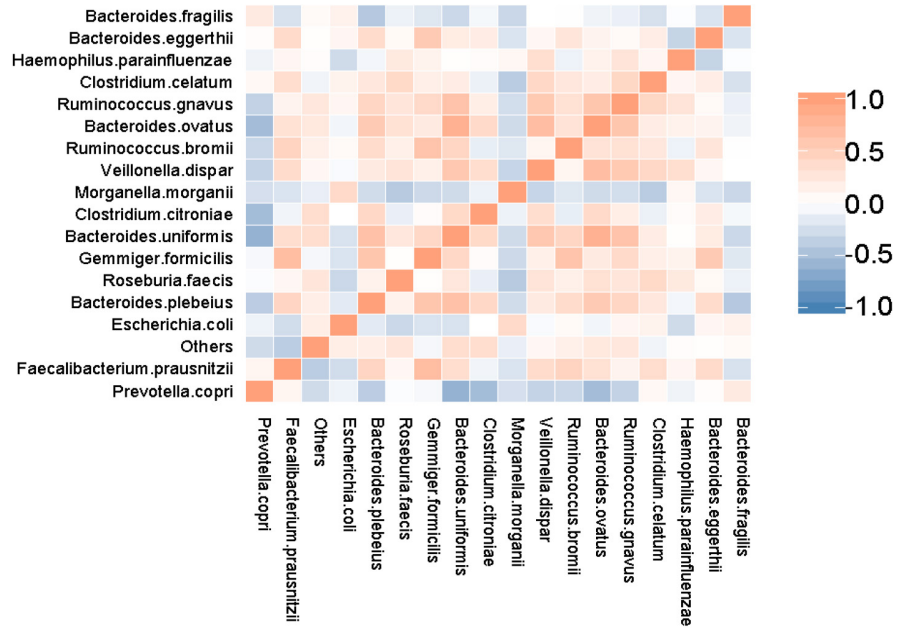

FIGURE 9 | Heatmap showing the results of the association analysis for the SAA and control groups.

10-dihydroxyoctadec-12-enoic acid were present at lower concentrations (**Table 3**). These metabolites are amino acids, steroids, keto acids, fatty acids, or hydroxyphenylacrylic acid, or derivatives thereof.

DISCUSSION

SAAs are a class of highly heterogeneous hematological diseases that have complex etiologies and pathogenesis. The clinical symptoms often include fatal anemia, hemorrhage, and infection. SAA is considered to be an immune disorder, and in previous studies, the number of activated $CD8^+$ T cells has been shown to be higher in patients with SAA (Sheng et al., 2014; Xiao et al., 2017). Hyperfunctional T cells have a significant inhibitory effect on bone marrow hematopoiesis *in vitro* (Young et al., 2010). Gidvani et al. (2007) aimed to identify single-nucleotide polymorphisms (SNPs) in genes encoding cytokines associated with autoimmune diseases, such as interleukin 6 (IL-6), IL-10, tumor necrosis factor α (TNF-α), interferon γ (IFN-γ), and transforming growth factor β1, and found that SNPs, especially of the genes encoding TNF-α and IFN-γ, were present in patients with SAA, which suggests that these genes might be involved in the pathogenesis of SAA. Solomou et al. (2006) measured the number of Th1 and Th2 cells in the peripheral blood of patients with SAA before and after IST and found that the Th1/Th2 ratio was abnormal. The abnormally large number of Th1 cells is considered to play an important role in the immunopathogenesis of SAA (Du et al., 2013). Solomou et al. (2007) studied the Tregs of patients with SAA and found that the expression of FoxP3 is lower in $CD4^+CD25^+$ cells, which suggests that patients with SAA have poor immune tolerance. In our previous studies, we showed that the mDCs of patients with SAA are hyperfunctional (Zonghong et al., 2011), that there were fewer natural killer cells in such patients, and that the number of natural killer cells recovered after IST (Liu et al., 2014). Therefore, SAA is an autoimmune disease characterized by hyperfunctional T lymphocyte–mediated bone marrow damage, which is associated with poor hematopoiesis and immune tolerance. However, the etiology of the defects in the immune system is unclear.

It has recently been shown that metabolism and the intestinal microbiota play important roles in autoimmune diseases. Small-molecule metabolites generated by gut bacteria have biological activities and affect host health (Nicholson et al., 2012). Metabolomic studies of the differences in metabolite concentrations that are associated with internal and external factors have aided understanding of the pathophysiology of diseases including systemic lupus erythematosus (Yan et al., 2016), rheumatoid arthritis (RA) (Narasimhan et al., 2018), and systemic sclerosis (Bengtsson et al., 2016).

In the present study, patients with SAA and healthy controls had differing intestinal microbial compositions. Specifically, *Enterobacteriales* was more abundant in the intestine of patients with SAA. Some previous studies have shown that larger numbers of *Enterobacteriales* are associated with poor intestinal barrier function, which permits antigens from the diet or bacteria to enter the circulation from the intestine and activate the immune system (Pedersen et al., 2018). It has been speculated that *Enterobacteriales* may be involved in the immune and metabolic defects involved in SAA. We also found larger numbers of *C. citroniae* and *C. cateriicrmis* and smaller numbers of *R. faecis*, *C. eutactus*, *C. clostridioforme*, *L. ruminis*, and *Dorea formicigenerans* in the intestines of patients with SAA. The

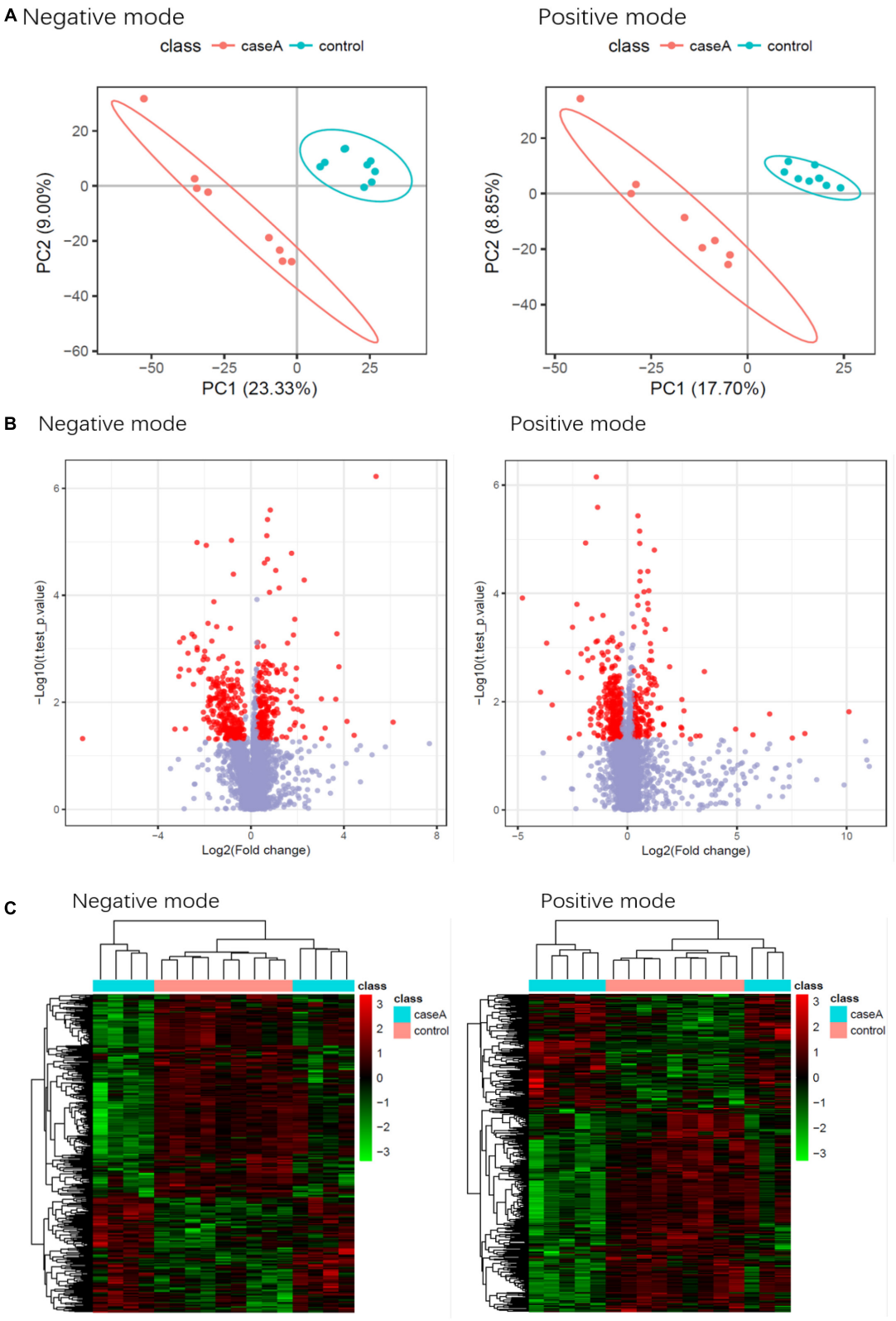

FIGURE 10 | Results of the metabolomic comparison of patients with SAA and healthy controls in both positive and negative mass spectrometric ion modes. **(A)** PLS-DA, **(B)** Visual volcanic map, **(C)** heatmap.

TABLE 3 | List of metabolites that were present at differing plasma concentrations in the SAA and healthy control groups.

Description	Molecular formula	m/z	Ion mode	Matching degree	Fold change
Coumaric acid	$C_9H_8O_3$	182.08	ESI^+	93.6	1.26
L-Phenylalanine	$C_9H_{11}NO_2$	188.07	ESI^+	59.7	1.68
Sulfate	H_2O_4S	96.96	ESI^-	86.9	1.36
L-Glutamic γ-semialdehyde	$C_5H_9NO_3$	132.07	ESI^+	68.2	0.80
Theobromine	$C_7H_8N_4O_2$	181.07	ESI^+	54.9	0.08
3a, 7a-Dihydroxy-5b-cholestane	$C_{27}H_{48}O_2$	405.37	ESI^+	54.7	0.52
γ-δ-Dioxovaleric acid	$C_5H_6O_4$	129.02	ESI^-	82.2	0.59
9, 10-DHOME	$C_{18}H_{34}O_4$	313.24	ESI^-	53.6	0.57

differences in the abundances of *C. citroniae* and *R. faecis* were particularly marked. *Roseburia* species, including *R. faecis*, can produce short-chain fatty acids (SCFAs), and especially butyrate. It has previously been shown that SCFAs have anti-inflammatory effects through regulation of immune cell chemotaxis and reactive oxygen species release, which inhibits the production of the proinflammatory molecules TNF-α, IL-1β, and nitric oxide, and the activity of nuclear factor κB. In addition, butyrate inhibits the production of IL-2 and lymphocyte proliferation (Säemann et al., 2000; Cavaglieri et al., 2003; Ni et al., 2010), maintains intestinal health and immune defense, participates in the regulation of Tregs, and plays an important role in the maturation of the immune system (Jost et al., 2013). It can also reduce injury to the colon and the symptoms of inflammatory bowel disease (Tamanai-Shacoori et al., 2017). Furthermore, butyrate generated by gut microbes can also enter the circulation and improve myeloid hematopoiesis. There is also evidence that *Roseburia* can produce bacteriocin-like substance, which is a polypeptide that has antibacterial activity and helps prevent infection with a variety of pathogens (Hatziioanou et al., 2013). Thus, a lower abundance of *R. faecis* might lead to greater production of proinflammatory factors in SAA. *C. citroniae* is a pathogen that is associated with bacteremia and celiac infection. Therefore, the greater abundance of *C. citroniae* may imply that patients with SAA are at a higher risk of infections that would activate the immune response. Taken together, these findings imply that the abnormal immune response that characterizes SAA might be ameliorated by measures that affect the composition of the intestinal microbiota.

The results of species association analysis in the two groups showed that there was strong competition between *P. copri* and *C. citroniae*, which may have implications for the immune system. *P. copri* is a member of the *Bacteroidetes*, which can produce succinate, a metabolic regulator and participant in proinflammatory responses. Previous studies have shown that the relative abundance of *P. copri* in the intestinal tract of patients with RA is high, but that in early RA it is lower than that in healthy individuals (Maeda et al., 2016). Therefore, the importance of *P. copri* in patients with RA requires further study. In addition, it has been shown that the relative abundance of *P. copri* is lower in the intestines of patients with psoriasis, which may be related to their abnormal immunity (Tamanai-Shacoori et al., 2017). In the present study, the relative abundance of *P. copri* was lower in the SAA group than in the healthy control group, which may have influenced the immune system of the SAA group. The lower abundance of *P. copri* and the higher abundance of *C. citroniae* broke the equilibrium competition relationship, which may also have had an influence on the immunity of patients with SAA.

Alpha diversity analysis showed that the composition of the microbiota of patients with SAA differed from that of normal controls, but probably owing to the small sample size, a significant difference was not identified. Therefore, future studies should be conducted that recruit larger numbers of patients and controls.

Previous metabolomic studies have generated insights into hematological diseases (Chen et al., 2014). In this previous study, a prognosis risk score was created using six metabolite markers that are indicative of upregulation of glycolysis and the tricarboxylic acid cycle, and an upregulation of glycolysis contributes to a lower sensitivity to cytarabine. In the present study, we found that L-phenylalanine, coumaric acid, and sulfate were present in higher concentrations in the plasma of patients with SAA, which suggests that these metabolites may be useful for the characterization of SAA. Phenylalanine is an essential aromatic amino acid that is necessary for the synthesis of neurotransmitters and hormones. However, high concentrations of phenylalanine are neurotoxic and increase the risk of cardiovascular disease (Wurtz et al., 2015), although the mechanisms involved have yet to be characterized. In the present study, the plasma phenylalanine concentration was high in patients with SAA, and this might have been responsible for bone marrow hematopoietic stem cell damage.

CONCLUSION

In conclusion, the plasma concentrations of certain metabolites and the composition of the intestinal microbiota are altered in patients with SAA. The abnormalities in the metabolites may be associated with the intestinal dysbacteriosis and might indicate potential molecular mechanisms for the immune defects that characterize SAA. Furthermore, these substances might represent candidate metabolic markers of SAA and/or suggest novel therapeutic targets.

DATA AVAILABILITY STATEMENT

The microbiome sequence data have been deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under BioProject PRJNA524870. The gene expression data generated by the NanoString analysis has been deposited in the GEO database under the accession number GSE127753.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Medical Ethics Committee of General Hospital of Tianjin Medical University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Gut Microbiota-Mediated Modulation of Cancer Progression and Therapy Efficacy

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The role of gut microbiota in the development of various tumors has been a rising topic of public interest, and in recent years, many studies have reported a close relationship between microbial groups and tumor development. Gut microbiota play a role in host metabolism, and the positive and negative alterations of these microbiota have an effect on tumor treatment. The microbiota directly promote, eliminate, and coordinate the efficacy of chemotherapy drugs and the toxicity of adjuvant drugs, and enhance the ability of patients to respond to tumors in adjuvant immunotherapy. In this review, we outline the significance of gut microbiota in tumor development, reveal its impacts on chemotherapy and immunotherapy, and discover various potential mechanisms whereby they influence tumor treatment. This review demonstrates the importance of intestinal microbiota-related research for clinical tumor treatment and provides additional strategy for clinical assistance in cancer treatment.

Keywords: gut microbiota, cancer, metabolism, gut immunity, immunotherapy, chemotherapy

IMPACT OF INTESTINAL MICROBIOTA ON TUMORIGENESIS

The homeostasis of the intestinal microbiota plays an important role in the normal physiological activities of the hosts, and microbiome imbalance greatly promotes tumorigenesis. In the past decade, substantial progress has been made in the investigation of the relationship between tumorigenesis and the role of microbiota. Investigations have shown that causes of cancer are associated with obesity, cardiovascular disease, type 2 diabetes, and carcinogenic chemicals, which are proven affected by the microbial community (Viennois et al., 2017; Cremonesi et al., 2018; Qin et al., 2018; Wu et al., 2019). Moreover, intestinal microbiota play a role in the pathogenesis of colorectal cancer. Studies have shown that microbiota indirectly affect the occurrence and development of colorectal cancer through inflammation and immune response (Honda and Littman, 2016; Chiaro et al., 2017). At present, the pathogenesis of colon cancer is mainly affected by epithelial gene mutation, mucosal integrity, intestinal microbiota, and inflammation. Gallimore and Godkin (2013) describe, in detail, the intestinal microorganism-mediated carcinogenic model and propose that the damage to the intestinal mucosal barrier integrity is key in the occurrence and development of colon cancer (Grivennikov et al., 2012; Gallimore and Godkin, 2013). Due to the destruction of the intestinal mucosal barrier, bacteria and their metabolites in the intestinal cavity are translocated to the lamina propria through the intercellular space of the epithelial cells,

thus triggering an adaptive inflammatory response and the release of cytokines, such as IL-1, IL-6, and IL-23. This will activate downstream Th17 cells, promoting the release of IL-17 and further activating the inflammatory and proliferative pathways of epithelial cells, such as the STAT3 and NF-κB pathways, in turn, promoting cancer cell proliferation and invasion. This results in further destruction of mucosal integrity, aggravation of inflammatory reaction, and repeated inflammation of the colon, which directly factors in the occurrence and development of colon cancer (**Figure 1**) (Gallimore and Godkin, 2013; Chen et al., 2017; Dai et al., 2019).

The mucous layer is the first line of defense of the intestinal mucous barrier. Most of the microbial symbiosis in the human body occurs in the intestinal epithelial barrier, which greatly influences intestinal health. Researchers have found that microbes promote the normal structure and function of the intestine in aseptic mice, that is, the intestinal mucosa of aseptic mice is considerably thin, and intestinal epithelial cell proliferation is significantly reduced. Moreover, the production of mucin and other intestinal epithelial cell derivatives is also impaired. The thinner mucin layer negatively affects the protective function of the epithelial barrier, which allows contact between the host's intestinal epithelium and exogenous substances, making it more vulnerable to foreign chemicals and pathogenic microbiota, thereby increasing the risk of colon cancer (Allaire et al., 2019).

The outer mucosal later is the habitat of symbiotic microbiota and the nutrient source of some microbiota. The microbial species inhabiting, and physiological functions of, the outer mucosal layer can affect the composition and structure of microbiota in the intestinal cavity. Nineteen strains from the phyla Bacteroidetes, Firmicutes, Actinomycetes, and Verrucobacteria have mucin-degrading ability (Tramontano et al., 2018); these strains mainly include *Akkermansia muciniphila* (Geerlings et al., 2018), *Barnesiella intestinihominis* (Desai et al., 2016), *Bacteroides thetaiotaomicron* (Xu et al., 2003; Comstock, 2009), *Bifidobacterium bifidum* (Turroni et al., 2011), *Bacteroides fragilis* (Hecht et al., 2017), *Bacteroides vulgatus* (Png et al., 2010), *Ruminococcus gnavus* (Owen et al., 2017), and *Ruminococcus torques* (Halmos et al., 2015). In addition to the physical mechanical barrier, the intestinal mucus layer also has an immuno-barrier function. Pathogenic microbiota are sensed by various types of pattern recognition receptors and antigen-presenting cells, and the colonic mucus is a repository of Immunoglobulin A (IgA). There are also some bactericidal proteins in the mucus that directly kill displaced microbiota (Xu et al., 2015; Honda and Littman, 2016; Chiaro et al., 2017; Allaire et al., 2019).

In addition to the direct effect on the intestinal tract, intestinal microbiota can indirectly affect tumor occurrence by influencing inflammation, immune function, and systemic metabolic function, in addition to their direct effect on the intestinal tract. Zhang Guodong's team found that triclosan (dichlorophenoxy chlorophenol) could change the intestinal microorganism composition to reduce intestinal micromicrobiota diversity and the abundance of beneficial bacteria (*Bifidobacterium*, a known bacteria with anti-colitis), thereby promoting tumorigenesis through the development of colitis. Experimental results have shown that triclosan can disrupt the intestinal barrier function in the body, increase immune cell infiltration, cause the transfer of TLR4 ligands (such as lipopolysaccharides and other bacterial products) from the intestinal tract to the systemic circulation,

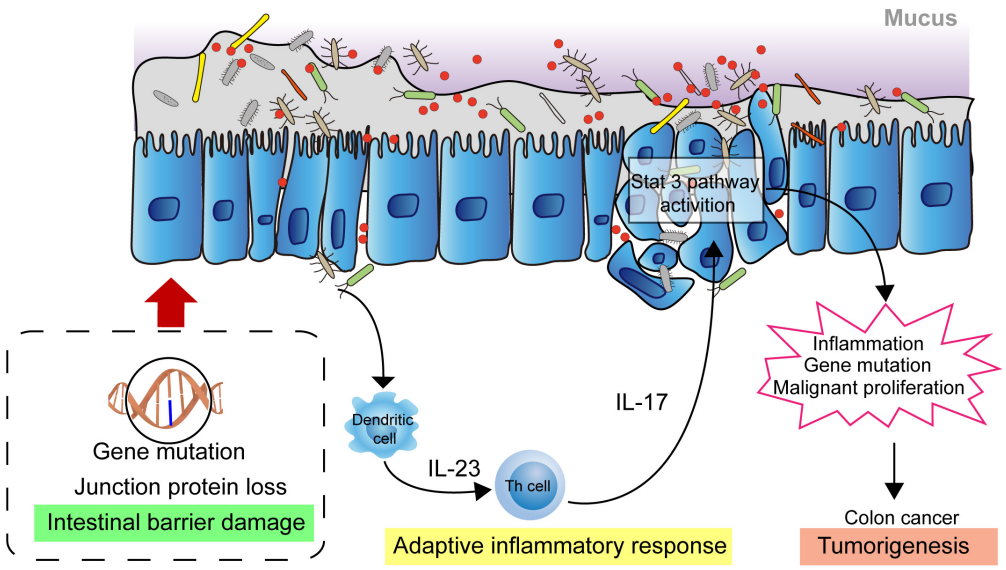

FIGURE 1 | Intestinal microbial facilitating colon cancer progression. The damaged intestinal mucosal barrier permits the transposition of intestinal bacteria and metabolites from the lumen to the lamina propria which activates adaptive immune response. The releasing cytokines drives development of Th17 cells response. The response activates the STAT3, NF-κB signaling pathway in epithelial cells, promoting cell proliferation and invasion, leading to further destruction of mucosal integrity and increased inflammatory response. These biological events contribute to the development of colon cancer.

and promote the activation of TLR4 signal, thus promoting the occurrence and development of colitis and colon cancer (Thaiss et al., 2016; Yang et al., 2018).

INTESTINAL MICROBIOTA AND THE HOST'S CO-METABOLISM REGULATE TUMORIGENESIS AND TUMOR PROGRESSION

The intestinal microbiota composition grows and develops with the host and is affected by the complex interaction of the host genome, nutrition, and lifestyle. Intestinal microbiota is involved in the regulation of various host metabolic pathways, which affects the co-metabolism of host-micromicrobiota interaction (Nicholson et al., 2012). The host will rely on gut microbiota to increase the production of digestive and metabolic enzymes. Intestinal microbiota produce various metabolic components, which include fermentation products of undigested food and endogenous compounds produced by the host, to construct a natural metabolite microenvironment-intestinal epithelial mucus layer, so that microbial metabolites can enter and interact with the host cell to affect the host immune response and disease occurrence and development (Rooks and Garrett, 2016). The microbial community in the human body can prevent pathogen growth by producing beneficial microbial metabolites; however, the imbalance of metabolites in the body will result in side effects that induce the occurrence and development of cancer (Del Carmen Martínez-Jiménez et al., 2018). Further research on the co-metabolism of host and microbiota is required to optimize treatments that control the intestinal microbiota and is a potential treatment strategy in the prevention and cure of various diseases.

The Impact of Intestinal Microbial Metabolites on Cancer Progression

The intestinal microbiota can use substances that cannot be digested by the small intestine, such as dietary fiber, and certain undigested sugars, proteins, and peptides. Consequently, the intestinal microbiota can also cooperate with the human body to form intestinal microbiota-host co-metabolites, such as short-chain fatty acids (SCFAs), vitamin H, and vitamin K (Lokody, 2014). These metabolites can be used as energy materials for intestinal microbes and can be transported to other parts of the body to stimulate cell growth, inhibit harmful microorganism growth, and participate in disease defense (Wu et al., 2016). When the composition, ratio, and quantity of the intestinal microbiota undergo pathological changes, it can lead to corresponding changes in the content and types of metabolites related to the microbiota, affecting the physiological functions of the host, and promoting the occurrence and development of tumors (Han et al., 2018). Therefore, changes in intestinal microbiota metabolites have important potential in tumor disease diagnosis.

Short-Chain Fatty Acids (SCFAs)

SCFAs are mainly produced by the microbial fermentation of undigested food, and its products are mainly acetate, propionate, and butyrate. Among the three major SCFAs, butyrate is the most important component that maintains colon health; it is primarily used as a direct energy source for colon cells. SCFAs plays a beneficial role in human intestinal health and affect colon health through various mechanisms. *In vivo* and *in vitro* studies have shown that SCFAs have anti-inflammatory and anticancer effects and plays an important role in maintaining the homeostasis of colon cell metabolism, protecting colon cells from external damage (McNabney and Henagan, 2017).

SCFAs can have a direct effect against cancer by inhibiting histone deacetylase (HDAC) and activating G protein-coupled receptors (GPCRs). HDAC inhibition by SCFAs is related to cell cycle arrest. Moreover, the microarray analysis of human colonic epithelial cells reveals that most butyrate-related genes are indeed involved. Butyrate can reduce the expression levels of the anti-apoptotic gene Bcl-2 and the pro-apoptotic protein Bax. Further, butyric and propionic acids can promote adenoma and cancer cell apoptosis by stimulating the expression of the cell cycle regulatory genes p53 and p21. Finally, butyric and propionic acids can promote cancer cell differentiation, inhibit colon cancer cell migration *in vitro*, and reduce the invasiveness of colon cancer cells. In addition, both activated GPR43 and GPR109a have anti-tumor effects by inhibiting proliferation and promoting the apoptosis of colon cancer cells, not related to HDAC inhibition (van der Beek et al., 2017). Furthermore, the high expression of SCFAs receptors in immune cells has indicated that SCFAs affect the expansion and production of Treg cells through the inhibition of SCFA-GPCR or HDAC, participating in intestinal immune regulation, and regulating and colon cancer development (El Kaoutari et al., 2013; Johansson et al., 2015).

Based on the above analysis, the role of SCFAs seems contradictory. It could provide energy for the growth and proliferation of normal cells, and inhibit cancer cell proliferation. However, current studies have shown that butyric acid generally could not reach the crypt cells, mainly providing energy for the colon cells in the anterior segment of the crypt. While the crypt structure is destroyed in the state of colon cancer, butyric acid could mediate the inhibitory effect of colon stem cells on colon cancer cells. SCFAs produced by intestinal microbiota play an active role in maintaining the normal state of the body, based on the cooperation between micromicrobiota and the body. Propionic and butyric acids may be involved in the above-mentioned tumor inhibition pathway (Alexander et al., 2017). Exploring the addition of probiotics and prebiotics in preventing or treating cancer can provide new ideas for its clinical treatment.

Amino Acids and Their Derivatives

In the distal human gut, proteins and peptides have three possible prospects: assimilated by the microbiota; as the substrate of microbial alienation metabolism, wherein its products enter the host portal circulation; or as intermediates of extensive microbial crosstalk and fecal excretion (Krautkramer et al., 2021). To a large extent, the degree of amino acid metabolism of intestinal microbiota depends on the utilization of substrate and cavity environments. It has been reported that the bacterial fermentation rate of protein (relative to carbohydrates) is greater in higher colonic pH and lower carbohydrate utilization

(Krautkramer et al., 2021). SCFA production caused by the microbial protein degradation being significantly lower than that of carbohydrates. In addition, the decrease of organic acids leads to a higher pH value of the lumen, which in turn alters microbiota structure and function (Ratzke and Gore, 2018). In contrast, the low lumen pH, which is a result of the presence of SCFAs, is considered to inhibit bacterial proteases; eventually, fermentable carbohydrates drive bacterial growth, and bacterial protein assimilation then increases at the expense of fermentation (Stephen and Cummings, 1980; Birkett et al., 1996). To date, due to the complexity of intestinal microbial content, the complex interdependence between many hosts and these substrate metabolic pathways, and the technical limitations of metabolite source classification (host and microbiota), has limited research on intestinal microbial histone degradation to a certain extent (Sridharan et al., 2014). However, in recent decades, intestinal microbiota has recovered large amounts of energy from proteins and peptides that escape host digestion; this results in the synthesis of various bioactive compounds, some of which are potentially toxic, including SCFAs, bifunctional chelating agents, ammonia, phenol, indole, amines, sulfides, and N-nitroso compounds (Smith and Macfarlane, 1996).

Tryptophan is an essential amino acid that ismetabolized into indole derivatives, 5- hydroxytryptophan (5-HT) and kynurenines (kynurenine and its derivatives) by different pathways. Indolepropionic acid (IPA) inhibit the early development of breast cancer by acting on AHR and PXR (Sari et al., 2020), which is converted by *Bacteroides* spp., *Clostridium* spp., *Lactobacillus* spp., *Parabacteroides distasonis, Peptostreptococcus* spp. etc. (Dodd et al., 2017; Agus et al., 2018). Recent studies have shown that 5-HT enhances the activation of NLRP3 inflammasomes by acting on its ion channel receptor HTR3A and promotes tumor progression in colitis associated colorectal cancer mouse models (Li et al., 2021). The microbiota is also involved in regulating host 5-HT, and Some species grown in culture can produce 5-HT (Tsavkelova et al., 2006). Kynurenines are ligands for arylhydrocarbon receptor (AhR) ligands to promote cell migration and immune tolerance, thereby driving cancer progression (Cervenka et al., 2017). Indoleamine 2, 3-dioxygenase 1 (IDO1) is the rate-limiting step of tryptophan conversion to kynurenines. The gut microbiota, such as *Lactobacillus* spp., *Pseudomonas aeruginosa, Pseudomonas*, *Fluorescens* (Vujkovic-Cvijin et al., 2013; Agus et al., 2018), promotes the expression of IDO1, and IDO1 activity can also regulate the composition of the microbiome.

Microorganism-Mediated Host Metabolism Affects Tumor Progression

Secondary bile acid is a primary bile acid catalyzed by intestinal microbiota, which is a substance that is converted by dehydroxyl group by the de-binding reaction. Secondary bile acids have a potential DNA-damaging ability, that is, a carcinogenic effect (Cao et al., 2017). In people with obesity, the dominant *Clostridium* members in the intestines can convert primary bile acids (such as chenodeoxycholic acid and cholic acid) into secondary bile acids (such as lithocholic acid and deoxycholic acid), which have a high affinity for bile acid receptors that affect multiple metabolism-associated processes. Hepatic sinusoidal endothelial cells expressed chemokine CXCL16 (CXCR6 ligand) to regulate the accumulation of NKT cells. Intestinal microbiota then enzymatically modify primary bile acidsto secondary bile acids, which could affect the process: primary bile acids increased the expression of CXCL16, while secondary bile acids had the opposite effect (Ma et al., 2018). Intestinal bacteria with this enzymatic reaction capacity have BSH enzymes, including *Lactobacillus* (Wang et al., 2012), *Bifidobacterium* (Kim et al., 2004), *Clostridium* spp. (Kim et al., 2004), *Listeria* (Begley et al., 2005), and *Enterococcus* (Wijaya et al., 2004). In addition, secondary bile acids can directly or indirectly affect the composition of intestinal microbiota. Current studies have shown that reduced abundance of *Clostridium labile* has an inhibitory effect on secondary bile acids, which can prevent liver tumors in mice (Winston and Theriot, 2016).

IMPACT OF INTESTINAL MICROBIOTA ON TUMOR IMMUNOTHERAPY AND CHEMOTHERAPY

Spontaneous tumor remission in patients with severe bacterial infection has been reported for the past two centuries. Coley, a surgical oncologist from the United States, used *Streptococcus pyogenes* extract named Coley's toxin to treat tumor patients at the late nineteenth century. Approximately 30% of lymphoma and sarcoma patients were cured, thus opening the door to tumor immunotherapy. In the history of tumor therapy development, chemotherapy agents emerged as the mainstay of present tumor therapy (Hoffman, 2012). Exploring the combination of intestinal microbiota with chemotherapeutic drugs and the interaction mechanism between them can provide better treatment innovations in clinical settings.

Impact of Intestinal Microorganism-Mediated Chemotherapy Drugs on Tumor Treatment

Since the discovery of nitrogen mustard cytotoxicity in World War II, researchers have gradually developed cytotoxic chemotherapeutic agents, such as chemotherapeutic drugs. Nowadays, chemotherapeutic drugs remain as the main treatment of tumors in most clinics (Einhorn, 1985). Lehouritis et al. (2015) observed the potential effect of bacteria on the effectiveness of chemotherapeutic drugs against cancer cells *in vitro*, whereby the activities of 10 out of 30 tested drugs were found to be specifically inhibited by one or two bacteria, and the correlation analysis of HPLC and mass spectrometry revealed that bacterial contact leads to the biotransformation of drugs. Therefore, experimental results show a complex and dynamic interaction between chemotherapeutic drugs and microbiota.

Intestinal microbiota directly affect drug absorption and metabolism, and indirectly affect oral drug metabolism by regulating host gene expression. Compared with ordinary mice,

it was found that the expression of certain members of the cytochrome P450 (Cyp450) gene family increased in aseptic mice livers. The expression of proteins from the Cyp2a, Cyp2b, and Cyp3a families, which are involved in heterogeneous steroid metabolism was increased, but that of other cytochromes was decreased. The fatty acid and arachidonic acid metabolism associated with members from the Cyp4a family involves heterogeneous biosensing receptors and transcription factors, such as androgen receptor, aryl hydrogen carbon receptor, and P450 oxidoreductase, which regulate target gene overexpression. Interestingly, the colonization of microbiota taken from routinely cultured ordinary mice in aseptic mice can restore the normal expression of related genes, and probiotic use in aseptic mice can also improve certain gene expression. These gene changes accelerate the metabolism of multiple drugs in aseptic mice (Jourová et al., 2017; Li et al., 2017). The role of microbiota in regulating drug metabolism and detoxification was also indirectly proven. Therefore, the heterogeneity of the therapeutic effect and toxicity of drugs on tumor patients can be exhibited from the differences in the composition and activity of intestinal microbiota among individual patients (Björkholm et al., 2009; Selwyn et al., 2015). In addition to oral drugs, several injected drugs are metabolized in the liver, and then excreted into the intestinal tract through bile, in which they are further metabolized and reabsorbed in the intestinal microbiota' environment.

Additionally, intestinal microbiota are involved in the biochemical conversion of various drugs, including reduction, hydrolysis and functional group removal, such as N-oxide cleavage, proteolysis, denitrification, amine formation, hydrolysis, thiazole ring opening, and acetylation (Wilson and Nicholson, 2017). Microbiota also reduce drug absorption through physical binding and separation. At present, it has been shown that more than 40 types of exogenous chemicals (non-natural foreign chemicals) are metabolized by intestinal microbiota. However, among anticancer drugs, only misonidazole, a radiosensitizer, and irinotecan (also known as CPT-11), a topoisomerase I inhibitor for hydrolysis and depolymerization of methotrexate, are affected by intestinal microbiota (Haiser and Turnbaugh, 2013).

Most chemotherapeutic drugs have no specificity and generally produce significant toxicity for all cells and tissues exhibiting accelerated renewal (Sancho-Martínez et al., 2012). Platinum anti-tumor drugs, such as oxaliplatin and cisplatin, kill tumor cells by inhibiting DNA replication and targeting the cell membranes and mitochondria. In addition, they can cause severe enterotoxicity, nephrotoxicity, ototoxicity, and peripheral neuropathy. Chemotherapeutic drugs have a strong toxic effect, particularly on intestinal mucosal cells which show acute regeneration, and are damaging to intestinal barrier function, moreover, this damage causes microbiota and pathogens to enter the mesenteric lymph nodes and blood circulation, leading to septicemia and systemic inflammation (Hooper and Macpherson, 2010). Therefore, generating better methods to combine and operate biospecific molecules on the surface of the chemotherapeutic drugs in order to safely and effectively deliver the drugs to the tumor site is a limitation of the current research. The latest research uses genetic engineering to modify bacterial protoplasts and develop nano-vesicles without toxic outer membrane components, which can aid in the specific targeting of the tumor tissue by chemotherapeutic agents to improve drug safety and efficacy (Kim et al., 2017).

Briefly, intestinal microbiota play a critical role in drug metabolism, and the interaction between chemotherapeutic drugs and intestinal microbiota has a major effect. A significant reference for the development of chemotherapeutic drugs can be provided through a deeper understanding of the role and function of intestinal microbiota in the pathology and treatment of cancer.

The Effect of Intestinal Microbiota on Tumor Immunotherapy

Tumor immunotherapy kills cancer cells and inhibits their proliferation through artificial intervention and mobilization of the body's own immune system. It is the fourth tumor therapy after surgery, radiotherapy, and chemotherapy, with great potential for further development. Particularly, immune checkpoint inhibitors (ICIs), particularly CTLA-4 and PD-1 protein inhibitors, and adoptive cell therapy show good prospects in various tumor treatment.

Recently, ICIs have played an important role in tumor therapy. CTLA-4 inhibitor, the first immune checkpoint inhibitor, was discovered in 2011, with the approval of ipilimumab by the United States FDA. In 2014, nivolumab was approved as the first PD-1 inhibitor on the market worldwide. Within the next few years, a number of ICIs, including the antibody against PD-1 and PD-L1, were approved. The main indications include metastatic non-small cell lung cancer, melanoma, and urothelial carcinoma. At present, five PD-1 and PD-L1 antibodies have been approved by the US FDA, namely, nivolumab, pembrolizumab, atezolizumab, avelumab, and durvalumab (Clarke et al., 2018; Gong et al., 2018) (**Table 1**).

With immunotherapy development, it has been found that small molecule drugs have greater advantages that might be complementary and potentially synergistic to large biological molecules in the immune system (Weinmann, 2016) (**Table 1**).

In addition, the role of microbiota is essential in immunotherapy. In 2015, the Gustave Roussy Cancer Center and Laurence found that gut microbiota composition determines the effectiveness of cancer immunotherapy represented by ICIs. In 2017, the two teams proved that microbiota *in vivo* play a decisive role in immunotherapy based on a large-scale analysis of patients with different types of cancer treated with PD-1 inhibitors (Sivan et al., 2015; Vétizou et al., 2015; Gopalakrishnan et al., 2018; Routy et al., 2018).

However, certain problems remain in tumor immunotherapy, such as the uncertainty of the curative effect, the narrow application scope, and immune system-related complications. Therefore, finding an optimal approach to immunotherapy is an important direction of clinical research. In 2015, Vétizou et al. discovered anticancer immunotherapy by CTLA-4 blockade that relies on the gut microbiota. Since then, the relationship between tumor immunotherapy and intestinal microbiota has become a

TABLE 1 | List of ICIs.

Drugs	Mechanisms of action	Principal indication	Approval times	References
Monoclonal antibody				
1. Ipilimumab	CTLA-4	Unresectable or metastatic melanoma	2011	Billan et al., 2020
2. Nivolumab	PD-1	Melanoma and other indications	2014	Yau et al., 2020
3. Pembrolizumab	PD-1	Melanoma and lung cancer and other indications	2016	Pestana et al., 2020
4. Atezolizumab	PD-L1	Urothelial carcinoma	2016	Alhalabi et al., 2019
5. Avelumab	PD-L1	Metastatic merkel cell carcinoma and locally advanced or metastatic urothelial carcinoma	2017	Kim et al., 2020
6. Durvalumab	PD-L1	Locally advanced or metastatic urothelial carcinoma	2017	Kim et al., 2020
Small-molecule drugs				
7. AUNP-12	PD-1-PD-L1 interaction	Inhibition of Tumor growth and metastasis	–	Weinmann, 2016
8. Inhibitor of IDO	Kynurenine pathway	Inhibit immune evasion system of tumor cells	–	Mándi and Vécsei, 2012; Dounay et al., 2015; Gostner et al., 2015; Zak et al., 2015
9. Inhibitor of CD39 or CD73	Adenosine pathway	Enhances the effect of tumor vaccines during T cell activation	–	Bastid et al., 2013; Antonioli et al., 2014; Muller-Haegele et al., 2014; Young et al., 2014; Bhattarai et al., 2015
10. STING Activators	STING	Activates innate immunity and T cell recruitment factors	–	Dubensky et al., 2013; Fridlender et al., 2013; Gravekamp and Chandra, 2015; Wang et al., 2015
11. Toll-Like Activators	Toll-Like Receptors	Activates dendritic cells and natural killer cells	–	Hamm et al., 2009; Holldack, 2014; Mancini et al., 2014; Pradere et al., 2014
12. SyAM-Ps	Prostate-specific membrane antigen and Fc g receptor	Effectively recruits immune cells and acts as cytotoxic agents	–	McEnaney et al., 2014

popular research topic (Vesely and Schreiber, 2013; Bachireddy et al., 2015; Garrett, 2015; Segre, 2015; Topalian et al., 2015; Drewes et al., 2016; Tran et al., 2017).

Immune Checkpoint Inhibitor Responses May Be Affected by Gut Microbiota Composition

Clinical studies have confirmed that the progression-free survival and overall survival of patients with malignant tumors using antibiotics are significantly shorter than those of patients undergoing immunotherapy that did not use antibiotics, indicating the important role of the intestine in the tumor immunotherapy process (Derosa et al., 2018; Routy et al., 2018). Intestinal microbiota analysis revealed that the abundance of various types of *bifidobacteria* spp. increased significantly in mice with slow tumor growth, and anti-PD-1 therapy could have a significant effect. Useful microbiota in mice could be transferred to mice that lack them by fecal microbiota transplantation (FMT), or through feeding in the same nest. In addition, the anti-tumor effect of PD-L1 blockade was improved in mice that had an unfavorable intestinal microbiota through the oral administration of *Bifidobacterium*-containing probiotics. This effect predominantly benefits from the enhancement of DC maturation, which increases tumor-specific $CD8^+$ T cell activity (Sivan et al., 2015). After anti-CTLA-4 treatment, the richness of intestinal micromicrobiota in mice changed significantly, mainly manifesting as a relative increase in *Bacteroidales* and *Burkholderiales* abundance, and a decrease in that of *Clostridiales*. Oral administration of *Bacteroides fragilis* with cyclophosphamide (*Bacteroides thetaiotaomicron*) or cephalosporin (*Burkholderia cepacia*) could trigger Th1 reaction and promote DC maturation, thus improving the efficacy of anti-CTLA-4 therapy. In germ-free and specific-pathogen-free mice treated with broad-spectrum antibiotics, the effect of anti-CTLA-4 therapy was significantly reduced, which could reverse the trend of the dominant microbiota of patients by FMT (Vétizou et al., 2015).

Researchers from the University of Texas MD Anderson Cancer Center have found that intestinal microbiota regulate the response of melanoma patients to PD-1 immunotherapy (Beaver et al., 2018). Through fecal microbial sample analysis and the study of different intestinal microorganism functions in the metagenome of patients, they found that there was a significant difference in intestinal micromicrobiota composition between melanoma patients who responded, and did not respond, to PD-1 immunotherapy and the immune response to tumors was significantly enhanced. To verify the conjecture, they transplanted the relevant beneficial bacteria and the feces of the respondent into the sterile mice with melanoma, and the same result was obtained.

Several species of intestinal bacteria have been associated with enhanced efficacy of immune-checkpoint blockade (ICB). *Akkermansia muciniphil*a was correlated with increased immune cell infiltration in lung and kidney cancers, as $CCR9^+CXCR3^+CD4^+$ T cells were recruited to the tumor bed in an interleukin-12-dependent manner and the ratio of $CD4^+$ T cells to $CD4^+FoxP3^+$ T cells (Tregs) was increased (Routy et al., 2018). Oral administration of pasteurized

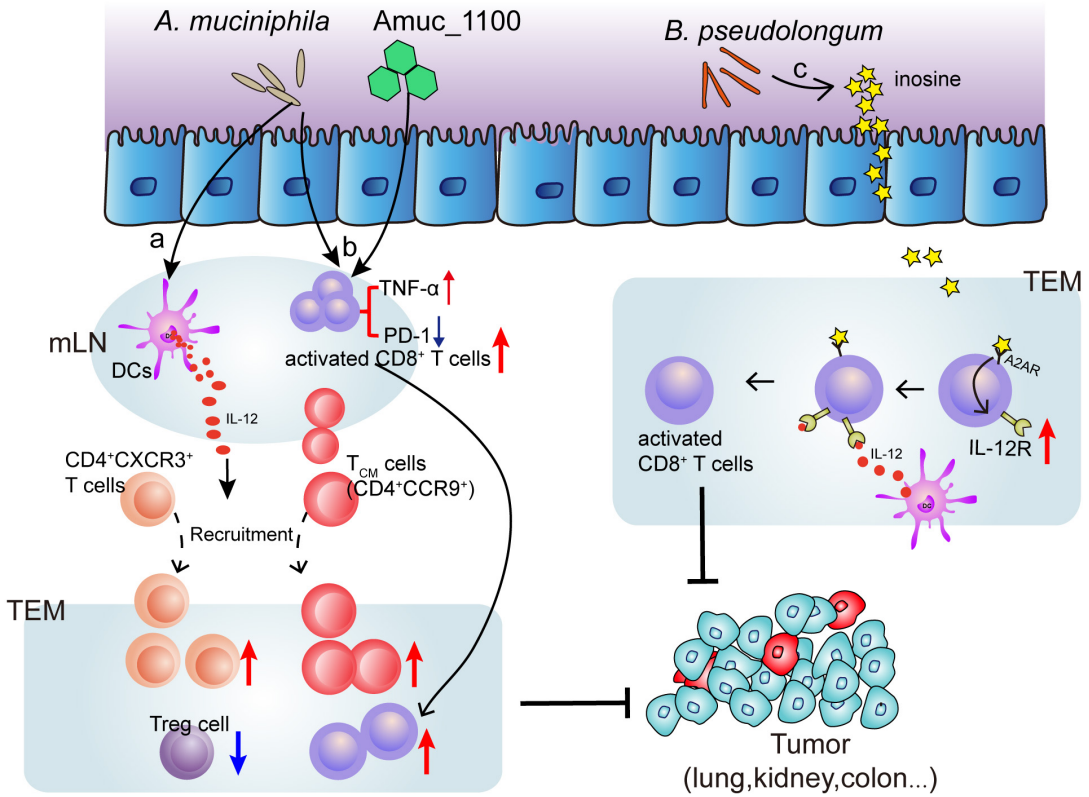

FIGURE 2 | Mechanism of bacteria (*Akkermansia muciniphila* and *Bifidobacterium pseudolongum*) induced ICB efficacy enhancement. **(a)** Oral supplementation with *A. muciniphila* accelerates IL-12 secretion by dendritic cells (DCs) leading to restoring the recruitment of $CXCR3^+CD4^+$ and $CCR9^+$ $CD4^+$ T lymphocytes into tumor microenvironment (TEM), so that restoring the efficacy of PD-1 blockade. **(b)** *A. muciniphila* or a A purified membrane protein (Amuc_1100) from *A. muciniphila* treatment activates $CD8^+$ T cells in mesenteric lymph nodes (mLN) and promote the recruitment of $CD8^+$ T cells into TEM, representing by TNF-α upregulation and PD-1 downregulation. **(c)** Inosine, the metabolite from *B. pseudolongum*, co-stimulates A2A receptor (A2AR) leading to increased expression of IL-12 receptor on $CD8^+$T cells, which further increases the T cell activation.

A. muciniphila or its purified membrane protein Amuc_1100 reduced colon infiltration of macrophages and cytotoxic T lymphocytes (CTL) and improve colitis in mice. Moreover, they increased the number of CTL in colon and mesenteric lymph nodes, up-regulate the expression of TNF-α, inhibit the expression of PD-1, and increase the activation of CTL, which has an inhibitory effect on colitis associated colorectal cancer in mice (Wang et al., 2020). Furthermore, Mager et al. (2020) isolated three bacterial species, including *Bifidobacterium pseudolongum*, *Lactobacillus johnsonii*, and *Olsenella species,* that significantly enhanced the efficacy of immune checkpoint inhibitors (anti-CTLA-4 and anti-PD-1). Notably, intestinal *B. pseudolongum* modulated enhanced immunotherapy response through the production of the metabolite inosine (Mager et al., 2020) (**Figure 2**). However, the mechanisms whereby the microbiome enhances anti-tumor immunity are unclear.

Intestinal Microbiota Can Cause Immune Checkpoint Blockade-Associated Toxicity

Immune checkpoint inhibitors enhance the activity of anti-tumor T cells and are widely used in cancer therapy, but also cause immune-related adverse reactions, especially in the gastrointestinal tract (Soularue et al., 2018). The effect of intestinal microbiota on toxicity was studied in animal models and clinical cohorts. The melanoma patients treated with anti-CTLA-4 were rich in *Bacteroidetes* and various genetic pathways, involving polyamine transport and B vitamin synthesis, and no colitis occurred. This lack of toxicity could be related to the known effects of these bacteria. Interestingly, the bacteria related to reaction and toxicity have different taxa, some of which have overlapping characteristics. According to related studies, there was a higher risk of colitis in patients with a higher abundance of *Faecalibacterium prausnitzii* and a lower abundance of *Bacteroides* after anti-CTLA-4 treatment (Chaput et al., 2017). However, other studies have shown that a greater abundance of *Ruminococcaceae* family (including *Faecalibacterium* spp.) have been found in patients who responded to ICIs therapy (Gopalakrishnan et al., 2018). In contrast, bacterial taxa related to a poor response to ICIs are included in the order Bacteroidales of Bacteroidetes; however, a higher abundance of these classifications usually reduces the incidence of toxicity. In addition, a series of studies have shown that the intestinal

microbiota can affect the therapeutic effects of immune checkpoints through a series of metabolites. Members of Firmicutes and *F. prausnitzii* produce SCFAs. These SCFAs enter the bloodstream through the damaged intestinal mucosa (Coutzac et al., 2020). It can reduce the effect of immune checkpoint suppression via the upregulation of CD80/CD86 on DCs and promoting the accumulation of tumor-specific T cells and memory T cells.

In summary, intestinal microbiota undeniably affect immunotherapy. Intestinal microbiota can regulate the anti-tumor immune response and the response to immune checkpoint inhibitors. These findings explain the therapeutic potential of intestinal microbiota in checkpoint blocking immunotherapy, which can greatly contribute to the treatment of tumors through further clinical and experimental studies (Gopalakrishnan et al., 2018).

The Role of Adoptive Cellular Immunotherapy Affected by the Intestinal Microbiota

Since the FDA first approved CAR-T therapy in 2017, adoptive cell therapy (ACT) has become a hotspot in current tumor immunotherapy research due to its remarkable efficacy in treating various tumors. Due to the recent approval of ACT, there are relatively few clinical studies on its effects on the intestinal microbiota. In 2007, Paulos et al. (2007) first demonstrated that in mouse tumor models, intestinal microbiota can be induced to translocate to the mesenteric lymph nodes under the action of systemic radiotherapy; then, the efficacy of ACT was enhanced through TLR4 signal transduction. After antibiotic treatment in mice, the efficacy of ACT was greatly reduced. After supplementation with bacterial lipopolysaccharide, the anti-tumor response of ACT was enhanced (Paulos et al., 2007). This phenomenon was found in clinical studies of metastatic melanoma patients, and the efficacy of ACT was better in patients who received radiotherapy pretreatment. Further, Uribe-Herranz et al. (2018) reported that in the process of ACT for treating mouse tumors, the treatment efficacy in vancomycin-treated mice was significantly better than that of untreated mice. However, the effect of ACT after treatment with neomycin and metronidazole was lower than that of untreated mice. Compared with the treated group, there was no significant change; 16S rRNA sequencing of mouse feces found that several genes from the members of Bacteroidetes were significantly different following vancomycin treatment, including bacteria from *Bacteroides* and *Parabacteroides*. Further research found that the mechanism underlying the action of ACT may involve the increased abundance of systemic CD8α + DCs, which further promotes IL-12 expression and enhances the efficacy of ACT. In summary, these results revealed a certain correlation between the intestinal microbiota and the therapeutic effects of ACT; however, the specific microbiota that exert these effects remain unknown. With the continuous expansion of ACT-related research and the wide range of clinical applications, more in-depth research will reveal potential gut microbiota-related targets for improving the therapeutic effects of ACT.

SUMMARY AND OUTLOOK

With the in-depth study of intestinal microbiota, the correlation between intestinal microbiota and tumorigenesis will be revealed. The intestinal microbiota plays an important role in tumorigenesis and tumor progression, and simultaneously affects the effectiveness of chemotherapy. In recent years, increasing attention has been paid to the relationship between intestinal microbiota and tumor-related health problems, which mainly benefits from the rapid development of microtechnology, including the second and third generations of high-throughput sequencing, such as 16S rRNA sequencing and PCR-DGGE, that can determine the DNA of microbial specimens. Real-time fluorescence quantitative PCR was used to quantitatively study the intestinal microbiota and explore the microbial diversity. The bacteria in intestinal microbiota were obtained by anaerobic culture, and intestinal microorganism function was studied at the strain level. Fecal transplantation technology verifies the mechanism of intestinal microbiota through the forward and reverse experiment.

In current studies, the effects of intestinal microbiota on tumors and immunity have been observed; however, the mechanism underlying these effects remain unclear. In addition, there are still challenges associated with studying how intestinal microbiota regulation improves the effects of tumor immunotherapy. At present, the components of intestinal microbiota that are most conducive to promoting anti-tumor immune response remain unclear. Furthermore, there are various treatments to modify the intestinal microbiota, which need to be carefully tested in clinical trials. After fully understanding these interactions, optimization of intestinal microbiota regulation for enhancing the host's anti-tumor immunity and for potentially improving immune surveillance can be achieved.

At present, only a few types of bacteria have been reported to be related to cancer, and great potential exists to explore the relationship between microbiota and tumorigenesis and tumor progression. The technologies to study these aspects are improving with the emergence of new treatments, such as the above-mentioned fecal transplantation, i.e., transplanting feces from healthy people to patients, which can have a good therapeutic effect and broaden the ideas of clinical treatment.

In future research, with improvements in microtechnology and analytical technology, we believe that more cancer-related target microbiota will be discovered, providing new methods and ideas for the clinical treatment of malignancies.

AUTHOR CONTRIBUTIONS

ZW and YL: conceptualization and design the review, review final version approval, and funding acquisition. PC, RD, YS, and WC: bibliographic research. PC, PS, and YY: writing—original draft preparation. PS, PC, YS, and ZW: the table and figure design. WC and YL: supervision. All authors contributed to the article and approved the submitted version.

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