

Identification of specific gut microbes and their therapeutic potential in ameliorating systemic lupus erythematosus in a mouse model

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ABSTRACT

Aims: The gut microbiome significantly influences autoimmune diseases, including systemic lupus erythematosus (SLE). This study aimed to characterize the gut microbiome and metabolome in SLE and evaluate the therapeutic potential of specific microbial supplementation in MRL/lpr mice.

Materials and methods: MRL/lpr mice, a well-established model for SLE, were used to analyze gut microbiome changes before and after SLE symptom onset. 16S rRNA sequencing and GC-MS-based metabolic profiling were performed to identify key microbial species and associated metabolites. Selected microbes were supplemented in MRL/lpr mice for 10 weeks, and their effects on SLE symptoms and Th17/Treg balance were evaluated.

Key findings: *Eisenbergiella massiliensis*, *Lacrimispora saccharolytica*, and *Hungatella xylanolytica* were significantly decreased in MRL/lpr mice following the onset of SLE symptoms. These microbes were strongly correlated with specific metabolites, including 5-cholestanol, cholesterol, *p*-cresol, and indole. Supplementation with these microbes alleviated SLE symptoms and modulated the Th17/Treg balance.

Significance: This study highlights the critical role of gut microbiota in immune regulation and SLE symptom relief. Targeted microbial supplementation may serve as a novel therapeutic strategy for managing SLE.

1. Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease characterized by persistent inflammation and abnormal T cell responses to self-antigens, leading to tissue damage in various organs, including the kidneys, skin, and central nervous system [1,2]. The exact pathogenesis of SLE remains incompletely understood, but it is clear that a complex interplay of environmental, immunological, and genetic factors contributes to disease development [3,4]. These factors converge to trigger a cascade of immunological abnormalities within the immune system, leading to the generation of autoantibody-producing B cells, autoreactive T cells, and the abnormal production of pro-inflammatory cytokines [5]. This dysregulation ultimately manifests as widespread

tissue damage, a hallmark characteristic of SLE [6]. MRL/lpr mouse, have proven invaluable in analyses of the pathogenesis of SLE [7]. Although detailed research has been carried out, the pathogenesis of SLE is still not completely clear [8].

Metabolomics has advantages of high throughput and high sensitivity and is helpful to clarify the pathology of disease and the mechanism of drug action [9]. It provides insights into biochemical interactions between organisms and external compounds, and is increasingly used to identify biomarkers for SLE. Recent research has focused on serum, plasma, urine, and feces from SLE patients [10–13], revealing the potential of metabolic profiling to distinguish SLE and lupus nephritis from healthy controls [14,15]. Additionally, studies have examined metabolic changes in MRL/lpr mice, a model for SLE, using

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serum samples analyzed by gas chromatography–mass spectrometry (GC–MS) [16]. However, there has been limited analysis of fecal samples from these mice, particularly comparing those from the gastrointestinal tract to naturally occurring feces. This study aims to explore significant substances by analyzing feces from both sources and serum in 16 weeks MRL/*lpr* mice.

The human microbiome, a complex ecosystem of trillions of microorganisms residing within our bodies, has emerged as a focal point of scientific inquiry, particularly in its potential role in various diseases [17]. This intricate community of microbes, encompassing bacteria, archaea, fungi, and viruses, interacts with our cells and tissues, influencing a wide range of physiological processes [18]. Various research has unveiled a compelling link between microbiome dysbiosis, an imbalance in the composition or function of the microbiome, and the development and progression of diverse diseases [19]. Interestingly, recent studies are accumulating evidence suggesting that the gut microbiome might also contribute to the development and progression of SLE [20]. Indeed, studies have shown that people with SLE have a reduced diversity of their gut microbiome and an increase in certain types of microorganisms compared to healthy individuals [21,22]. These changes are believed to enhance immune system activity and contribute to inflammation. However, studies have not yet elucidated which gut microbiomes are responsible for the changes in the immune system in SLE patients. We have previously demonstrated that the gut microbiota compositions of Feces samples and GI tract samples differ [23]. Using Feces samples and GI tract samples, we have also developed our own unique method to select and evaluate disease-modulating candidate microorganisms through differential analysis between comparison groups [24]. Using this method, we aimed to identify specific gut microbes that modulate SLE.

In this study, we used MRL/*lpr* mice before and after symptom onset to identify potential diagnostic biomarkers by comparing the metabolome using GC–MS. Moreover, we selected potential therapeutic gut microbial strains by comparing the abundance of the gut microbiome and confirmed their efficacy in suppressing SLE symptoms.

2. Methods

2.1. Study design

All animal protocols were approved by the Institutional Animal Care and Use Committee of the Korea Basic Science Institute (KBSI-IACUC-23-36), and were conducted according to the Guide for the Care and Use of Laboratory Animals issued by the Laboratory Animal Resources Commission of that Institute. Ten female MRL/MpJ-*Fas*^{*lpr*} (MRL/*lpr*) mice at 5-week-old and ten MRL/*lpr* mice at 15-week-old were purchased from SLC (Shizuoka, Japan). The mice were housed in groups of five in cages in a specific-pathogen-free facility at the Animal Facility of Aging Science (KJ309) of Korea Basic Science Institute, with access to sterilized food and water ad libitum. The mice were housed under a 12-h light/12-h dark cycle, at a temperature of 22 ± 1 °C and humidity of 40–50 %. After one week of acclimation, their weight was recorded, and feces and urine were collected individually. Following 12 h of fasting, anesthesia was induced using 3 % isoflurane in 100 % oxygen, supplied through a rodent respirator. Blood was collected via retro-orbital sampling, and euthanasia was carried out by cervical dislocation while anesthesia was maintained. The abdomen was surgically opened to obtain the kidney, spleen, and samples from the gastrointestinal tract. The spleens were measured for size and weight and used in subsequent experiments. All procedures were performed in accordance with ethical guidelines for animal research.

For microbial efficacy evaluation experiments, twenty-five female 5-week-old MRL/*lpr* mice were purchased from SLC (Shizuoka, Japan). *Eisenbergiella massiliensis* (DSM 101499) and *Hungatella xylanolytica* (DSM 3808) were purchased from DSMZ GmbH (Braunschweig, Germany), and *Lacrimispora saccharolytica* (GMB 2344) was purchased from

GM Bank (Jeonju, Korea). The mice were divided into five groups ($n = 5$ per experimental group): CTRL (feeding saline solution), EM (feeding *E. massiliensis*), LS (feeding *L. saccharolytica*), HX (feeding *H. xylanolytica*) and Mix (feeding mixture of *E. massiliensis*, *L. saccharolytica*, and *H. xylanolytica*). After one week of acclimation, mice were administered an antibiotic/antifungal mixture, azithromycin (15 mg/kg), neomycin (25 mg/kg), ciprofloxacin (20 mg/kg), and miconazole (30 mg/kg) daily for 2 days to disrupt the balance of their endogenous microbiota [25]. Afterwards, the mice in each group were fed 100 µL of the corresponding microorganisms twice a day until the mice were 16 weeks old. To check for SLE symptoms, when the mice were 16 weeks old, blood and urine were collected, and the spleen and kidneys were checked after sacrifice, as described previously.

2.2. Analyses of biochemical parameters

Blood was centrifuged for 5 min at 3000 rpm to obtain serum. Albumin (ALB), urea-nitrogen (BUN), creatinine (CRE), glucose (GLU), high-density lipoprotein cholesterol (HDL—C), total cholesterol (TCHO), and uric acid (UA) levels were analyzed using an automated serum analyzer (FUJI DRI CHEM NX3500i, Fujifilm, Tokyo, Japan). Data was carried out using a student's *t*-test with SPSS version 29 (Chicago, IL, USA). Data were presented as mean $\pm$ standard error of the mean (S.E.M). The value of $p < 0.05$ was considered statistically significant.

2.3. Enzyme-linked immunosorbent assay

Blood was collected from the orbital sinus, and serum samples were stored at -20 °C until use. Serum levels of anti-double stranded DNA (dsDNA) IgG antibodies and IgG2a were measured using enzyme-linked immunosorbent assay (ELISA) kits (Condex #3031 and Bethyl Laboratories #E99–107, respectively). Absorbances were determined using a Sunrise ELISA microplate reader (Tecan, Switzerland). The levels of IL-10 and IL-17 A in the cultured supernatants of splenocytes were analyzed using ELISA. Briefly, capture antibodies (purified anti-mouse IL-10, IL-17A) were coated onto the plate overnight. The supernatants were then added and incubated at room temperature for 4 h. Subsequently, detection antibodies (Biotinylated anti-mouse IL-10 and IL-17A) were applied, followed by incubation with Avidin-peroxidase for 1 h at room temperature. Finally, 100 µL of ABTS containing 0.1 % H_2O_2 was added, and the absorbance was measured using a microplate reader (Molecular Devices, Sunnyvale, CA, USA). ELISA data were carried out using a student's *t*-test with SPSS version 29 (Chicago, IL, USA). Data were presented as mean $\pm$ standard error of the mean (S.E.M). The value of $p < 0.05$ was considered statistically significant.

2.4. Urine albumin and creatinine assays

Urine samples were collected using aseptic microtubes. Urine albumin and creatinine concentrations were measured using a mouse albumin ELISA assay (Bethyl Laboratories #E99–134) and a creatinine assay (R&D systems #KGE005), respectively, according to the manufacturer's instructions. Data was carried out using a student's *t*-test with SPSS version 29 (Chicago, IL, USA). Data were presented as mean $\pm$ standard error of the mean (S.E.M). The value of $p < 0.05$ was considered statistically significant.

2.5. Flow cytometry analysis of T cell populations on peripheral blood

For DNT analysis, RBCs were removed using an ACK lysis buffer after orbital blood collection. Fc receptor was blocked with anti-mouse CD16/32 and then stained with anti-Thy1.2-PE Cy7, anti-CD4-FITC, and anti-CD8-PE. Flow cytometry was performed using a FACS Canto II (BD Biosciences, San Jose, USA) and the data were analyzed using FlowJo software ver 10.8.1 (TreeStar, OR, USA). Cytometry analysis was carried

out using a student's *t*-test with SPSS version 29 (Chicago, IL, USA). Data were presented as mean $\pm$ standard error of the mean (S.E.M). The value of $p < 0.05$ was considered statistically significant.

2.6. Histological analysis

The kidneys were frozen in OCT (Optimal cutting temperature, Surgipath FSC 22, Leica, Wetzla, Germany) prior to histological analysis. Cryosectioned tissue (6 μ m) was prepared using a Freezing microtome (Amos Scientific, Melbourne, AU). The section was washed with PBS to remove OCT and then stained using an H&E stain kit (Hematoxylin and Eosin, Abcam, MA, USA) according to the manufacturer's instructions. The fluorescence image was stained with fluorescent-conjugated anti-Thy1.2-PE Cy7 antibodies (BD biosciences, NJ, USA) at 4 °C overnight. The fluorescence image was obtained by EVOS M7000 ($\times 10$ and $\times 40$ air objectives) and TCS NT5 (Leica, Germany) at the Korea Basic Science Institute (KJ302, Gwangju, Korea). Subsequently, the image was analyzed using ImageJ ver.1.53c and Celleste image analysis software ver.5.0 (Thermo, MO, USA). Histological analysis was carried out using a student's *t*-test with SPSS version 29 (Chicago, IL, USA). Data were presented as mean $\pm$ standard error of the mean (S.E.M). The value of $p < 0.05$ was considered statistically significant.

2.7. Flow cytometry analysis of Treg and Th17 populations on splenocytes

Splenocytes were stimulated with anti-CD3 ϵ (1 μ g/mL, BD biosciences, NJ, USA) for 96 h. Following overnight culture with Brefeldin A (1 μ g/mL, Sigma, MO, USA), the cells were stained with anti-mouse CD16/32 (Fc block, BD biosciences, NJ, USA), anti-CD4-FITC (BD biosciences, NJ, USA), and anti-CD25-APC Cy7 (BD biosciences, NJ, USA) for Treg and Th17 analysis. Intracellular staining was performed using the Foxp3/transcription factor staining buffer set (eBioscience, San Diego, CA) according to the manufacturer's instructions. The cells were then stained with anti-Foxp3-PE (BD biosciences, NJ, USA) and anti-IL-17A-PE (BD biosciences, NJ, USA), and analyzed using flow cytometry (FACS Canto II, BD Biosciences, San Jose, USA). The data were visualized using FlowJo software ver 10.8.1 (TreeStar, OR, USA). Cytometry analyses were carried out using a student's *t*-test with SPSS version 29 (Chicago, IL, USA). Data were presented as mean $\pm$ standard error of the mean (S.E.M). The value of $p < 0.05$ was considered statistically significant.

2.8. Fecal and serum Metabolomic analysis preparation

Lyophilized fecal samples were first homogenized and then measured in 20 mg portions, which were placed into 2 mL Eppendorf tubes. Following this, 1 mL of HPLC grade Methanol was added to each tube, and the contents were vortexed for 1 min. Subsequently, the samples underwent sonication for 10 min and were then centrifuged at 2530 g for five minutes at 10 °C. The resulting supernatant was filtered using polytetrafluoroethylene (PTFE) syringe filters (Whatman, Maidstone, UK). Finally, the filtrate solution (100 μ L) was dried under nitrogen. A derivatization process, informed by previously established protocols [26]. For derivatization, nitrogen-dried stool samples were treated with a mixture comprising 10 μ L of 2-fluorobiphenyl in pyridine, 30 μ L of 20,000 μ g/mL methoxylamine hydrochloride in pyridine, and 50 μ L of BSTFA (*N,O*-bis[trimethylsilyl]trifluoroacetamide; Sigma-Aldrich, St. Louis, MO, USA) containing 1 % trimethylchlorosilane. Additionally, 10 μ L of 4 mM 2-fluorobiphenyl (TCI America, Portland, OR, USA) was included as an internal standard. The mixtures were then subjected to incubation in a 65 °C water bath for 60 min.

Serum samples were thawed on ice and subjected to centrifugation at 13,000 g for 10 min at 4 °C. The resulting supernatant (100 μ L) was loaded onto an Amicon filter designed for proteins, which was washed with 0.1 N NaCl. Subsequently, 30 μ L of the filtered liquid was combined

with 120 μ L of cold methanol and vortexed for 1 min. The mixture was then sonicated for 10 min and centrifuged at 20,000 g for 10 min at 4 °C. The resulting supernatant was carefully transferred to a 1.5 mL vial (100 μ L) and dried under nitrogen.

For derivatization, 30 μ L of 20,000 μ g/mL methoxylamine hydrochloride in pyridine, 50 μ L of BSTFA (*N,O*-bis[trimethylsilyl]trifluoroacetamide; Sigma-Aldrich, St. Louis, MO, USA) containing 1 % trimethylchlorosilane, and 10 μ L of 2000 μ g/mL myristic-d27 acid (Tokyo Chemical Industry Co., Japan) in pyridine (Sigma-Aldrich, St. Louis, MO, USA) as an internal standard was added to the dried serum samples and the mixtures were incubated in 65 °C water bath for 60 min.

2.9. GC-MS analysis

For quality control (QC), a mixture containing an equal volume (10 μ L) of each sample was analyzed. GC – MS analysis was performed after the samples were transferred into vials with an insert according to previously described methods. An Agilent GC (7890A, Agilent Technologies, CA) model equipped with a DB-5 MS column (30 m $\times$ 0.25 mm I.D. $\times$ 0.25 μ m df, Agilent Technologies, CA), an MSD detector (5975C, Agilent Technologies, CA), and ChemStation software was used for the analysis. For quality control (QC), a mixture containing an equal volume (10 μ L) of each sample was analyzed. GC – MS analysis was performed after the samples were transferred into vials with an insert according to previously described methods. An Agilent GC (7890A, Agilent Technologies, CA) model equipped with a DB-5 MS column (30 m $\times$ 0.25 mm I.D. $\times$ 0.25 μ m df, Agilent Technologies, CA), an MSD detector (5975C, Agilent Technologies, CA), and ChemStation software was used for the analysis. Fecal samples initial oven temperature was set to 70 °C, subsequently increased to 75 °C (at a rate of 1 /min), then to 135 °C (at 5.63 °C /min), 141 °C (at 1 °C /min), and finally to 300 °C (at 5.63 °C /min). An autosampler (7683B, Agilent Technologies, CA) was used for injecting 1 μ L of the sample at a split ratio of 1:10. The helium gas flow rate was maintained at 1.0 mL/min; the voltage of the electron multiplier was set to 1553 V; and the auxiliary, MS source, and quadrupole temperatures were set to 280, 230, and 150 °C, respectively.

Serum samples initial oven temperature was set to 70 °C and then increased to 310 °C at 5 °C/min. An autosampler (7683B, Agilent Technologies, CA) was used for injecting 1 μ L of the sample at a split ratio of 5:1. The helium gas flow rate was maintained at 1.0 mL/min; the voltage of the electron multiplier was set to 1553 V; and the auxiliary, MS source, and quadrupole temperatures were set to 280, 230, and 150 °C, respectively.

Fecal and serum metabolites were identified by comparing their retention times and mass fragments with those in the Human Metabolome Database (HMDB; <http://www.hmdb.ca/>), Golm Metabolome Database (GMD; <http://gmd.mpimp.golm.mpg.de/>).

2.10. GC-MS data processing and statistical analysis

The data obtained from the GC-MS analysis were processed using MS-DIAL (version 4.90, alpha version, RIKEN, Japan), with metabolite identification following established protocols. Raw metabolite data were converted to Abf files using Abf Converter (<https://www.reifycs.com/AbfConverter/index.html>) for compatibility with MS-DIAL. To ensure accurate peak detection, the tetramethylsilane mass peak with masses of 73 and 147 was excluded. Parameters for peak detection included a retention time tolerance of 0.5 min and a mass-to-charge ratio (*m/z*) tolerance of 0.5 Da. Normalization was achieved by dividing each area intensity by the intensity of the internal standard (IS). Statistical analyses including the Mann-Whitney test, and *t*-test were conducted using SPSS software (version 28, IBM, Somers, CT). Orthogonal partial least squares discriminant analysis (OPLS-DA) was performed using SIMCA software (version 15.0; Umetrics, Umeå, Sweden). Preprocessing techniques such as mean centering, UV scaling, and Pareto scaling were applied, and permutation tests were conducted 100

times to validate the OPLS-DA models. For the generation of heat maps analysis, we utilized Metaboanalyst 6.0 (<http://www.metaboanalyst.ca/>).

2.11. Metabolome and microbiome correlation data processing and statistical analysis

Spearman correlation analysis was carried out using R software (version. 4.3.3, R Core Team, 2023) to examine the interrelations between the gut microbiome and metabolome that were selected as functional constipation-specific substances, and to compute the correlation coefficient between the differential metabolome and the gut microbiota. The results with an absolute correlation coefficient ($|r_{\rho}|$) >0.49 and a significance level (p -value) <0.05 were selected and imported into Excel. For the visualization of associations among the metabolome and gut microbiome, we created relevant network diagrams using Cytoscape 3.9.1 (National Resource for Network Biology, USA).

2.12. Fecal DNA extraction and 16S rRNA gene sequencing

To isolate genomic DNA from bacterial samples, samples were suspended in lysis buffer (200 mM NaCl, 200 mM Tris-HCl (pH 8.0), 20 mM EDTA) and subjected to bead-beating for cell disruption. Following this, genomic DNA was partitioned from the lysate into the aqueous phase using a mixture of Phenol:Chloroform:Isoamylalcohol. The DNA was subsequently precipitated with 3 M sodium acetate and isopropanol, rinsed with 70 % ethanol to remove impurities, and finally re-suspended in TE buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA) for further analysis.

Metagenome sequencing analyses of the gut microbiome DNA were processed and sequenced by a commercial company, ebiogen, Inc. (Republic of Korea). They employed the Illumina 16S Metagenomic Sequencing Library protocols. Briefly, this involved amplifying the 16S rRNA gene V3-V4 region using specific primers for each sample. To add multiplexing indices and Illumina sequencing adapters, a subsequent limited-cycle amplification step was performed. The amplified libraries were then normalized and pooled using PicoGreen, and size-verified using the Agilent TapeStation DNA ScreenTape D1000 system (Agilent Technologies, Santa Clara, CA, USA). Finally, paired-end sequencing was performed on the Illumina MiSeq platform (Illumina, San Diego, CA, USA). Sequence data was subsequently processed using DADA2 software (version 1.10.1) to account for potential amplicon errors [27].

2.13. Data and statistical analyses

Statistical analyses were performed using established protocols as previously described [28–30]. Briefly, the QIIME 2 platform was used for data processing and analysis. Following denoising and quality control, the taxonomic classification of sequencing data was performed using Q2-Feature classifier. Alpha-diversity metrics were calculated using the “q2-diversity” plugin with appropriate sampling depth. Normalized OUT data was then imported into R (version 3.6.1) using the phyloseq package (version 1.46.0) for visualization of alpha (within-group) and beta (between-group) diversity. Alpha-diversity comparisons were performed using the Kruskal-Wallis rank sum test. For beta-diversity analysis, Bray-Curtis dissimilarity matrices were generated from log-transformed OTU data using the vegan package in R. Non-metric multidimensional scaling (NMDS) was then performed to visualize community dissimilarities. The ADONIS test (vegan package) with 999 permutations was used to assess the statistical significance of factors influencing Bray-Curtis distances. Finally, unsupervised filtering with a 5 % abundance threshold identified the most abundant taxa for heatmap generation using average linkage hierarchical clustering and Bray-Curtis distance metrics.

2.14. Construction of phylogenetic tree and co-occurrence network

Phylogenetic trees and Co-occurrence networks were constructed for each experimental group following previously established methods [28–30]. To build the phylogenetic trees, taxa that could not be classified to the species level were assigned a new classification based on their NCBI accession numbers using the R package Taxonomizr (0.10.7). Subsequently, 16S rRNA sequences were aligned using default ClustalW parameters. These alignments served as the basis for constructing maximum-likelihood phylogenetic trees in MEGAX with 500 bootstrap replicates. Finally, all phylogenetic trees were visualized using the iTOL online platform.

To investigate microbial co-occurrence patterns for each experimental group, co-abundance networks were constructed using a permutation-renormalization-bootstrap approach. Unnormalized abundance data for each experimental group was uploaded separately to CoNet, a Java Cytoscape plugin. CoNet employed multiple ensemble correlation methods to identify significant co-occurrences of operational taxonomic units (OTUs) across samples. OTUs present in less than three samples were excluded from the analysis. The final network was generated by combining five similarity measures (Spearman and Pearson correlations, Mutual Information Score, Bray-Curtis dissimilarity, and Kullback-Leibler Dissimilarity) calculated by CoNet. P -values from these measures were merged using Brown's method and subsequently adjusted for multiple comparisons with the Benjamini-Hochberg correction (adjusted P -value < 0.05). Edges in the network represent significant co-abundance relationships between OTUs identified by at least two of the five similarity measures. Finally, the igraph package in R was used to visualize the co-occurrence network structure. This package employs the Louvain algorithm to identify network communities, optimizing the modularity score within each network.

2.15. Differential abundance analysis

Gut microbiomes with different abundance between groups before and after the onset of SLE symptoms were identified by DESeq2 and LEfSe methods. DESeq2 analysis was performed using DESeq2 software (version 1.42.0), as described previously [31,32]. Taxa with a presence in <0.5 % of samples were excluded from the analysis to ensure robust comparisons. LEfSe analysis was performed using microbiomeMarker software (version 1.8.0), following previously established methods [33].

3. Results

3.1. Comparison of SLE-mimic symptom expression according to age in MRL/lpr mice

MRL/lpr mice were used as an SLE model due to their manifestation of SLE-like characteristics, including splenomegaly, lymphadenopathy, and glomerulonephritis [34]. These mice exhibit age-dependent worsening of SLE symptoms, nearly normal before 6 weeks, showing increased autoimmune responses around 12 weeks, and developing severe symptoms after 16 weeks [35]. Prior to metabolome and gut microbiome comparisons, we confirmed SLE symptom onset in 16 weeks MRL/lpr mice (Fig. 1a). Body weight was higher in 16 weeks mice than in 6 weeks mice, which is expected to be due to age-related growth differences (Supplementary Fig. 1a). Serum biochemical analyses showed higher total cholesterol (TCHO) in 16 weeks group, while other markers, including albumin (ALB), urea-nitrogen (BUN), creatinine (CRE), and glucose (GLU), showed no significant differences (Supplementary Fig. 1b-h). One of the main symptoms of SLE is kidney inflammation, which is mainly caused by autoantibodies and immunoglobulins [36,37]. The severity of proteinuria, a key symptom of renal inflammation, was assessed in 6 weeks and 16 weeks MRL/lpr mice. The albumin/creatinine ratio (ACR) tended to be higher in the 16 weeks group than in the 6 weeks group, but the difference was not statistically

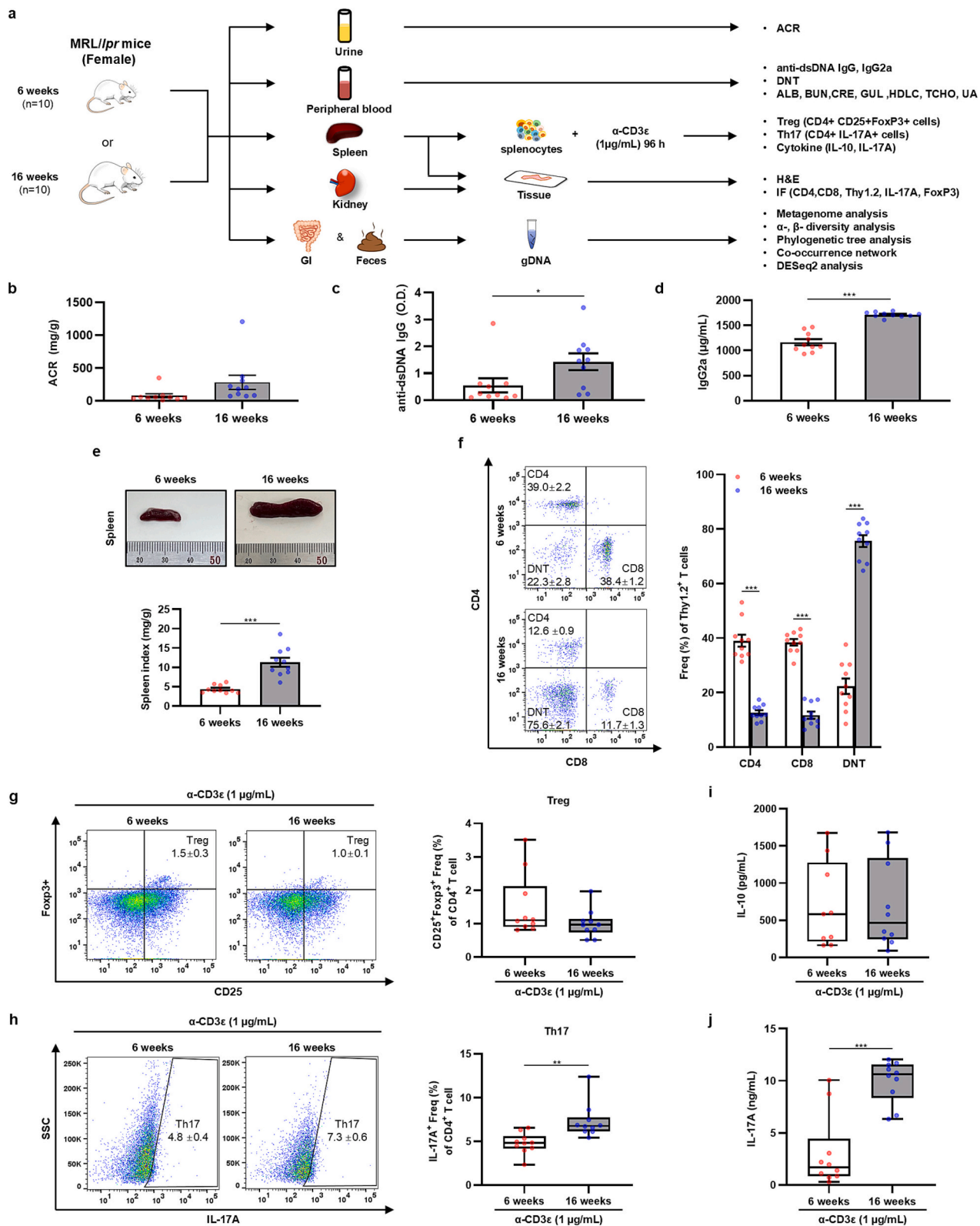


Fig. 1. Comparison of MRL/*lpr* mice before and after SLE onset. (a) The schematic diagram of the experimental strategy. (b) Albumin/creatinine ratio (ACR) measured from urine samples of MRL/*lpr* mice at the 6 weeks and 16 weeks group. (c) Levels of anti-dsDNA antibodies (total IgG) in the sera from MRL/*lpr* mice at the 6 weeks and 16 weeks group. (d) Levels of IgG2a in the sera from MRL/*lpr* mice at the 6 weeks and 16 weeks group. (e) Representative images of spleen and spleen index in MRL/*lpr* mice at the 6 weeks and 16 weeks group. (f) Flow cytometry was performed to analyze peripheral blood mononuclear cells obtained from MRL/*lpr* mice from preclinical (6 weeks, $n = 7$) to diseased (16 weeks, $n = 7$) stages. (g–j) They were analyzed for different T cell populations using flow cytometry. Inducing an imbalance of Th17 and Treg cells dependent on aging. Total splenocytes from MRL/*lpr* mice were cultured with stimulation from anti-CD3 ϵ antibodies (1 μ g/mL) for 96 h. Flow cytometry data showing the proportion of Treg (CD4 $^{+}$, CD25 $^{+}$, Foxp3 $^{+}$) (g) and Th17 (CD4 $^{+}$, IL-17A $^{+}$) (h) expressed as dot plots (left panels) and bar graphs (right panels). Levels of IL-10 (i) and IL-17A (j) in the supernatants were analyzed using the same conditions as the splenocytes in the flow cytometry data. The values represent the mean $\pm$ S.E.M. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

significant (Fig. 1b). Autoimmune symptoms were assessed through anti-dsDNA IgG and IgG2a levels [38,39], both of which were significantly elevated in the 16 weeks group (Fig. 1c,d). The spleen index, calculated as the spleen weight relative to body weight, was used to evaluate splenomegaly severity. The 16 weeks group exhibited a significantly higher spleen index than the 6 weeks group (Fig. 1e). The MRL/*lpr* mice exhibited pathological features such as splenomegaly, increased double-negative T cells (DNT) in the blood, an imbalance between Th17 and Treg, and renal lymphocyte infiltration at the time of SLE expression [40]. Histological kidney analysis showed normal tissue in 6 weeks mice, whereas immune cell infiltration and fibrosis were

observed in 16 weeks mice (Supplementary Fig. 2). Flow cytometry analysis revealed age-related changes in blood T cell populations. At 6 weeks, MRL/*lpr* mice exhibited 39.0 % CD4 and 38.4 % CD8 T cells, with only 22.3 % double-negative T (DNT) cells. In contrast, 16 weeks mice displayed a significant increase in DNT cells (75.6 %), while CD4 and CD8 T cells decreased to 12.6 % and 11.7 %, respectively (Fig. 1f). SLE exhibits an imbalance between Treg and Th17 cells due to a decrease in Tregs and an increase in Th17 cells, resulting in an immune-inflammatory response [41–44]. Splenocytes stimulated with anti-CD3 ϵ antibody for 96 h showed no significant difference in regulatory T cells (Tregs: CD4⁺, CD25⁺, Foxp3⁺) between the 6 weeks and 16 weeks

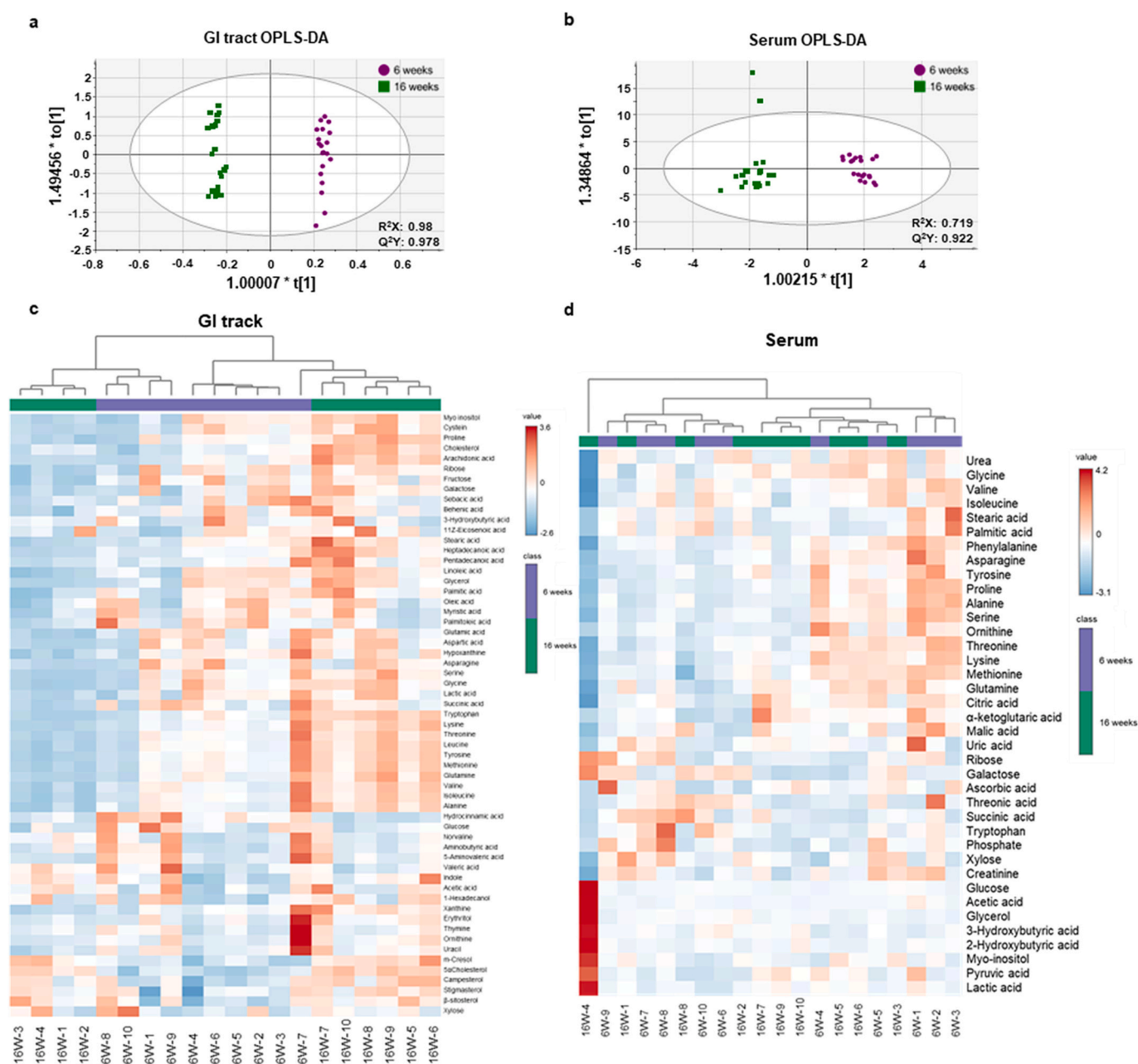


Fig. 2. OPLS-DA derived score plots, heatmap analysis and correlation network analysis of the 6 weeks and 16 weeks groups MRL/*lpr* mice. (a,b) OPLS-DA derived score plots of MRL/*lpr* mice GI tract samples (a) and serum samples (b) based on relative levels of identified metabolites in the 6 weeks and 16 weeks groups, respectively. Score vector (t) is displayed with the first and second predictive component as t [1] and t [2]. Predicted Y value plots (a) and (b) from sevenfold cross-validation for verification of the OPLS-DA model. The predicted Y values from the sevenfold cross-validation are shown as violet circle and green box and for the 6 weeks and 16 weeks groups, respectively. Misclassified samples are represented with a circle according to the threshold line value of 0.5 in the Y-axis. (c,d) Heatmap analysis of MRL/*lpr* mice GI tract (c) and serum (d) metabolites identified by GC-MS. Relatively high and low levels of metabolites are displayed in red and blue, respectively.

group, while Th17 cells (CD4⁺, IL-17A⁺) were significantly increased in 16 weeks mice (Fig. 1g,h). Corresponding cytokine analyses confirmed that IL-10 levels remained similar, but IL-17A were elevated in 16 weeks mice (Fig. 1i,j).

3.2. Metabolic profiling of GI-tract, fecal, and serum samples from MRL/lpr mice in relation to SLE progression

Metabolic profiling of gastrointestinal (GI) tract, fecal (Feces), and serum samples from the 6 weeks and 16 weeks groups of MRL/lpr mice were performed by GC-MS analysis. Orthogonal partial least squares discriminant analysis (OPLS-DA) revealed clear separation between two groups, indicating SLE-induced metabolic differences (Fig. 2a,b and Supplementary Fig. 3a). The OPLS-DA models were evaluated based on the statistical parameters such as R²X and Q²Y values. In addition, the results of the heat map analysis (Fig. 2c,d and Supplementary Fig. 3b) showed that the 16 weeks group had lower levels of 29 metabolites in GI tract samples (Fig. 2c), 33 metabolites in feces samples (Supplementary Fig. 3b), and 25 metabolites in serum samples compared to the 6 weeks group (Fig. 2d). Main SLE-specific metabolites were identified from the OPLS-DA derived VIP values (cut-off values >1 as follows; 14 metabolites including 1 amino acid, 4 fatty acids, 5 steroids, 1 sugar and 3 others in GI tract samples (Supplementary Table 1), 19 metabolites including 7 amino acids, 2 fatty acids, 1 organic acid, 2 sugar alcohols, 5 sugars, and 2 others in feces samples (Supplementary Table 2), and 16 metabolites including 10 amino acids, 1 fatty acid, 2 organic acids, 1 sugar alcohol, and 2 others in the serum sample (Supplementary Table 3).

3.3. Comparison of gut microbiome composition associated with SLE progression in MRL/lpr mice

The composition of cecal microbiota in MRL/lpr mice varies with age and SLE-like phenotype manifestation [45]. To examine gut microbial changes associated with SLE onset, GI tract and feces samples were analyzed (Supplementary Table 4). Microbiome composition was assessed at the phylum, class, and order levels, revealing that *Firmicutes*, *Clostridia*, and *Lachnospirales* were significantly lower in the 16 weeks group in GI tract samples (Fig. 3a-c; Supplementary Tables 5–7). However, no significant differences were found in fecal microbiome composition (Supplementary Fig. 4a-c). SLE-like phenotype Acquisition correlated with increased gut bacterial diversity. α -diversity analyses using Fisher's alpha (Fisher) and Shannon's index (Shannon) indicated higher richness and evenness in the 16 weeks group (Fig. 3d, Supplementary Fig. 4d, and Supplementary Table 8). β -Diversity analysis using non-metric multi-dimensional scaling (NMDS) plots showed distinct gut microbiota clustering between groups (Fig. 3e and Supplementary Fig. 4e). Phylogenetic analysis of 16S rRNA sequences revealed a slight increase in species diversity in the GI tract (177 in control vs. 179 in SLE) and a minor decrease in feces samples (230 in control vs. 231 in SLE) (Fig. 3f,g and Supplementary Fig. 4f,g). Co-occurrence network analysis showed that the gut microbiota community was more diverse in the 16 weeks group (34 communities in GI tract sample, 37 communities in feces sample) compared to the 6 weeks group (27 communities in GI tract sample, 31 communities in feces sample) (Fig. 3h,i, Supplementary Fig. 4h,i and Supplementary Table 9). Thus, it can be concluded that the gut microbiota community in the 16 weeks group became more imbalanced compared to the 6 weeks group.

3.4. Identification of gut microbial species with differences in abundance associated with SLE progression in MRL/lpr mice

After identifying the association between the gut microbiome and SLE, we aimed to determine specific gut microbial species associated with SLE suppression. The linear discriminant analysis (LDA) effect size (LEfSe) method was further used to compare bacterial abundances

between groups (Fig. 4a and Supplementary Fig. 5a). Twenty-two species in GI tract and feces samples respectively were identified as significant by both the Kruskal-Wallis test adjusted for multiple testing ($p < 0.05$) and the effect size analysis (LDA score > 3). Further, the associative relationship of SLE with gut microbiome at the species level was analyzed using the DESeq2 method (Fig. 4b, Supplementary Fig. 5b and Supplementary Table 10). DESeq2 analysis identified seven bacterial species in GI tract samples and eight bacterial species in feces samples that showed significantly different abundance between the 6 weeks and 16 weeks groups. In GI tract samples, *Hungatella xylanolytica*, *Lacrimispora saccharolytica*, and *Eisenbergiella massiliensis* were significantly enriched in the 6 weeks group, while in feces samples, *Kineothrix alysoides*, *Acetatifactor muris*, *Eisenbergiella massiliensis*, *Phocaeicola sartorii*, and *Anaeroplasmabactoclasticum* were enriched (Fig. 4b, Supplementary Fig. 5b, and Supplementary Table 10). However, when directly comparing the relative abundances of these species (Fig. 4c, Supplementary Fig. 5c, and Supplementary Table 10), only *Muribaculum intestinale* and *E. massiliensis* in GI tract samples, and *Clostridium saudiense*, *Turicibacter sanguinis*, *A. muris*, *E. massiliensis*, *P. sartorii*, and *A. abactoclasticum* in feces samples showed significant differences between the two groups. In both LEfSe and DESeq2 analyses, in GI tract samples, *E. massiliensis* and *L. saccharolytica* were abundant in the 6 weeks group. Similarly, in feces samples, *A. abactoclasticum*, *P. sartorii*, *E. massiliensis*, and *A. muris* were abundant in the 6 weeks group (Fig. 4 and Supplementary Fig. 5). These findings suggest that these microorganisms may contribute to suppressing SLE progression.

3.5. Correlation network analysis of gut microbiota and metabolites observed in GI tract and feces samples from MRL/lpr mice

Based on the clear changes in gut microbiome diversity and composition in GI tract samples compared to feces samples as observed in Fig. 3, we reasoned that GI tract samples may better reflect gut microbiome differences according to SLE symptoms. In addition, DESeq2 and LEfSe analyses of GI tract samples in Fig. 4 showed that *E. massiliensis*, *L. saccharolytica*, and *H. xylanolytica* were decreased with SLE onset.

We applied Spearman correlation analysis to the selected microbiome and metabolome and considered the correlation to be significant if $|\text{rho}| > 0.49$, $p < 0.05$. Based on our findings, we selected 7 metabolites (5-aminovaleric acid, 5 α -cholestanol, cholesterol, indole, oleic acid, palmitoleic acid, and *p*-cresol) and 25 gut microbes from the GI tract samples of the MRL/lpr mice (Supplementary Fig. 6a). We also selected 12 metabolites (3-hydroxybutyric acid, 5-aminovlaeric acid, acetic acid, arachidic acid, caproic acid, glycerol, glycine, hippuric acid, myo-inositol, phenylalanine, proline, and serine) and 39 gut microbes from feces samples (Supplementary Fig. 6b). *E. massiliensis* was negatively correlated with 5 α -cholestanol, indole, and *p*-cresol in GI tract samples. Acetic acid was negatively correlated in feces samples. *L. saccharolytica* was negatively correlated with cholesterol, and *H. xylanolytica* was negatively correlated with 5 α -cholestanol in GI tract samples (Table 1). Based on this evidence, we hypothesized that *E. massiliensis*, *L. saccharolytica*, and *H. xylanolytica* may have symptom-alleviating functions in SLE.

3.6. Mixed gut microbiota suppressed the onset of SLE-mimicking symptoms in MRL/lpr mice

We compared the gut microbiota of 6- and 16-week-old MRL/lpr mice to identify microorganisms that could potentially suppress SLE development. However, it remained unclear whether these microbial differences actively influenced SLE progression or simply reflected age-related changes. To clarify this, we administered the selected microorganisms to MRL/lpr mice and assessed their effects on SLE symptoms. Fifty 6-week-old MRL/lpr mice received antibiotics and antifungals for two days before being divided into five groups: saline control (CTRL),

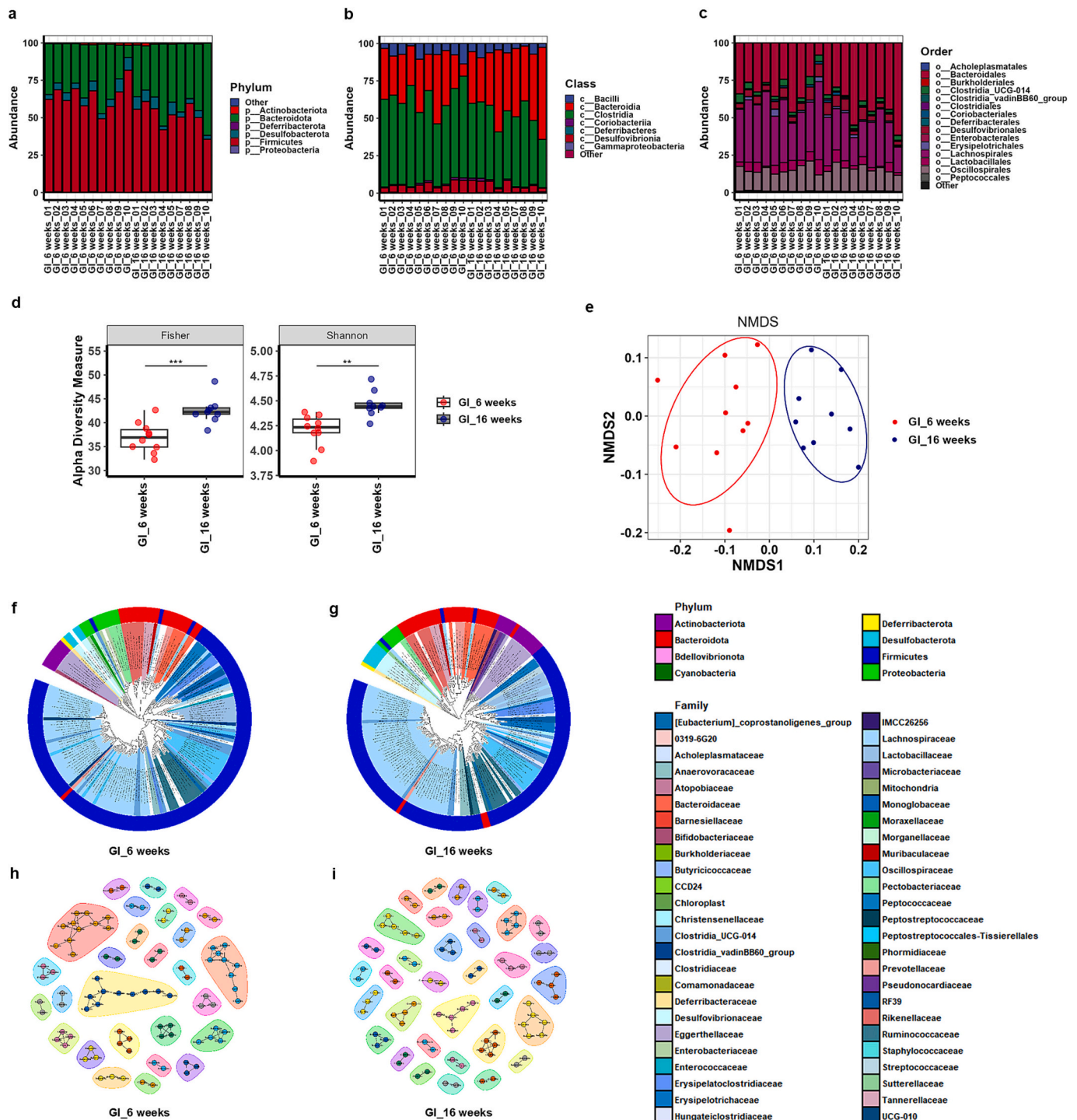


Fig. 3. Comparison of GI tract gut microbial diversity of the 6 weeks and 16 weeks groups MRL/*lpr* mice. (a-c) Comparisons of the composition of the gut microbiome of the 6 weeks and 16 weeks groups MRL/*lpr* mice. The composition of GI tract gut microbiomes of each group was compared at the phylum (a), class (b), and order (c) levels. (d) The α -diversity index of GI tract gut microbiome of the 6 weeks and 16 weeks groups MRL/*lpr* mice was measured using the Fisher and Shannon methods. (e) The β -diversity plot of the GI tract gut microbiome of the 6 weeks and 16 weeks groups MRL/*lpr* mice measured using nonmetric multidimensional scaling (NMDS) plot. (f,g) Comparisons of the maximum-likelihood phylogenetic tree comprising taxa of gut microbiome. The phylogenetic tree of the GI tract gut microbiome of the 6 weeks group (f) and the 16 weeks group (g) are shown. The circular dendrogram rings represent phylum levels, with corresponding taxa depicted in the inner layer. The circular ring (phylum) and inner layer (family) color legends are on the left. (h,i) Color-coded network graphs of gut microbiome, illustrating co-occurrence and mutual exclusion interactions among OTUs. The co-occurrence networks of the GI tract gut microbiome of the 6 weeks group (h) and the 16 weeks group (i) are shown. The network was developed using class-level OTUs. Additional network information is provided in Supplementary Table 9. $^{**}p < 0.01$; $^{***}p < 0.001$.

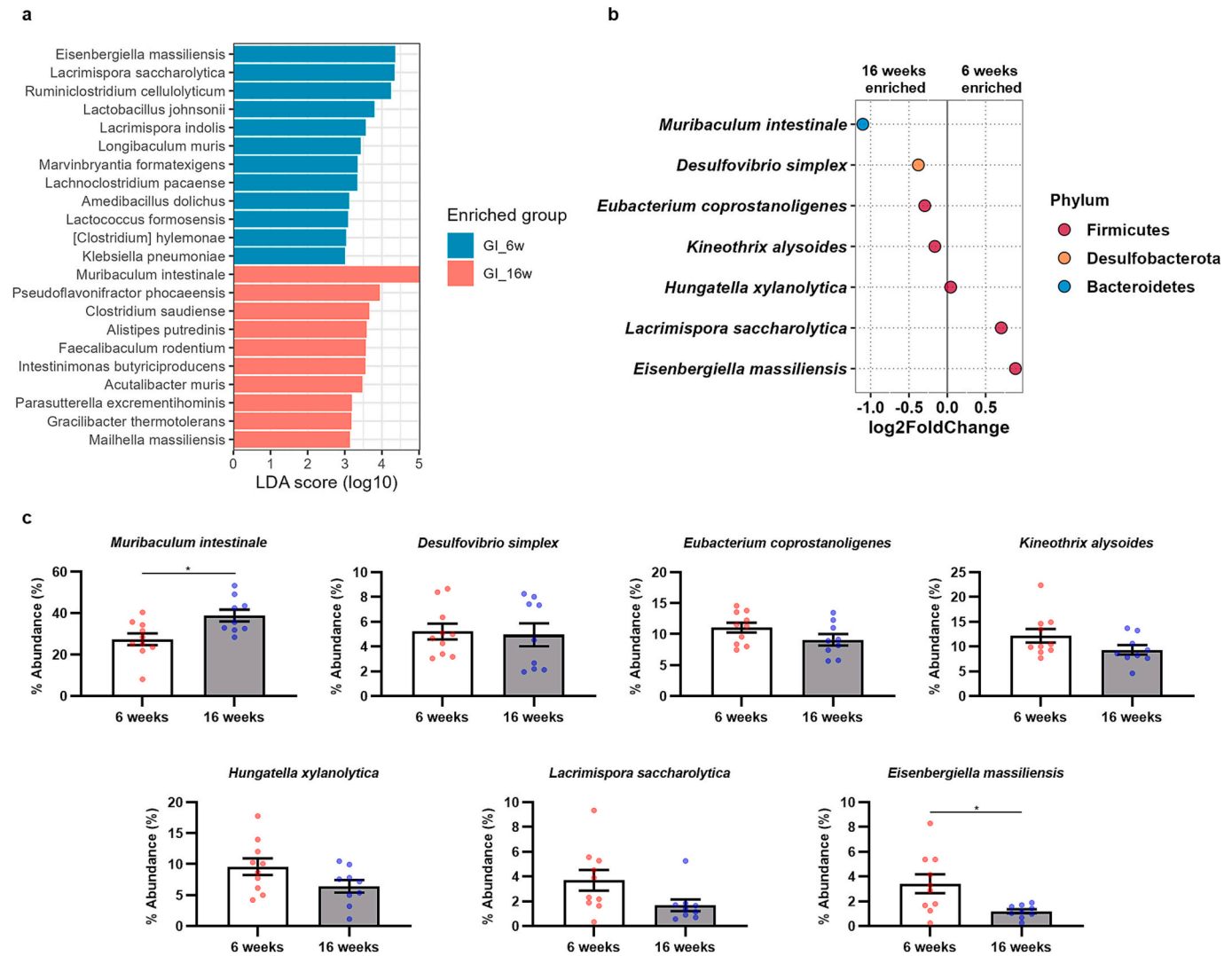


Fig. 4. Differential analysis of GI tract gut microbial species of the 6 weeks and 16 weeks groups MRL/*lpr* mice. (a) LefSe analysis of the 6 weeks and 16 weeks groups MRL/*lpr* mice (LDA score of >3). (b) DESeq2 analysis of the 6 weeks and 16 weeks groups MRL/*lpr* mice. LefSe and DESeq2 methods are based on the relative abundance table. (c) % Abundance of selected gut microbiomes through differential analysis in GI tract samples from each group. **p* < 0.05.

Table 1
Correlation analysis between selected microbes and metabolites in GI sample (the 16 weeks group) of MRL/*lpr* mouse.

Metabolites	<i>p</i> -value	<i>r</i>
<i>Eisenbergiella massiliensis</i>		
5 α -Cholestanol	0.015	−0.558
Indole	0.033	−0.495
<i>p</i> -Cresol	0.007	−0.605
Acetic acid*	0.0303	−0.502
<i>Lacrimispora saccharolytica</i>		
Cholesterol	0.017	−0.547
<i>Hungatella xylanolytica</i>		
5 α -Cholestanol	0.026	−0.516

* The values in the table are Spearman's *p*-value and *r* value from correlation analysis. Specific microbiome correlated metabolite in MRL/*lpr* mouse feces sample.

E. massiliensis (EM), *L. saccharolytica* (LS), *H. xylanolytica* (HX), and a mixture of the three (Mix). SLE symptoms were assessed when the mice were 16 weeks old (Fig. 5a). There was no significant difference in body weight between the groups (Fig. 5b). The CTRL group had higher food intake, while water intake was similar across groups (Supplementary

Fig. 7a,b). Serum biochemical analysis showed decreased uric acid (UA) in the LS group, decreased creatinine (CRE) in the HX group, and decreased glucose (GLU) in the Mix group, with no significant differences in other factors (Supplementary Fig. 7c-i). Microbial administration did not significantly change ACR (Fig. 5c), anti-dsDNA IgG levels (Fig. 5d), serum IgG2a level (Fig. 5e), spleen index (Fig. 5f). Histological kidney analysis revealed T cell (Thy1.2⁺ cells) infiltration and fibrosis in the CTRL and EM groups, with reduced symptoms in LS, HX, and Mix groups (Supplementary Fig. 8). We further assessed immunological changes. All treatment groups significantly suppressed the increase in DNT cells and showed a trend toward preserving CD4 and CD8 T cells (Fig. 5g). The EM group showed a notable increase in Treg (30 %) (Fig. 5h), but IL-10 levels were not significantly different from those in the CTRL group (Fig. 5i). Th17 levels significantly decreased in the Mix group (23 %) (Fig. 5j), and IL-17A levels also declined (36 %), though not significantly (Fig. 5k). These findings suggest that microbial administration we discovered has therapeutic potential in alleviating SLE-related immunological abnormalities in MRL/*lpr* mice.

4. Discussion

Systemic Lupus Erythematosus (SLE) is a chronic autoimmune

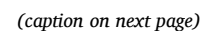


Fig. 5. Evaluation of the efficacy of intestinal microorganisms in suppressing SLE-mimicking symptoms in MRL/*lpr*. (a) The schematic diagram of the experimental strategy. (b) The body weight of each group before (6 weeks) and after (16 weeks) intake of gut microorganisms. (c) Albumin/creatinine ratio (ACR) measured from urine samples of each group after intake of gut microorganisms. (d) Levels of anti-dsDNA antibodies (total IgG) in the sera of each group after intake of gut microorganisms. (e) Levels of IgG2a in the sera of each group after intake of gut microorganisms. (f) Representative images of spleen and spleen index of each group after intake of gut microorganisms. (g) Flow cytometric analysis of Thy1.2⁺ gated cells were performed to identify T cell subsets, including DNT (CD4⁺, CD8⁺), CD4⁺, and CD8⁺ cells from spleens of 16 weeks mice in each group after intake of gut microorganisms. (h–k) Quantification of T cell subsets in the spleens of MRL/*lpr* mice after intake of gut microorganisms. Cumulative results showing the percentage of Treg (h, CD4⁺, CD25⁺, and Foxp3⁺) and Th17 (i, CD4⁺ and IL-17A⁺) in MRL/*lpr* mice after intake of gut microorganisms. Splenocytes from MRL/*lpr* mice after intake of gut microorganisms were isolated and stimulated with anti-CD3ε (1 μg/mL) for 96 h. Then the supernatants were tested for IL-10 (j) and IL-17A (k) levels. The values represent the mean ± S.E.M. **p* < 0.05; ***p* < 0.01; ****p* < 0.001. CTRL: saline buffer intake group; EM: *Eisenbergiella massiliensis* intake group; LS: *Lacrimispora saccharolytica* intake group; HX: *Hungatella xylanolytica* intake group; Mix: Group that consumed a mixture of three microorganisms, *E. massiliensis*, *L. saccharolytica*, and *H. xylanolytica*.

disorder where the immune system attacks the body's own tissues, leading to inflammation and organ damage [1,2]. In MRL/*lpr* mice, a model of SLE, symptoms like splenomegaly, proteinuria, and increased autoantibodies develop as the mice age [34,35]. At 6 weeks, the mice showed mild symptoms, with minimal kidney damage and low autoantibody levels. By 16 weeks, these symptoms worsen, with significant kidney inflammation, immune cell infiltration, and an imbalance between Th17 and Treg cells (Fig. 1). This progression indicates that 6 weeks represents the pre-disease phase, while 16 weeks marks the onset of full SLE-like symptoms in mice.

Age significantly influences gut microbiota composition and diversity. In MRL/*lpr* mice, age-related microbial changes could confound the interpretation of disease-associated shifts. Younger mice (6 weeks) show a balanced microbiota, while older mice (16 weeks) exhibit reduced diversity, potentially affecting immune responses and disease progression [45]. These age-related changes can complicate distinguishing between disease-specific and age-related microbial alterations. To address this, future studies should use age-matched, genetically unmodified MRL/MpJ mice as controls, which do not develop lupus. This will help clarify the impact of age-related microbiota shifts on immune system changes and disease development.

Immune cell infiltration in the kidney was assessed using Thy1.2, which effectively captured inflammatory changes in lupus nephritis (Supplementary Fig. 2). However, since Thy1.2 is also expressed in fibroblasts and mesangial cells, incorporating markers such as CD3 could enhance the specificity of T cell analysis [46]. Additionally, immune infiltration is a key factor in lupus nephritis, alongside immune complex deposition (IgG, C3), which may exacerbate renal damage [47]. While our study did not focus on immune complex accumulation, integrating this analysis in future research could provide a more comprehensive understanding of lupus-related kidney pathology.

Our study provides important insights into the immunological shifts occurring in MRL/*lpr* mice, particularly in relation to Treg, Th17, and DNT cell populations. These findings are supported by previous studies demonstrating the role of gut microbiota in shaping immune responses and promoting immune homeostasis in autoimmune conditions [48]. Among these subsets, DNT cells play a particularly crucial role in lupus pathogenesis, as they expand abnormally in MRL/*lpr* mice due to *Fas* mutations, contributing to systemic autoimmunity. This pronounced DNT expansion may overshadow potential shifts in conventional CD4⁺ and CD8⁺ T cell populations. These immune cell subsets are crucial in lupus pathogenesis, and our findings support the hypothesis that gut microbiota alterations contribute to their dysregulation. However, given the complexity of SLE immunopathogenesis, other T cell subsets, such as Th1 (CD4⁺IFN-γ⁺) and Th2 (CD4⁺IL-4⁺), may also play significant roles, requiring further investigation. Additionally, although microbiota changes according to SLE progression were observed, we did not directly analyze their correlations with immune cell populations due to the limited range of immune subsets included in this study. Future research incorporating a broader spectrum of immune cells could provide a more comprehensive understanding of the microbiota-immune system interplay.

This study utilized GC–MS analysis to investigate the metabolic profiles of MRL/*lpr* mice to differentiate between the 6 weeks and 16

weeks groups. The analysis revealed significant differences in metabolite levels between the two groups. Specifically, Supplementary Tables 1, 2, and 3 highlight that amino acid levels were notably lower in the 16 weeks group. GC–MS analysis (Supplementary Tables 1–3) and OPLS-DA score plots (Fig. 2a,b) further distinguished metabolic profiles based on disease presence, with the 6 weeks and 16 weeks groups showing distinct patterns. In the 16 weeks group, glucose, glycerol, lactic acid, and 3-hydroxybutyric acid levels were relatively low in GI tract samples but high in serum samples (Supplementary Tables 1–3). Elevated blood glucose levels, influenced by dietary glycemic index, have been shown to adversely affect the immune system and are associated with metabolic syndrome [49–53]. Glycerol, known to induce renal failure and promote inflammation [54–56], and lactic acid, which is linked to inflammation [57–62]. 3-Hydroxybutyric acid, which enhances butyric acid production in the gut, was elevated in serum of patients with inflammatory bowel disease [63–68]. The increased levels of those metabolites in serum might closely be associated with SLE symptom. The study's findings are aligned with previous research on metabolic changes in SLE and inflammatory diseases. Elevated and reduced amino acid levels in fecal and serum samples from SLE patients were consistent with observed results [69–72]. These observations highlight the potential for using metabolomic profiles to identify therapeutic targets for SLE. In summary, the study suggests that changes in the levels of glucose, glycerol, lactic acid, 3-hydroxybutyric acid, lysine, methionine, and tryptophan may be integral to the pathophysiology of the MRL/*lpr* mouse model of SLE, offering potential avenues for therapeutic intervention.

Previous studies have shown significant gut microbiota changes in various lupus-prone mouse models, including MRL/*lpr* and NZB/W F1 mice. MRL/*lpr* mice exhibit decreased *Lactobacillaceae* and increased *Lachnospiraceae* during disease progression [45], while NZB/W F1 mice show increased *Lactobacilli* linked to more severe lupus symptoms [73]. Additionally, both models share an increased *Bacteroidetes* and reduced *Firmicutes*/*Bacteroidetes* ratio, associated with disease severity [74]. In our study, 16S rRNA sequencing of MRL/*lpr* mice revealed that *Lactobacillaceae* decreased in GI tract samples, but increased in feces samples at 16 weeks. *Lachnospiraceae* showed the opposite trend, differing from previous reports. We also observed a decrease in *Firmicutes* and an increase in *Bacteroidota* in GI tract samples, consistent with findings in SLE patients and lupus mouse models [74,75]. As seen in our study, the gut microbiota composition differed between GI tract and fecal samples, suggesting that discrepancies between our results and previous studies may be due to differences in sampling methods. Many prior studies primarily focused on fecal microbiota, which may not fully represent microbial alterations occurring within the gut [23]. This highlights the importance of considering sample type when investigating gut microbiota changes in lupus research. Sex-dependent differences in gut microbiota and immune responses have also been reported in lupus models [76]. Given that lupus is more prevalent in females, hormonal influences on microbiota composition and immune regulation may play a crucial role in disease progression [77]. Although our study focused on female MRL/*lpr* mice, male BXSB (B6.Cg-BxSB-Yaa) mice develop severe autoimmune reactions due to the Y-linked autoimmune accelerator (*Yaa*) mutation [78], making them a valuable model for studying male-

specific lupus mechanisms. Future research should explore sex-specific microbial and metabolic changes to better understand their role in lupus pathogenesis.

Differential analysis of the GI tract samples suggests that *Eisenbergiella massiliensis*, *Lacrimispora saccharolytica*, and *Hungatella xylanolytica* may have potential therapeutic efficacy for SLE (Fig. 4b). Furthermore, microbiome-metabolome network analysis indicates that *E. massiliensis*, *L. saccharolytica*, and *H. xylanolytica* are associated with metabolites such as 5 α -Cholesterol, Cholesterol, indole, and *p*-cresol that differ between the two groups (Table 1). Cholesterol and its derivative, 5 α -cholestanol, accumulate in excess and contribute to inflammation, stress, and the development of various immune diseases [79]. Indole is consumed by intestinal bacteria and used as a beneficial substance in inflammation [80]. Exposure to *p*-cresol leads to liver damage and impaired hepatic circulation and loss of barrier function [81].

In this study, we revealed correlations between 5 α -cholestanol, cholesterol, *p*-cresol, and indole and those three microbes. However, we did not perform the metabolic profiling of GI tract or fecal samples after administration of selected microbes in this study. To establish a causal relationship between selected microbes and metabolites, it is necessary to combine experiments that lower metabolites by inhibiting specific pathways that synthesize metabolites such as 5 α -cholestanol, cholesterol, *p*-cresol, and indole with experiments that administer selected three independent microbes. We will perform the intervention, microbial, and metabolic profiling studies in the future to suggest a causal relationship as to whether the changes in metabolites were directly induced by the microbial intervention or indirectly due to network adjustments. The results of this study could be regarded as fundamental potential to develop microbiome-based medicines by identifying unique microbes and metabolites in SLE patients in future.

Our findings also highlight the potential of microbiota-based interventions for SLE. Notably, our study revealed significant associations between specific bacterial species and immune markers. Previous studies have shown that gut microbiota play a critical role in shaping immune responses by modulating T cell subsets and cytokine production. Certain gut microbiota, including *E. massiliensis*, have been associated with regulatory T cell differentiation and IL-10 secretion in inflammatory disease models, potentially through mechanisms involving short-chain fatty acids (SCFAs) or other microbial metabolites [82]. Consistent with these findings, *E. massiliensis* administration in our study was linked to an increase in Treg proportions, suggesting its potential role in enhancing anti-inflammatory responses (Fig. 5h). In contrast, the mixture of *E. massiliensis*, *L. saccharolytica* and *H. xylanolytica* supplementation led to a reduction in Th17 levels, which may indicate their immunomodulatory effects in suppressing lupus-associated inflammation (Fig. 5j). These findings align with previous reports demonstrating that gut microbiota can influence T cell differentiation and cytokine production, ultimately impacting immune balance in autoimmune diseases [78]. Probiotics and prebiotics have been increasingly explored as therapeutic options for autoimmune diseases, and previous studies have demonstrated their efficacy in modulating immune responses in lupus [83,84]. In our study, administration of *E. massiliensis*, *L. saccharolytica*, and *H. xylanolytica* significantly reduced DNT levels while modulating Treg and Th17 cell populations (Fig. 5). These results suggest that targeted microbiota modulation may offer a promising strategy for restoring immune balance in SLE. However, not all microbial changes observed in lupus-prone mice may be beneficial. Interestingly, *Muribaculum intestinale* and *Desulfovibrio simplex* were increased in 16-week-old MRL/*lpr* mice, suggesting potential involvement in disease progression. Although *M. intestinale* has previously been associated with induced immune response [85], its expansion was unexpectedly accompanied by heightened immune responses in our study, implying a possible immunostimulatory role in this context. Similarly, *D. simplex*, a sulfate-reducing bacterium, may promote intestinal inflammation via hydrogen sulfide production [86,87]. These findings highlight the possibility that some microbial shifts during lupus

progression may contribute to pathogenesis rather than protection.

Our study showed that supplementation with candidate microbes effectively modulated immune cell populations, including DNT, Treg, and Th17 cells. However, no significant effects were observed on ACR, IgG2a levels, or spleen index (Fig. 5). This may be due to the need for a longer intervention period to impact tissue damage and autoantibody production. Additionally, our candidate microbes may primarily influence T cell differentiation rather than directly regulating B cell activity. Since the spleen index reflects systemic immune activation, local gut immune modulation alone may not be sufficient to reduce splenomegaly. These findings suggest that while gut microbiota contribute to immune balance, microbial interventions alone may have limitations in fully mitigating SLE pathology, highlighting the need for prolonged treatment or combinatorial approaches.

Taken together, our study provides strong evidence that gut microbiota alterations contribute to immune dysregulation in lupus. Our findings demonstrate distinct immunological and metabolic changes in MRL/*lpr* mice, reinforcing the relevance of this model for studying lupus pathogenesis. While our results highlight key microbiome-immune interactions, additional research incorporating functional assays and microbiota modulation experiments will be essential to fully elucidate their mechanistic contributions to SLE. The observed associations between specific bacterial species and metabolic signatures provide further evidence for the microbiota's role in SLE pathogenesis, consistent with previous microbiome studies in autoimmune diseases. By identifying key microbial strains and their associated metabolites, this research provides a foundation for developing microbiome-based therapeutic strategies.

While our study provides valuable insights into the link between gut microbiota and SLE progression, it has several limitations. First, as our findings are based on MRL/*lpr* mice, they may not fully replicate the complexities of human SLE, given species differences in gut microbiota composition and function. Second, the lupus therapeutic candidate microbes have different origins [88–90], with some not typically found in the human gut, making their relevance to SLE patients uncertain. Further research using human-derived strains is needed to validate their therapeutic potential. Despite these limitations, our study lays the groundwork for microbiota-targeted interventions in SLE.

CRedit authorship contribution statement

Ji-Seon Ahn: Writing – review & editing, Writing – original draft, Visualization, Software, Project administration, Methodology, Formal analysis, Data curation. **Ye-Been Lee:** Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Formal analysis, Data curation. **Eui-Jeong Han:** Writing – review & editing, Validation, Investigation. **Yu-Jin Choi:** Validation, Investigation. **Da-Hye Kim:** Validation, Investigation. **Seung Ki Kwok:** Validation, Investigation. **Hyung-Kyoon Choi:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Hea-Jong Chung:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization.

Ethics approval statement

All animal experiments were conducted in accordance with the ARRIVE guidelines and approved by the Institutional Animal Care and Use Committee of the Korea Basic Science Institute (KBSI-IACUC-23-36).

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Hea-Jong Chung reports financial support was provided by National Research Foundation. HyungKyoon Choi reports financial support was provided by Ministry of Food and Drug Safety. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.lfs.2025.123684>.

Data availability

Metabolomics raw data have been uploaded to the figshare database and can be accessed at <https://doi.org/10.6084/m9.figshare.26892547.v1> [91]. Sequence data generated in this study was uploaded to the NCBI SRA database. It can be accessed via the accession number PRJNA1146986 [92]. The FACS gating strategies are available in Supplementary Fig. 9.

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