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Gut microbiota mediated the effects of high relative humidity on lupus in female MRL/lpr mice

Chaochao Wang[1†], Yongqiang Lin[1†], Leiming Chen[1] and Hui Chen[2*]

Abstract

Introduction The relationship between humidity and systemic lupus erythematosus (SLE) has yielded inconsistent results in prior research, while the effects of humidity on lupus in animal experiments and its underlying mechanism remain inadequately explored.

Methods The present study aimed to investigate the impact of high humidity (80 ± 5%) on lupus using female and male MRL/lpr mice, with a particular focus on elucidating the role of gut microbiota in this process. To this end, fecal microbiota transplantation (FMT) was employed to transfer the gut microbiota of MRL/lpr mice under high humidity to blank MRL/lpr mice under normal humidity (50 ± 5%), allowing for an assessment of the effect of FMT on lupus.

Results The study revealed that high humidity exacerbated lupus indices (serum anti-dsDNA, ANA, IL-6, and IFN- g, and renal pathology) in female MRL/lpr mice but had no significant effect on male MRL/lpr mice. The aggravation of lupus caused by high humidity may be attributed to the increased abundances of the *Rikenella*, *Romboutsia*, *Turicibacter*, and *Escherichia-Shigella* genera in female MRL/lpr mice. Furthermore, FMT also exacerbated lupus in female MRL/lpr mice but not in male MRL/lpr mice.

Conclusion In summary, this study has demonstrated that high humidity exacerbated lupus by modulating gut microbiota in female MRL/lpr mice. The findings underscore the importance of considering environmental factors and gut microbiota in the development and progression of lupus, particularly among female patients.

Keywords Systemic lupus erythematosus, Humidity, Gut microbiota, MRL/lpr mice, Fecal microbiota transplantation

Background

Systemic lupus erythematosus (SLE) is a chronic inflammatory autoimmune disease that predominantly affects females and involves aberrant activation and apoptosis of T/B cells, as well as the deposition of autoimmune complexes in multiple organs [1]. SLE can cause damage to various organs, including the skin, joints, central nervous system, kidneys, liver, and peripheral nervous system [2]. Although the pathogenesis and etiology of SLE remain unclear, it is widely believed that genetic susceptibility and diverse environmental factors contribute to its onset [3].

The climate is a crucial environmental factor that impacts health and is associated with various diseases, including rheumatoid arthritis [4], asthma [5], and acute diarrhoeal illnesses [6]. SLE activity has been closely linked to climate factors such as wind velocity, sunshine duration, ambient temperature, and precipitation [7–11], However, the relationship between relative humidity and

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SLE activity remains unclear and inconclusive [10–12]. Therefore, this study aims to investigate the effects of relative humidity on lupus disease in animals for the first time.

The gut microbiota plays a vital role in host physiology, encompassing metabolism, inflammation, immunity, and neurology. Alterations of the gut microbiota may precede the transition from normal homeostasis to disease states in the host [13]. Dysbiosis or disturbance of the gut microbiota has been observed in both SLE patients [14, 15] and animal models [16, 17]. Dysbiosis of the gut microbiota is believed to initiate systemic immune dysregulation in SLE by translocating beyond its niches, molecular mimicry, epitope spreading, and bystander activation, thereby promoting systemic inflammation [18]. Furthermore, dysbiosis of the gut microbiota has been associated with the impact of relative humidity on disease development [19, 20]. Thus, this study aimed to explore whether the gut microbiota mediates the effects of high humidity on lupus development in MRL/lpr mice.

In this study, we housed 10-weeks-old female and male MRL/lpr mice in two distinct humidity environments (50 ± 5% and 80 ± 5%) to investigate the impact of humidity on lupus development. Furthermore, we transplanted fresh fecal matter from MRL/lpr mice under an environmental 80 ± 5% humidity into those under an environmental 50 ± 5% humidity to explore whether the gut microbiota mediates the effects of environmental humidity on lupus. Our findings may provide novel insights into how environmental factors trigger lupus development.

Materials and methods

Animals

For the SLE animal model in this study, we utilized the MRL/MpJ-Fas lpr (MRL/lpr) mouse strain, comprising both male and female mice purchased from Shanghai SLAC Laboratory Animal Co., Ltd. The mice were reared under standard conditions with a 12-h light/dark cycle, constant temperature of 25 ± 1 °C, and humidity of 50 ± 5%. They were provided ad libitum access to food and water until they reached 10 weeks old. All animal experiments were conducted in accordance with the Institutional Animal Care and Use Committee of China.

Study design

Eighteen 10-week-old female or male MRL/lpr mice were divided into three groups: (1) Model group (MT, N = 6 mice/group): MRL/lpr mice received a daily oral gavage of 200 μL sterile water and were housed in an environment with 50 ± 5% humidity; (2) High humidity group (HT, N = 6 mice/group): MRL/lpr mice received a daily oral gavage of 200 μL sterile water and were housed in an environment with 80 ± 5% humidity; (3) FMT group (FMT, N = 6 mice/group): MRL/lpr mice received a daily oral gavage of 200 μL fecal suspension from the MRL/lpr mice in the HT group and were housed in an environment with 50 ± 5% humidity. Fresh fecal samples were collected daily from the mice in the HT group using a sterile micro-centrifuge tube: the mouse was immobilized and its tail lifted, while gentle pressure was applied to the lower abdomen to facilitate defecation. The fresh fecal samples were suspended in a sterile water solution at a weight/volume ratio of 5:1, followed by filtration through a 20-μm filter to eliminate large particles. The filtered samples were then transplanted into the FMT group on the same day. To minimize disruption to gut microbiota, all three groups of mice underwent identical procedures with regards to their diet, water intake, and fecal collection.

Environmental humidities were maintained using a man-made climate box (RXZ-380 A Ningbo Jiangnan Instrument Factory, China) throughout the 6-week experimental period. The time course, grouping information, and humidity fluctuations were depicted in Fig. 1. At the end of the trial, blood samples were collected from the eye socket vein of each mouse, and centrifuged at 3000 rpm for 10 min at 4 °C. Fecal samples were collected from the colon and stored at −80 °C for further analysis. Colon and kidney tissues were immediately isolated, fixed in 4% formalin, and processed.

Assessment of lupus activity

The levels of anti-nuclear antibodies (ANA) and anti-double-stranded DNA (anti-dsDNA) in mouse serum were measured using the mouse anti-nuclear antibody (IgG) ELISA Kit (CAS: CSB-E12912m, CUSABIO, Wuhan, China) and mouse anti-dsDNA antibody (IgG) ELISA Kit (CAS: CSB-E11194m, CUSABIO, Wuhan, China), respectively. Additionally, the concentrations of interleukin 6 (IL-6) and interferon gamma (IFN-γ) in mouse serum were measured using the Mouse IL-6 ELISA Kit (CAS: EK206/3–96, Multisciences, Hangzhou, China) and mouse IFN-γ High Sensitivity ELISA kit (CAS: EK280HS-96, Multisciences, Hangzhou, China), respectively. Kidney tissue was harvested from exsanguinated mice, flushed with 1 × PBS, and dissected longitudinally. After overnight fixation in 4.0% formaldehyde, the tissues were decalcified using EDTA decalcification solution, embedded in paraffin, and sectioned into 5 μm slices. The sections were stained with hematoxylin and eosin (H&E) to assess kidney tissue damage.

Assessment of intestinal barrier

In cases where the intestinal mucosa function is compromised, an increase in serum levels of diamine oxidase (DAO) and D-lactic acid can indicate a breakdown

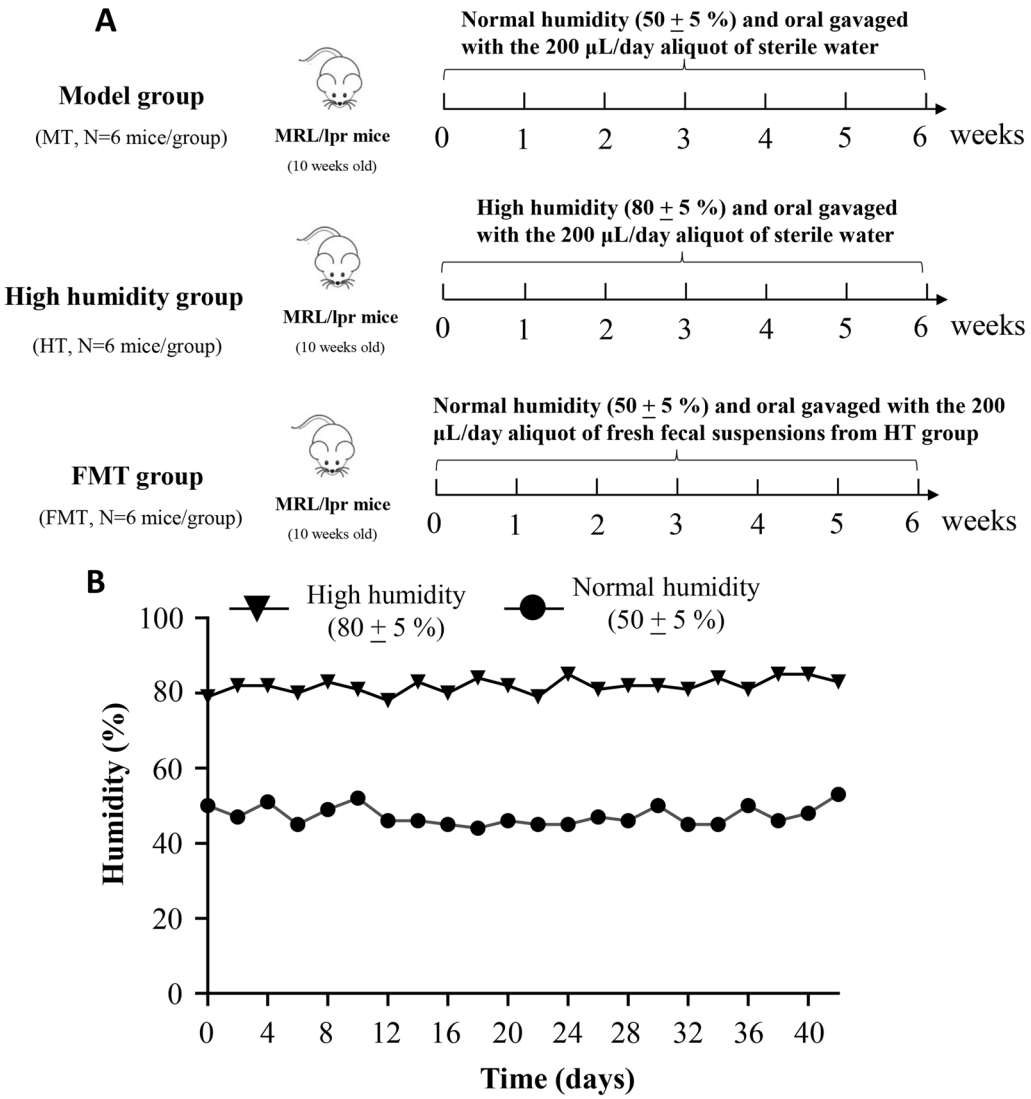

Fig. 1 Overview of group information (**A**) and variation of humidity in the man-made climate box (**B**)

in intestinal barrier function. The assay kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) were utilized to measure the serum levels of DAO (CAS: A088-2-1) and D-lactic acid (CAS: A019-2-1).

Additionally, the maintenance of intestinal barrier integrity relies heavily on the presence of tight junction proteins ZO-1 and occludin. To evaluate their expression levels, we fixed colon segments in formalin, embedded them in paraffin, sectioned them into 5 μm slices, and conducted immunofluorescence assays. The sections were deparaffinized, rehydrated, and blocked with 3% hydrogen peroxide for 25 min at room temperature to inhibit endogenous peroxidase activity. Following a 30-minute incubation in BSA, the tissue sections were subjected to immunostaining using either goat anti-ZO-1 primary antibody (1:300, Servicebio GB111402) or goat anti-occludin primary antibody (1:200, Servicebio GB111401). Subsequently, the slides were treated with HRP-conjugated goat anti-rabbit IgG secondary antibody (1:500, Servicebio GB23303) for 50 min at room temperature and mounted with Prolong Gold Antifade containing DAPI (Servicebio GB012). Fluorescence microscopy (NIKON ECLIPSE C1, Tokyo, Japan) with the imaging system (NIKON DS-U3, Tokyo, Japan) was employed to capture images. The acquired images were processed using ImageJ software (National Institutes of Health, Bethesda, MD). Control slides stained without primary antibody and absorptive

controls where primary antibody was applied with excess peptide were included.

Gut microbiota analysis

For gut microbiota analysis, genomic DNA was extracted from stool samples using a QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The qualified DNA was then amplified with broad-range bacterial primer pairs targeting the V3-V4 region of the 16 S rRNA gene. The amplicons obtained were subjected to purification and quantification prior to pooling and paired-end sequencing on an Illumina MiSeq PE300 platform (Illumina, San Diego, USA) by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). The raw sequencing reads have been deposited in the NCBI Sequence Read Archive (SRA) database under Accession Number PRJNA949349.

Following demultiplexing, the sequences were underwent quality filtration using fastp (0.19.6) [21] and were merged with FLASH (v1.2.11) [22]. The high-quality sequences were subsequently subjected to de-noising via DADA2 [23], a plugin in the Qiime2 [24] (version 2020.2), utilizing recommended parameters to generate amplicon sequence variants (ASVs). To minimize the effects of sequencing depth on beta diversity measures, the number of sequences from each sample was rarefied to 40,000, with an average Good's coverage of 97.90%. Taxonomic assignment of ASVs was performed using the Naive bayes consensus taxonomy classifier implemented in Qiime2 and the SILVA 16 S rRNA database (v138).

Bioinformatic analysis of the gut microbiota was conducted using the Majorbio Cloud platform (https://cloud.majorbio.com). The similarity among the microbial communities in different samples determined by non-metric multidimensional scaling (NMDS) based on Bray-Curtis dissimilarity using the Vegan v2.5-3 package. The Wilcoxon rank-sum test was then performed to identify the significantly abundant genera of bacteria between two groups.

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 8.0 (GraphPad Software Inc., San Diego, CA, USA). Depending on the results of the normality test and variance homogeneity, we applied either one-way ANOVA or the rank-sum test for data analysis. Additionally, we controlled for the false discovery rate (FDR) by adjusting p-values using the Benjamini-Hochberg method. A statistically significant result was considered when the adjusted p-value was less than 0.05.

Results

Effects of high humidity or FMT on lupus activity in MRL/lpr mice

Figure 2 demonstrates that the impact of high humidity on lupus activity exhibits gender-specific differences in MRL/lpr mice. High humidity could significantly increase serum levels of anti-dsDNA, ANA, IL-6, and IFN-g in female MRL/lpr mice. Histologically, high humidity significantly induced severe renal dysfunction in female MRL/lpr mice, as evidenced by increased inflammatory cell infiltration, glomerular crescent formation and diffuse endocapillary proliferation observed upon histological examination of H&E-stained kidney sections. However, there was no significant difference in lupus indices between male MRL/lpr mice under normal humidity and high humidity. Both female and male MRL/lpr mice exhibited consistent effects on lupus indices with FMT as high humidity. These findings indicate that gut microbiota mediates the exacerbation of lupus activity induced by high humidity in female MRL/lpr mice.

Effects of high humidity or FMT on intestinal barrier in MRL/lpr mice

There is increasing evidence suggesting a correlation between the development of lupus and compromised intestinal barrier function [25]. High humidity were found to significantly elevate serum levels of DAO and D-lactic acid in both female and male MRL/lpr mice, while FMT did not demonstrate any significant impact (Fig. 3A and B). Immunofluorescence staining revealed that neither high humidity nor FMT significantly impacted the expression of ZO-1 in colon tissues in both female and male MRL/lpr mice. However, there was a noticeable decrease in occludin expression (Fig. 3C, F). Generally, occludin expression as a transmembrane protein precedes that of the cytoplasmic protein ZO-1 [26]. Therefore, it is possible that occludin may be more sensitive to changes than ZO-1 during inflammation. These findings indicate that high humidity and FMT may compromise the integrity of intestinal barrier by downregulating occludin expression, which could contribute to the pathogenesis of lupus in MRL/lpr mice.

Alterations in microbial compositions caused by high humidity or FMT

To investigate whether the exacerbation of lupus by high humidity or FMT is associated with gut microbiota, bacterial 16 S rRNA v3-v4 regions in colon feces were sequenced. NMDS analysis based on Bray-Curtis distance revealed significant differences in the overall composition of gut microbiota among the three groups

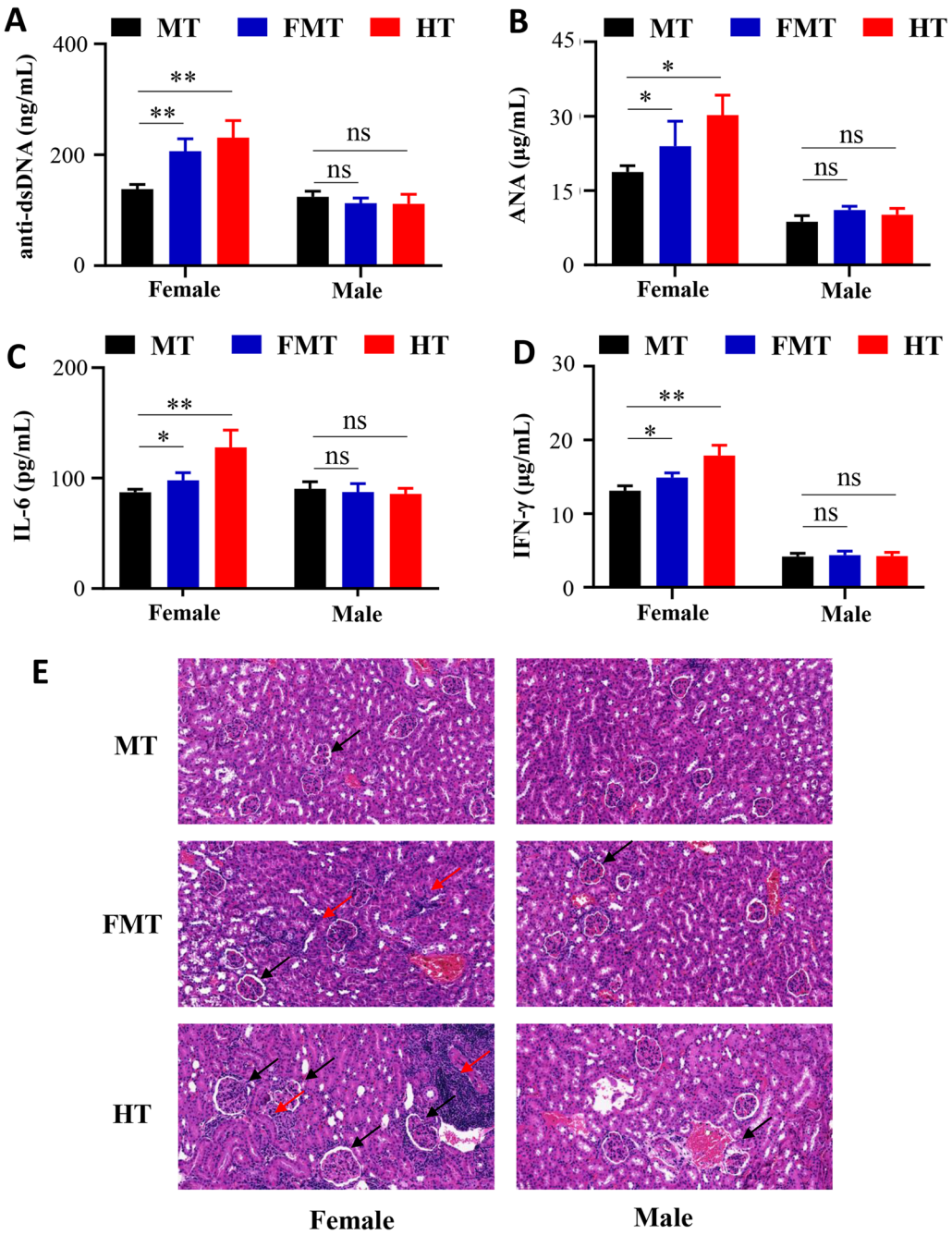

Fig. 2 Assessment of lupus activity in MRL/lpr mice. Effects of high humidity or FMT on serum anti-dsDNA (**A**), ANA (**B**), IL-6 (**C**), and IFN-g (**D**). Representative images of H&E-stained histology of kidney tissues (**E**). MT: model group; FMT: fecal microbiota transplantation group; HT: high humidity group. "**" $p < 0.01$, "*" $p < 0.05$, "ns" $p > 0.05$. Black arrows indicate glomerulonephritis; Red arrows indicate inflammatory infiltrates. N = 6 mice/group for serum autoantibody and proinflammatory analysis; N = 3 mice/group for H&E-stained histology analysis

in both female ($R^2 = 0.369$, $P = 0.001$, Fig. 4A) and male ($R^2 = 0.196$, $P = 0.027$, Fig. 4B) MRL/lpr mice. The scatter plot based on NMDS scores demonstrated distinct separation among the samples of the three groups in female MRL/lpr mice, while exhibiting overlapping samples in male MRL/lpr mice (Fig. 4A and B).

Subsequent analysis of the sequencing data allowed for a comprehensive investigation into the bacterial composition in fecal samples. At the phylum level,

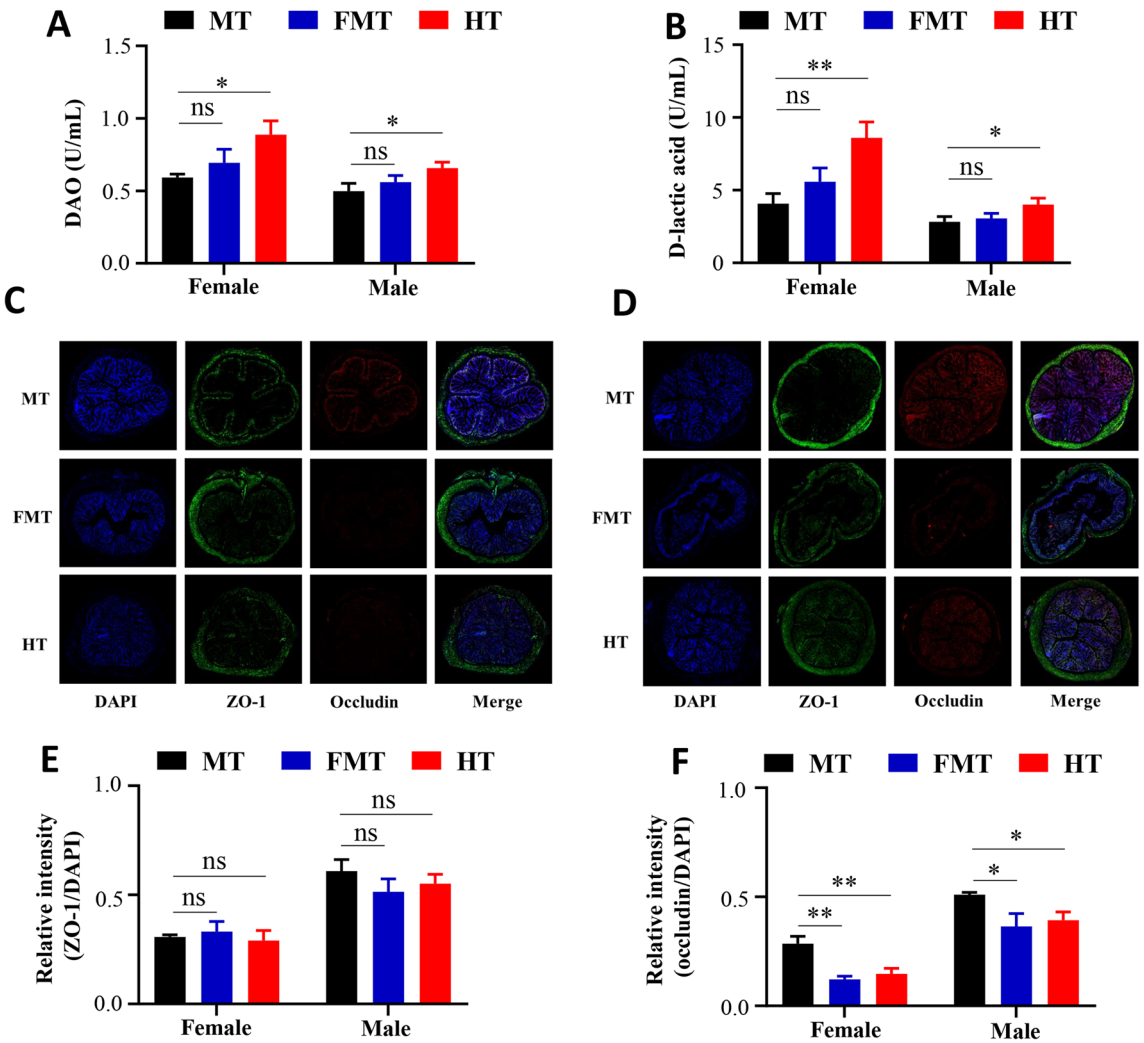

Fig. 3 Assessment of intestinal barrier in MRL/lpr mice. Effects of high humidity or FMT on serum DAO (**A**) and D-lactic acid (**B**). Representative immunofluorescence images of ZO-1, occludin, and DAPI in colon tissues of female (**C**) and male (**D**) MRL/lpr mice. Relative immunofluorescence intensity of ZO-1 (**E**) and occludin (**F**) analyzed by Image J software. MT: model group; FMT: fecal microbiota transplantation group; HT: high humidity group. "**" $p < 0.01$, "*" $p < 0.05$, "ns" $p > 0.05$. N = 6 mice/group for serum DAO and D-lactic acid analysis; N = 3 mice/group for immunofluorescence analysis

Bacteroidetes and Firmicutes were identified as the two most abundant phyla across all groups. Notably, high humidity resulted in an increase in the Firmicutes abundance and a decrease in the Bacteroidetes abundance within female MRL/lpr mice, whereas not in male MRL/lpr mice. Further examination at the genus level revealed 15 bacterial genera with an average relative abundance exceeding 2%. Genus norank_f_*Muribaculaceae* was the most prevalent genus across all groups.

Altered genera caused by high humidity or FMT in MRL/lpr mice

To examine changes in gut microbial genera and species induced by humidity, we conducted Wilcoxon rank-sum tests (Fig. 5). Inconsistent humidity-induced changes were observed between female and male MRL/lpr mice. In females, high humidity caused significant increases in four genera (*Rikenella*, *Escherichia-Shigella*, *Romboutsia*, and *Turicibacter*) and decreases in three genera (*Lachnospiraceae* UCG-006, *Tuzzerella*, and g_unclassified_f_*Rikenellaceae*) (Fig. 5A). In males, high humidity increased the relative abundance of *Lachnospiraceae* UCG-001 and reduced the relative

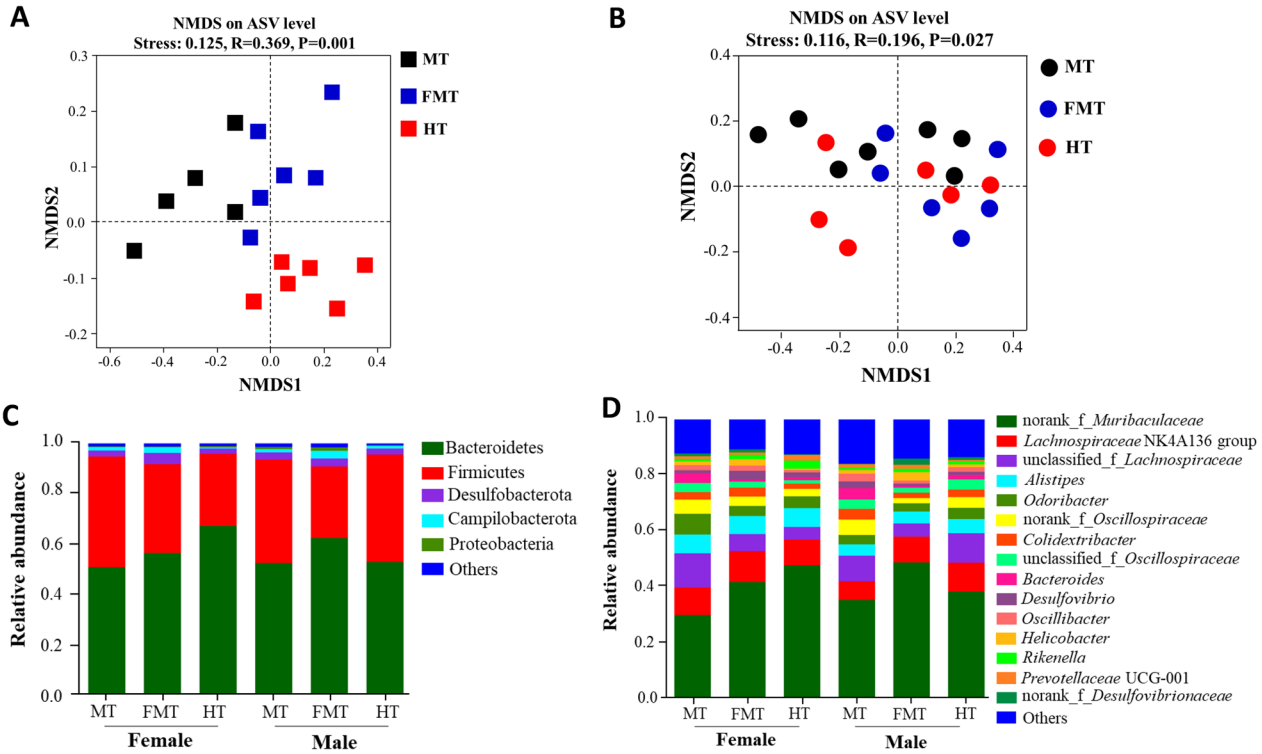

Fig. 4 Alteration in microbial compositions caused by high humidity or FMT. NMDS scores based on Bray-Curtis distance of gut microbiota in female (**A**) and male (**B**) MRL/lpr mice. Relative percentage abundance of the identified phylum (**C**) and genus (**D**) of MRL/lpr mice in six groups. N = 6 mice/group for gut microbiota analysis

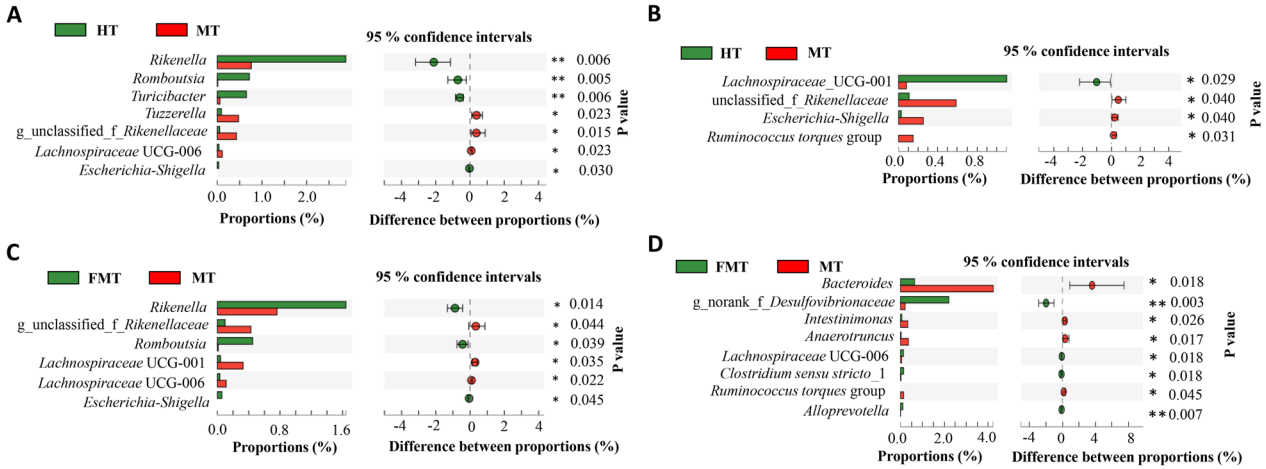

Fig. 5 Altered genera caused by high humidity or FMT in MRL/lpr mice. Different genera between MT and HT groups in female (**A**) and male (**B**) MRL/lpr mice. Different genera between MT and FMT groups in female (**C**) and male (**D**) MRL/lpr mice. MT: model group; FMT: fecal microbiota transplantation group; HT: high humidity group. N = 6 mice/group for gut microbiota analysis

abundances of *Ruminococcus torques* group, *Escherichia-Shigella*, and g_unclassified_f_*Rikenellaceae* (Fig. 5B).

Furthermore, FMT induced significant changes in the relative abundance of six genera in female MRL/lpr mice and eight genera in male MRL/lpr mice. Notably, *Lachnospiraceae* UCG-006 exhibited divergent responses to FMT between female and male MRL/lpr mice (Fig. 5C and D). Moreover, there was a considerable overlap between the genera affected by FMT and

humidity in female MRL/lpr mice, with upregulation of *Rikenella*, *Romboutsia*, and *Escherichia-Shigella* as well as downregulation of g_unclassified_f_*Rikenellaceae* and *Lachnospiraceae* UCG-001 (Fig. 5A and C). In male MRL/lpr mice, FMT significantly altered the composition of gut microbiota by increasing the relative abundance of *Clostridium sensu stricto* 1, g_norank_f_*Desulfovibrionaceae*, and *Alloprevotella*, while decreasing the relative abundance of *Bacteroides*, *Intestinimonas*, *Anaerotruncus*, and *Ruminococcus torques* group (Fig. 5D).

Discussion

Climate factors have been associated with the risk of SLE [8, 10], yet conflicting findings exist regarding the association between humidity and SLE. Moreover, no experimental investigations have been conducted to elucidate the impact of humidity on SLE or its underlying mechanisms involved. This study pioneering in presenting evidence that high humidity can affect lupus activity in MRL/lpr mice.

High humidity (80 ± 5%) significantly exacerbated lupus in female MRL/lpr mice, while it had no significant effect in male MRL/lpr mice. The gender-based disparity in the progression of lupus in MRL/lpr mice may account for the inconsistent impact of high humidity on lupus activity. At 16 weeks of age, female MRL/lpr mice exhibited peak lupus activity, while male MRL/lpr mice was in early stage of lupus disease [27]. Additionally, the varying impact of high humidity on lupus activity highlights the gender bias present in SLE [28]. Prior research has established a correlation between gut microbiota and lupus disease progression [16], as well as gender disparities in lupus [29]. There, the modifications to gut microbiota may serve as one of the underlying mechanisms that account for the diverse impacts of high humidity on lupus in female and male MRL/lpr mice. Specifically, the differential changes in gut bacterial genera induced by high humidity between male and female MRL/lpr mice could elucidate why high humidity exacerbates lupus symptoms in females.

The study revealed that the genera *Rikenella*, *Romboutsia*, and *Turicibacter* exhibited significant increases in female MRL/lpr mice under high humidity, while no noticeable changes were observed in male MRL/lpr mice. Notably, previous studies have demonstrated a positive correlation between the genus Rikenella and lupus activity in both lupus mice [30] and SLE patients [16]. Furthermore, *Rikenella* has been demonstrated to exhibit a positive correlation with inflammatory factors and is associated with chronic systemic inflammatory disorders, indicating it contribution to the promotion of inflammation [31–33]. Meanwhile, the potential pathobiont *Romboutsia* has been observed to increase in abundance in various diseases, such as irritable bowel syndrome [34], ulcerative colitis [35], and depression [36]. During the summer season, high humidity and temperature may result in an increase intestinal water content, which could potentially lead to a rise in the abundance of *Romboutsia* [37]. Genus *Turicibacter* is consistently observed among the top ten genera with the highest levels and serves as a biomarker for MRL/lpr mice [30] and SLE patients [38]. Furthermore, an elevation in *Turicibacter* has been positively correlated with the deterioration of lupus nephritis in SNF1 lupus-prone mice when exposed to acidic water [39]. Conversely, genus *Escherichia-Shigella* displayed a contrasting pattern of alteration due to high humidity between female and male MRL/lpr mice. Previous research has demonstrated the enrichment of *Escherichia-Shigella* in SLE patients [40, 41]. As a well-known pathogen, *Escherichia-Shigella* has been reported to modulate both innate and adaptive immune responses [42]. In summary, alterations in gut microbiota have been linked to the exacerbation of lupus symptoms in female MRL/lpr mice under high humidity.

FMT is a classical method for further elucidating the role of gut microbiota following observations of alterations in its composition [43–45]. In this study, FMT was employed to investigate the contribution of gut microbiota to lupus exacerbation under high humidity conditions. The findings revealed that FMT exacerbated lupus in female MRL/lpr mice but not male counterparts, possibly due to increased abundances of genus *Rikenella*, *Romboutsia*, and *Escherichia-Shigella*. These results suggest that gut microbiota serves as a mediator for the impact of high humidity on lupus in female MRL/lpr mice.

Conclusion

This study presents the initial evidence that high humidity can exacerbate lupus in female MRL/lpr mice by modulating gut microbiota. These findings illuminate the underlying biology of how high humidity impacts lupus and provide hypotheses for future investigations into the influence of climate on SLE. However, the limitations of this study were as follows: (1) this study failed to account for gender-based disparities in the impact of humidity on lupus; (2) this study did not utilize germ-free mice to investigate the mechanism underlying the association between lupus exacerbation and four microbial genera (*Rikenella*, *Romboutsia*, *Turicibacter*, and *Escherichia-Shigella*); (3) This study did not elucidate the mechanism by which high humidity exacerbates lupus from the perspective of the host. Nonetheless, this research contributes to a more profound comprehension of the reciprocal

communication between gut microbiota and ambient humidity in the progression of SLE.

Abbreviations

SLE	Systemic lupus erythematosus
FMT	Fecal microbiota transplantation
NMDS	Non-metric multidimensional scaling
DAO	Diamine oxidase
ASVs	Amplicon sequence variants
MT	Model group
HT	High humidity group
H&E	Hematoxylin and eosin
ANA	Anti-nuclear antibodies
Anti-dsDNA	Anti-double-stranded DNA
SRA	Sequence read archive
FDR	False discovery rate

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Author contribution
HC and CW contributed toward conceiving the research. CW conducted animal experiments. CW, YL and LC assisted in conducting the experiments. HC and CW analyzed the data and drafted the manuscript. All authors contributed to the article and approved the submitted version.

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Availability and data materials
Raw sequencing reads of 16 S rRNA sequencing described have been deposited in the NCBI Sequence Read Archive under accession number: PRJNA949349.

Declarations

Ethics approval and consent to participate
All animal handling and experimental procedures were performed following local ethical committees and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering and to reduce the number of animals used. All procedures performed in this study involving animals were approved by the Ethics Committee of Wenzhou Hospital of Integrated Traditional Chinese and Western Medicine.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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