Dynamics of Gut Microbiota in Autoimmune Lupus

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Gut microbiota has been recognized as an important environmental factor in health, as well as in metabolic and immunological diseases, in which perturbation of the host gut microbiota is often observed in the diseased state. However, little is known on the role of gut microbiota in systemic lupus erythematosus. We investigated the effects of host genetics, sex, age, and dietary intervention on the gut microbiome in a murine lupus model. In young, female lupus-prone mice resembling women at childbearing age, a population with the highest risk for lupus, we found marked depletion of lactobacilli, and increases in *Lachnospiraceae* and overall diversity compared to age-matched healthy controls. The predicted metagenomic profile in lupus-prone mice showed a significant enrichment of bacterial motility- and sporulation-related pathways. Retinoic acid as a dietary intervention restored lactobacilli that were downregulated in lupus-prone mice, and this correlated with improved symptoms. The predicted metagenomes also showed that retinoic acid reversed many lupus-associated changes in microbial functions that deviated from the control. In addition, gut microbiota of lupus-prone mice were different between sexes, and an overrepresentation of *Lachnospiraceae* in females was associated with an earlier onset of and/or more severe lupus symptoms. *Clostridiaceae* and *Lachnospiraceae*, both harboring butyrate-producing genera, were more abundant in the gut of lupus-prone mice at specific time points during lupus progression. Together, our results demonstrate the dynamics of gut microbiota in murine lupus and provide evidence to suggest the use of probiotic lactobacilli and retinoic acid as dietary supplements to relieve inflammatory flares in lupus patients.

The mammalian gut harbors trillions of microorganisms known as the microbiota (1). Increasing evidence in recent years suggests that the host microbiota and immune system interact to maintain tissue homeostasis in healthy individuals (2–6). The importance of microbiota for the host is highlighted by altered immune responses in the absence of commensal bacteria, where both systemic and gut-specific lymphoid tissues are generally smaller and less developed (7). Higher susceptibility to infectious pathogens (8) and, in some cases, attenuated symptoms in autoimmune disorders (2), have been observed in mice raised under germfree conditions. Indeed, perturbation of the host microbiota, especially that in the gut, is shown to be associated with many diseases. These include inflammatory bowel disease (9, 10), metabolic syndrome (e.g., obesity [11–15] and type 2 diabetes [16], and autoimmune diseases, e.g., type 1 diabetes [17–20], rheumatoid arthritis [5, 21–23], and multiple sclerosis [24, 25]).

However, whether gut microbiota differ in systemic lupus erythematosus (lupus) versus healthy individuals has not been reported. Lupus is an autoimmune disorder characterized by the generation of autoantibodies, recruitment of autoreactive or inflammatory T cells, and abnormal production of proinflammatory cytokines (26, 27). The hallmark of the disease is severe and persistent inflammation that leads to tissue damage in multiple organs, including kidneys, lungs, joints, heart, and brain (26). According to the Lupus Foundation of America, about 2 million Americans currently live with the disease. The prevalence ranges from 20 to 200 cases per 100,000 persons, with higher prevalence for people of African, Hispanic, or Asian ancestry. Although the disease affects both males and females, women of childbearing age are diagnosed nine times more often than men. African-American women suffer from more severe symptoms and a higher mortality rate. The cause of lupus is unclear and there is currently no cure. To date, treatment and prevention of disease flares have relied on long-term use of immunosuppressants where side effects, such as susceptibility to infections, are of particular concern (28). There is a need for better understanding of the disease and for better approaches in lupus treatment and management.

Here, we report the dynamics of gut microbiota in a murine lupus model. Disease-, sex-, and time-dependent differences were identified. Using vitamin A as a potential intervention to reduce lupus nephritis (29–32), we also showed that specific changes of the gut microbiota strongly correlated with disease attenuation. Predicted metagenomes associated with murine lupus and vitamin A treatments were also described.

MATERIALS AND METHODS

Mice. MRL/Mp (MRL), MRL/Mp-*Fas*[lpr] (MRL/lpr, stock number 000485), C56BL/6 (B6), and B6.MRL-*Fas*[lpr] (B6/lpr) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred and maintained in a specific-pathogen-free facility according to the requirements of Institutional Animal Care and Use Committee at Virginia Polytechnic Institute and State University. All four strains had been housed in the same room under the same condition for at least 12 months prior to the initiation of the study. The breeding rotations were 3 months for MRL and MRL/lpr due to the short life span of MRL/lpr and 6 months for mice of B6 background. All-*trans*-retinoic acid (RA) and all-*trans*-retinyl palmitate

(RP) were purchased from Sigma (St. Louis, MO) and prepared and used in the dark to avoid exposure to light. Both retinoids were dissolved in canola oil (vehicle) and administered orally, directly into the mouth, to mice from 6 to 14 weeks of age. For RA treatment, 6 mg RA/kg of body weight was given per day. For the daily VARA (vitamin A-retinoic acid) treatment, 11.2 mg RP/kg of body weight (equivalent to 6 mg retinol/kg) was mixed with 0.6 mg RA/kg of body weight (10% of the amount of retinol) before being given to the mice. Mice were weighed twice weekly, and the retinoid doses were adjusted accordingly.

Microbiota sampling, DNA extraction, and PCR. Fecal microbiota samples were obtained by taking individual mice out of their cage and collecting a fecal pellet. Colonic microbiota samples were collected within 20 min after euthanasia. To avoid cross-contamination, each microbiota sample was collected by using a new pair of sterile tweezers. All samples were stored at −80°C till being processed at the same time. Sample homogenization and cell lysis was aided by mixing 0.1-g portions of samples with 0.1-mm sterile zirconia beads using a bead-beater (BioSpec Mini-Beadbeater-16) for 45 s. DNA was extracted by a phenol-chloroform method [14]. The V4 region (ca. 252 bp) of 16S rRNA gene was PCR amplified with 515F and 12-base GoLay barcoded 806R primers [33]. The purified amplicons were sequenced bidirectionally (paired-end 150 bp) on an Illumina MiSeq at Argonne National Laboratory.

Taxonomy assignments and diversity analyses. Contigs were made from the paired-end reads by using the fastq-join program with a minimum overlap of 10 bp and minimum percent identity of 95% [34]. High-quality reads with Phred score of ≥20 were obtained by using QIIME (Quantitative Insights Into Microbial Ecology) [35]. Chimeric sequences were identified with usearch [36] and removed from further analysis. A total of 2,021,399 nonchimeric sequences were mapped into 1,005 operational taxonomic units (OTU) in the Greengene reference database (version 13.5, gg13.5) with the program uclust [36]. Sequences without a match to gg13.5 were clustered into new OTU at 97% similarity. Taxonomy was assigned by using a naive Bayes classifier [37] trained with the Greengenes taxonomy with more than 200,000 reference sequences [38]. A phylogenetic tree was constructed from aligned sequences representing each OTU. For beta diversity calculations, microbiota used for comparison were randomly sampled to the same depth coverage. Principal coordinates were calculated from unweighted UniFrac metrics [39]. Alpha diversity metrics, including phylogenetic diversity [40] and the Shannon index, were calculated by using QIIME.

Renal function. Proteinuria was measured weekly with Chemstrip 2GP from 12 to 14 weeks of age (Roche, Indianapolis, IN). A scale of 0 to 4 was used for proteinuria, corresponding to no protein, trace amounts of protein (5 to 20 mg/dl), and 30, 100, and ≥500 mg/dl total protein, respectively. When mice were euthanized at 14 weeks of age, kidneys were fixed in formalin for 24 h, paraffin embedded, sectioned, and stained with hematoxylin and eosin at the Histopathology Laboratory at Virginia-Maryland Regional College of Veterinary Medicine. Slides were read with an Olympus BX43 microscope. All slides were scored in a blinded fashion by a certified veterinary pathologist. Glomerular lesions were graded on a scale of 0 to 3 for each of the following five categories: increased cellularity, increased mesangial matrix, necrosis, the percentage of sclerotic glomeruli, and the presence of crescents. Renal function was calculated as follows: (the composite glomerular score ÷ 5) + the average proteinuria score in the last 3 weeks.

Metagenomic prediction. Bacterial metagenomes were predicted using PICRUSt [41] by comparing 16S to database gg13.5 and then to Integrated Microbial Genomes (IMG). The OTU were mapped to the gg13.5 database at 97% similarity by using the QIIME's pick_closed_otus script. OTU abundance was normalized by using the 16S rRNA gene copy numbers from known bacterial genomes. The normalized OTU were used for metagenomes prediction in PICRUSt. The predicted Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologs [42] was summarized to level 3 functional categories and compared among groups by using the Statistical Analysis of Metagenomic Profile package [43]. Differentially represented

gene families were identified by two-sided Welch's *t* test with Storey's false-discovery-rate correction.

Statistics. Two-sample comparison was performed by unpaired Student *t* test and Mann-Whitney test. For >2 groups, one-way analysis of variance (ANOVA) was performed. If the overall effect is significant, pairwise comparisons were made by using unpaired *t* test with *P* values adjusted for Tukey's honest significance differences (TukeyHSD). Two-way ANOVA was used to determine the effect of sex and strain. Correlation analysis (Pearson for continuous variables or Spearman for categorical variables) was performed. Principal coordinate analysis was tested for significance by a permutational multivariate method PERMANOVA [44]. All statistics were calculated by using R version 3.0.2.

Nucleotide sequence accession numbers. The 16S rRNA gene sequences from the present study were deposited in the Metagenomics RAST server under the accession number 4557629.3. The original sequences were also deposited in the Sequence Read Archive under accession number SRP045474.

RESULTS

Lupus-associated gut microbial composition and diversity. We have used the classical lupus-prone MRL/Mp-*Fas^lpr* (MRL/lpr) mice to model systemic lupus erythematosus [45]. Mice homozygous for the mutation *Fas^lpr* show systemic autoimmunity, lymphadenopathy, and glomerulonephritis [46], which are similar to lupus-associated manifestations in humans. Female MRL/lpr mice die at an average age of 18 weeks. Starting at about 12 weeks of age, the levels of circulating autoantibodies and immune complexes increase greatly in MRL/lpr mice. The control mouse strain, MRL/Mp (MRL), does not show lupus-like symptoms before 18 weeks of age [47].

Women of childbearing age have the highest risk for lupus [26]. We thus compared the gut microbiota of female MRL versus MRL/lpr mice at 5 weeks of age that mimic this population ($n = 3$ per strain). We found a clear distinction in the microbiota revealed by principal coordinate analysis (Fig. 1A). UniFrac distances between mouse strains were significantly higher than distances within strains (TukeyHSD test, $P < 0.005$, Fig. 1B), indicating an overall phylogenetic difference. The gut microbiota of lupus-prone MRL/lpr mice were characterized by a significant reduction in the family *Lactobacillaceae* (under "Firmicutes_ Bacilli") and a concurrent increase in the family *Lachnospiraceae* (under "Firmicutes_Clostridia"), which together accounted for more than 65% of all bacteria in the distal gut (Fig. 1C and D). A total of 105 operational taxonomic units (OTU) representing all *Lactobacillaceae* sequences were further classified to genus *Lactobacillus*, with the majority (92 of the 105 OTU) being uncultured species of this genus (see Table S1, highlighted rows, in the supplemental material). The *Lachnospiraceae* family comprises a phylogenetically diverse group of anaerobes defined as *Clostridium* cluster XIVa [48] that includes butyrate-producing genera such as *Butyrivibrio* and *Roseburia* [49]. The predominant *Lachnospiraceae*-affiliated OTU were uncultured (see Table S2, highlighted rows, in the supplemental material). Other lupus-enriched families included *Ruminococcaceae* (uncultured) and *Rikenellaceae* (genus *Alistipes*) (Fig. 1D). Rare OTU (defined as <0.1% of total bacteria from all samples) accounted for <3.5% of individual microbiota (see Fig. S1 in the supplemental material). Among them, the RF9 group ("Tenericutes_Mollicutes"), *Clostridiales* family XIII, and the *Streptococcaceae* were significantly enriched in lupus-prone mice (see Fig. S1 in the supplemental material).
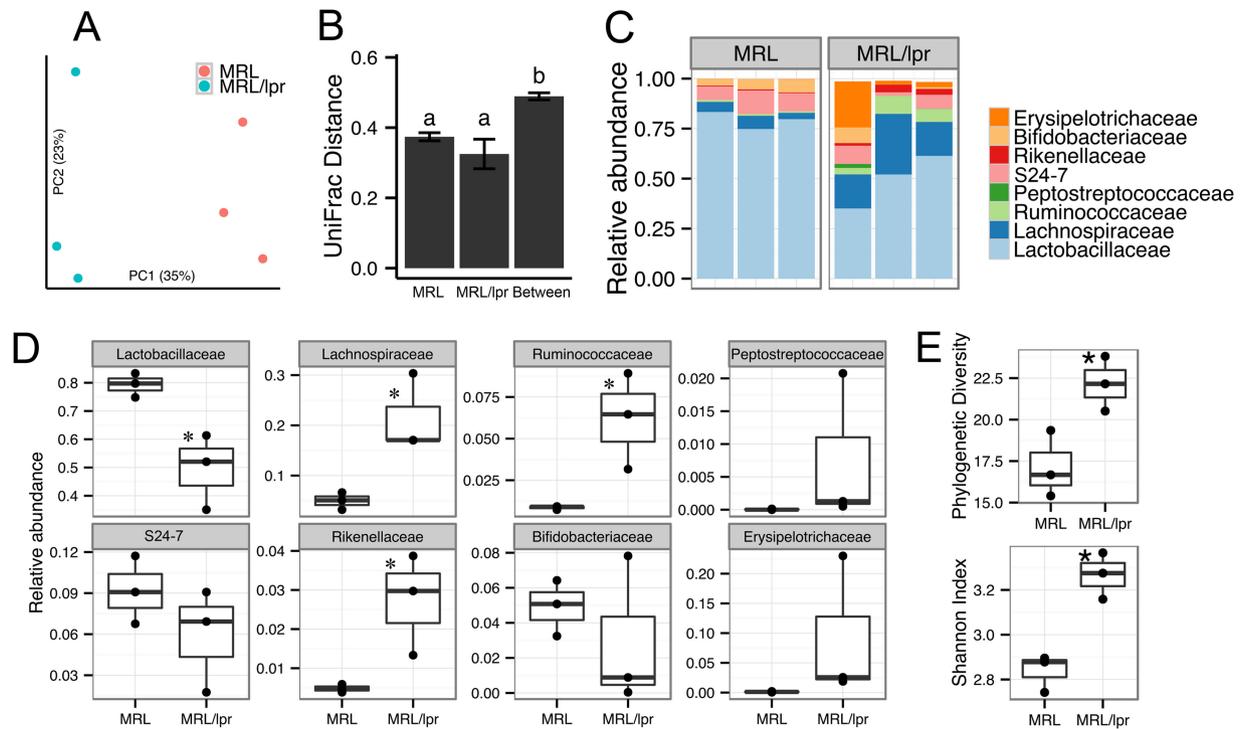
FIG 1 Differences in gut microbiota between healthy and lupus-prone mice. (A) Microbiota separation on the first two principal coordinates calculated from unweighted UniFrac distances. MRL, healthy control mice; MRL/lpr, lupus-prone mice. Fecal samples from 5-week-old female mice were used. The number of sequences in each of the six samples was 7,800. (B) UniFrac distances within and between mouse strains. Error bars indicate the standard errors of the mean for $n = 3$ (within MRL), $n = 3$ (within MRL/lpr), and $n = 9$ (between the two strains). Statistical significance was determined by one-way ANOVA, followed by pairwise t test with TukeyHSD-adjusted P values. (C) Taxonomy breakdown at the family level for abundant ($>0.1\%$) bacterial OTU. (D) Bacterial abundance comparison between MRL and MRL/lpr microbiota. (E) Diversity indices. Statistical comparison was based on an unpaired Student t test ($n = 3$ in each group; *, $P < 0.05$; **, $P < 0.01$).

Overall, gut microbiota in lupus-prone mice had a higher diversity measured as Faith's phylogenetic diversity and Shannon's entropy (Fig. 1E). These results show that lupus-prone mice have distinct bacterial composition and diversity compared to healthy controls, suggesting that lupus could alter gut microbiota.

Microbiota dynamics associated with sex and age in murine lupus. Lupus is a sex-specific disease affecting more women than men (26). In the MRL/lpr model, while lupus develops spontaneously in both females and males, female mice exhibit accelerated disease (50). We thus analyzed the gut microbiota separately in female and male mice (Fig. 2A and see Fig. S2A in the supplemental material). To try to minimize the cage effect as a confounding factor, we randomly selected one mouse of each sex from four different litters for each mouse strain (MRL or MRL/lpr, $n = 4$ per strain). In 14-week-old females, comparing lupus-prone MRL/lpr mice to MRL controls, significantly lower levels of *Lactobacillaceae* and higher levels of *Lachnospiraceae* were noted (Fig. 2A), a finding consistent with our observations in 5-week-old females (Fig. 1D). In males, however, the two strains of mice were not significantly different from each other in their gut microbial composition (Fig. 2A). Since sex and disease status appeared to interact, we also compared female and male mice within each mouse strain. In MRL mice, females had significantly higher *Lactobacillaceae* and *Streptococcaceae* levels and lower *Lachnospiraceae* and *Clostridiaceae* levels than did male mice (Fig. 2A and see Fig. S2B in the supplemental material). In contrast, in the lupus-prone MRL/lpr group, female mice had higher levels of *Lachnospiraceae* and *Bac-*

teroidetes S24-7, and lower levels of *Bifidobacterium* and *Erysipelotrichaceae*, whereas the levels of *Lactobacillaceae* were comparable between female and male mice (Fig. 2A). Principal coordinate analysis showed distinct microbiota between sexes, albeit the difference depended on mouse strains (see Fig. S2C in the supplemental material). Although no sex-dependent difference in bacterial diversity was observed in control mice, female lupus-prone mice had significantly higher diversity than their male counterparts (see Fig. S2D in the supplemental material). These results suggest that the gut microbiota of lupus-prone mice are different between sexes and that female-specific changes in certain bacterial phylotypes (such as the higher abundance of *Lachnospiraceae*) may be associated with an earlier onset of and/or more severe lupus symptoms in female MRL/lpr mice.

We next determined time-dependent changes of gut microbiota in murine lupus. Because MRL/lpr mice have a short life span of approximately 18 weeks (47), we chose to investigate the time course in another lupus-prone model with the same *Fas^lpr* mutation. B6.MRL-*Fas^lpr* (B6/lpr) mice, which are of C57BL/6 (B6) background, have a life span of at least 7 months. Both MRL/lpr and B6/lpr mice start to develop lupus-like symptoms at about 2 months of age, but disease progression is slower in B6/lpr mice (51–53). We found that the gut microbiota of female B6/lpr mice was distinct from healthy B6 mice, as shown by principal coordinate analysis (Fig. 2B, $n = 3$ per strain). In this pair of mouse strains we observed dynamic changes in microbiota composition over a 5-month time period (Fig. 2C). Notably, B6/lpr mice had
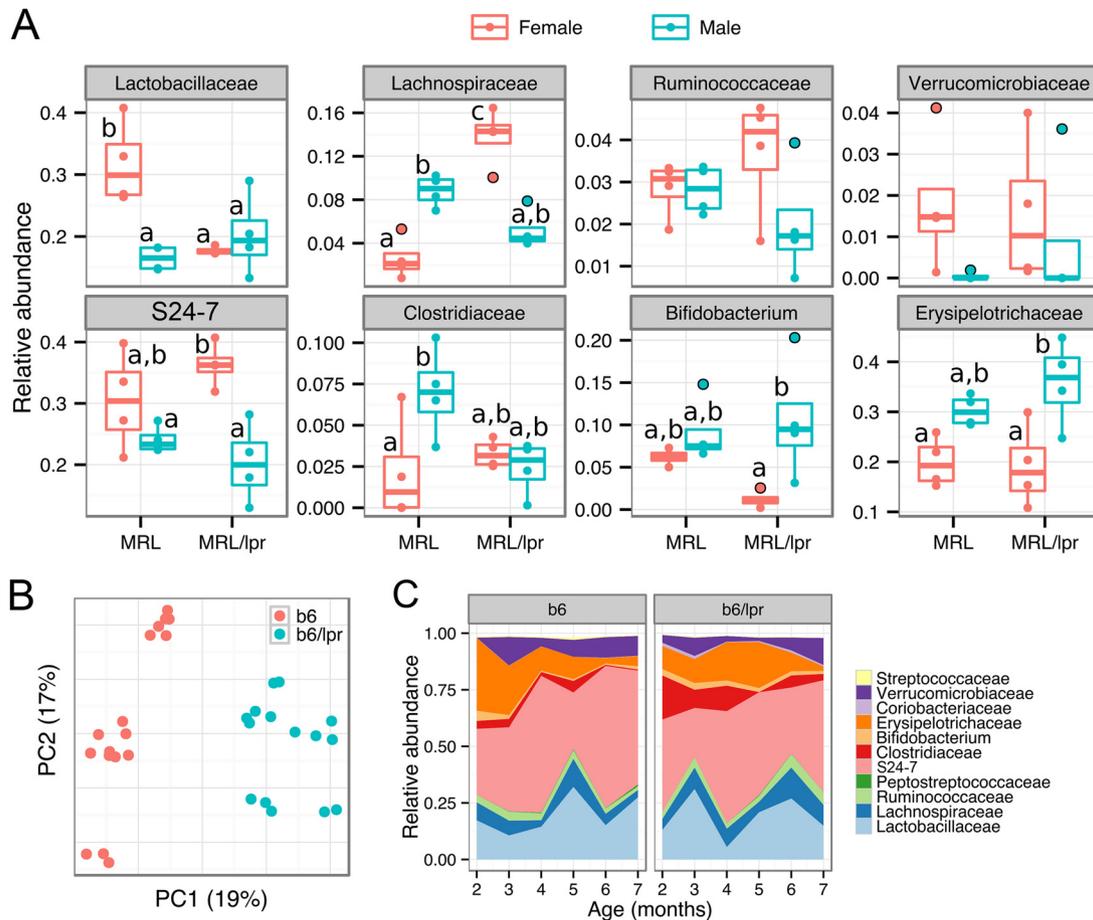
FIG 2 Microbiota dynamics associated with sex and age. (A) Sex-associated microbiota differences. Colonic contents from 14-week-old MRL and MRL/lpr mice were used. Statistical significance was determined by two-way ANOVA, followed by pairwise *t* test with TukeyHSD-adjusted significance ($n = 4$ in each group). (B and C) Age-associated microbiota differences. (B) Fecal microbiota separation on the first two principal coordinates calculated from unweighted UniFrac distances. b6, female healthy control mice; b6/lpr, female lupus-prone mice. (C) Time-dependent changes of fecal microbiota in b6 and b6/lpr strains. Abundant bacterial OTU ($>0.1\%$) were summarized ($n = 3$ per group).

higher levels of *Clostridiaceae* than did B6 mice from 2 to 5 months ($P < 0.05$, unpaired *t* test), a time frame wherein B6/lpr mice have just started to show lupus-like symptoms. The family *Clostridiaceae* includes the butyrate producers in *Clostridium* cluster I (48). In addition, the abundance of *Lactobacillaceae* oscillated in the guts of both B6/lpr and B6 mice, whereas *Lachnospiraceae* appeared to be more abundant in B6/lpr mice during the last month ($P = 0.003$, Fig. 2C). These results suggest that the composition of gut microbiota change over time in both healthy and lupus-prone mice. Certain bacterial phylotypes, such as *Clostridiaceae* and *Lachnospiraceae* (both butyrate producers), are more abundant in the guts of B6/lpr mice at specific time points during lupus progression.

Restoration of lactobacilli and improvement of lupus symptoms with RA. Vitamin A has been shown to reduce lupus nephritis (29–32). However, while orally administered vitamin A may come directly in contact with commensal bacteria in the intestinal tract during absorption, little is known about whether and how this nutrient affects gut microbiota. We thus investigated the effects of vitamin A (retinol) and a major physiological active metabolite of vitamin A, retinoic acid (RA), on gut microbiota in the MRL/lpr mouse model. We treated female MRL/lpr mice, orally

and daily, with RA at 6 mg/kg of body weight (suspended in canola oil as the vehicle) from 6 to 14 weeks of age. RA of comparable doses and with similar treatment time frames was previously reported to reduce spleen and lymph node sizes and improve renal pathology in murine lupus models (29, 30). To mimic natural vitamin A supplementation, where retinol (retinyl palmitate) is the primary ingredient, we also treated female MRL/lpr mice daily with 11.2 mg retinyl palmitate/kg of body weight (equivalent to 6 mg retinol/kg) mixed with 0.6 mg RA/kg of body weight (10% of the amount of retinol) from 6 to 14 weeks of age. The small amount of RA in this formulation, which we refer to as VARA, has been shown to enhance the storage of retinol in the body (54). Because the vehicle itself (canola oil, which contains polyunsaturated fatty acids) may affect the gut microbiota (55–57), MRL/lpr mice treated with canola oil were used as the diseased control, whereas MRL mice treated with canola oil were used as the healthy control. To try to minimize the cage effect as a confounding factor, we randomly selected female MRL/lpr mice from four different litters and housed them in four cages. Each cage had one mouse randomly assigned to vehicle treatment, one with RA, and a third with VARA. The MRL mice were from two different litters.
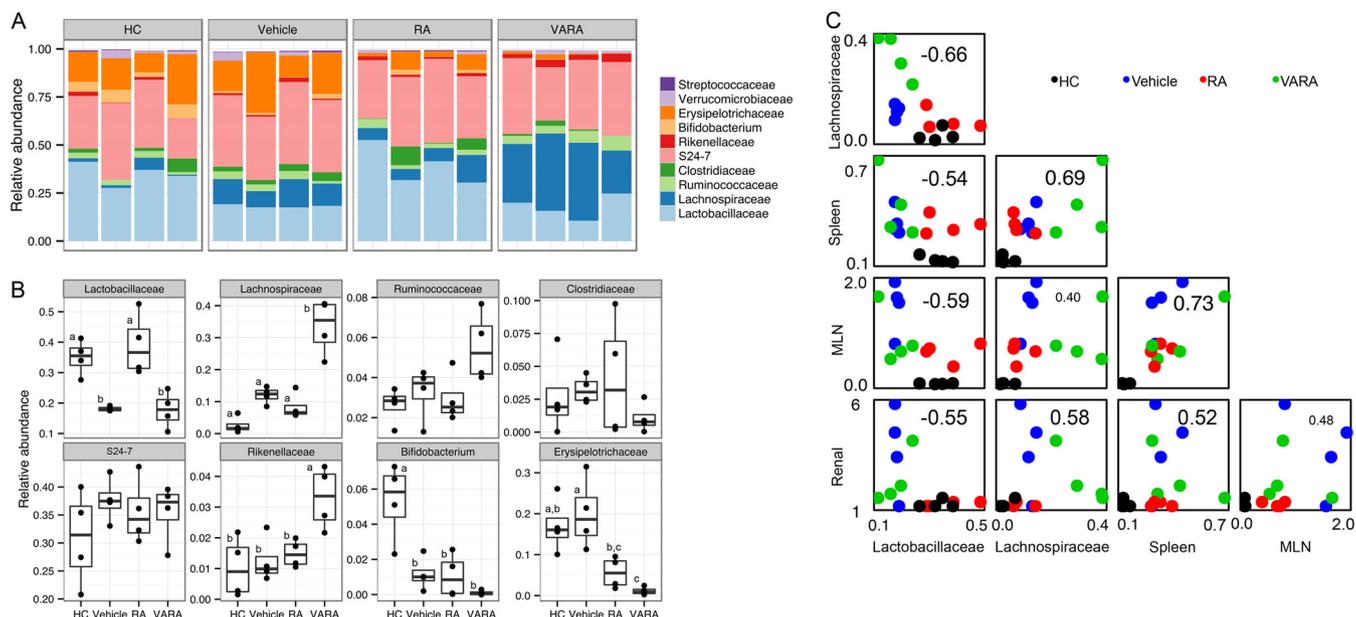
FIG 3 Changes of bacterial abundance with vitamin A treatments. (A) Taxonomy breakdown at the family level abundant (>0.1%) bacterial OTU. HC, MRL healthy control treated with canola oil; vehicle, lupus-prone MRL/lpr treated with canola oil; RA, MRL/lpr treated with retinoic acid (6 mg/kg of body weight); VARA, MRL/lpr treated with retinol (6 mg retinol/kg of body weight) and retinoic acid (0.6 mg/kg of body weight, which facilitates retinol storage). All mice were female and treated orally from 6 to 14 weeks of age. Mice were sacrificed at 14 weeks of age and colonic contents were collected ($n = 4$ per group). (B) Comparison of individual families shown in panel A. Statistical significance was determined by one-way ANOVA, followed by a pairwise t test with TukeyHSD-adjusted significance. (C) Correlation between bacterial abundance and lupus disease indices. Spleen, spleen weight in grams; MLN, mesenteric lymph node weight in grams; renal, renal function estimated from proteinuria and glomerular scores as described in Materials and Methods. Correlation coefficients are shown on each individual plot. For example, the correlation between *Lactobacillaceae* and *Lachnospiraceae* was −0.66. Spearman's correlation tests were used for pairs involving ranked renal function data; all other correlations were calculated by using the Pearson method. Raw data can be found in the supplemental material.

Colonic contents were collected after treatment and the microbiota were analyzed.

Gut microbiota after vitamin A treatments were not significantly different from the vehicle control among MRL/lpr groups based on principal coordinate analysis (see Fig. S3A in the supplemental material, $n = 4$ per group). However, we noted a number of significant differences in the taxonomy breakdown at the family level for abundant bacterial OTU. Strikingly, we found that RA restored *Lactobacillaceae* (Fig. 3A and B) that was downregulated in MRL/lpr mice (Fig. 1D, Fig. 2A, and Fig. 3B). VARA, on the other hand, did not have such an effect. Rather, it significantly increased the levels of *Lachnospiraceae* and *Rikenellaceae* (Fig. 3B), two bacterial families that were already higher in MRL/lpr than MRL mice, at least for 5-week-old mice (Fig. 1D). Importantly, we observed strong correlations between the abundance of two bacterial families, *Lactobacillaceae* and *Lachnospiraceae*, and lupus disease indices (Fig. 3C and see Table S3 in the supplemental material). Lupus disease parameters, lymphadenopathy (increased spleen and mesenteric lymph node weights) and glomerulonephritis (impaired renal function indicated by increasing scores), correlated negatively with the abundance of *Lactobacillaceae* and positively with *Lachnospiraceae* in the gut (Fig. 3C; $P < 0.05$, except for lymph node weight versus *Lachnospiraceae*, where $P = 0.124$). This suggests that RA improved lupus symptoms while restoring intestinal colonization of *Lactobacillaceae*, whereas VARA worsened the symptoms while increasing the abundance of *Lachnospiraceae* that were already higher in lupus-prone mice. Whether retinoid-mediated changes of gut microbiota directly affected lupus symptoms or they were by-products of the effects of vitamin A on lupus requires further investigation.

Both RA and VARA also decreased the levels of *Erysipelotrichaceae* compared to the vehicle control in MRL/lpr mice (Fig. 3B). In addition, lupus-prone mice had significantly lower levels of *Bifidobacterium* compared to the healthy control, and neither RA nor retinol restored the abundance of this bacterial family (Fig. 3B). Lower representation of *Erysipelotrichaceae* and *Bifidobacterium* could potentially make available more substrates for other commensal bacteria, such as *Lactobacillaceae* in the case of RA treatments and *Lachnospiraceae* in the case of VARA treatments (Fig. 3B). Consistent with prior observations (Fig. 1E), vehicle-treated MRL/lpr microbiota had higher diversity than vehicle-treated MRL microbiota based on Shannon's entropy analysis, whereas neither RA nor VARA treatments changed this phenomenon (see Fig. S3B in the supplemental material) despite their effects on lupus symptoms, suggesting that microbial diversity in the gut may depend on genetic predisposition of the mouse strains but not their phenotype.

Changes of microbiome metabolic functions with lupus and vitamin A treatments. To investigate microbiome functions associated with lupus, we used PICRUSt to infer putative metagenomes from 16S rRNA gene profiles of 14-week-old female MRL and MRL/lpr mice (41). Comparing the predicted gut microbiome of lupus-prone mice to that of the control, categories representing genes important for genetic information processing (e.g., DNA replication and repair, protein synthesis) and nucleotide metabolism were significantly lower in MRL/lpr microbiome (Fig. 4A), indicating reduced turnover of bacterial DNA, RNA, and protein synthesis (58). This was accompanied by a significant increase in cell motility genes (e.g., bacterial chemotaxis and flagellar assembly) in the gut microbiome of lupus-prone mice
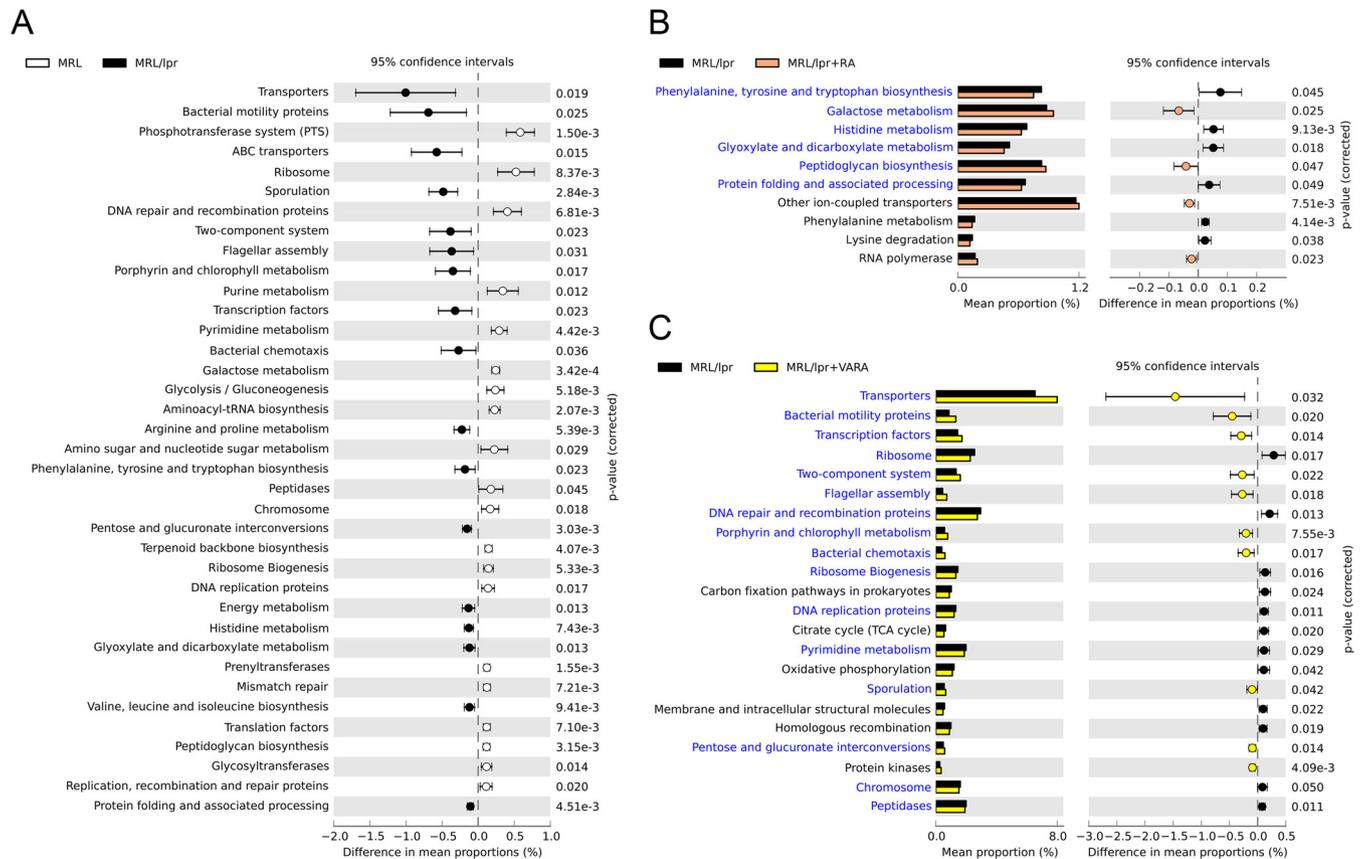
FIG 4 Bacterial metagenomes predicted from microbial community identities. Gene functional categories were from level 3 of KEGG pathways. Gene functions with a significant difference are shown ($P < 0.05$). (A) Differences between MRL control and lupus-prone MRL/lpr mice. The effect size was 0.10%. ABC transporters, ATP-binding cassette transport systems. (B) Effect of RA treatment on lupus-prone bacterial metagenomes. The effect size was 0.02%. (C) Effect of retinol treatment (VARA) on lupus-prone bacterial metagenomes. The effect size was 0.08%.

(Fig. 4A), suggesting that the bacteria may be actively accessing substrates and/or adjusting their locations (59). Sporulation genes also increased with lupus disease, possibly due to the increase of spore-forming clostridia (Fig. 2A). In the carbohydrate metabolism category, galactose metabolism and glycolysis decreased, while glyoxylate and dicarboxylate metabolism increased with lupus disease (Fig. 4A). Lupus-associated increases in membrane transport (e.g., ATP-binding cassette transporters), signal transduction (two-component system), and amino acid metabolism were also observed (Fig. 4A).

To assess whether vitamin A treatments could affect gut microbiome functions in lupus-prone mice, we performed a similar analysis between vehicle- and RA-treated MRL/lpr mice (Fig. 4B), as well as between vehicle- and VARA-treated MRL/lpr mice (Fig. 4C). Metabolic functions that overlapped with those in Fig. 4A were identified and color-coded in blue. Interestingly, we found that RA reversed many lupus-associated changes in microbial functions that deviated from the MRL control (Fig. 4B). These included genes in carbohydrate metabolism (e.g., galactose metabolism, glyoxylate and dicarboxylate metabolism) and amino acid metabolism (e.g., histidine metabolism, and phenylalanine, tyrosine and tryptophan biosynthesis). In contrast, VARA worsened lupus-associated changes on all overlapped functional categories, including cell motility, membrane transport, DNA replication and repair, protein synthesis, signal transduc-

tion, and sporulation (Fig. 4C). Together, these results suggest that microbiome metabolic functions in the gut could change with lupus disease. RA treatment, which attenuated symptoms, reversed lupus-associated changes in microbial gene functions. VARA, on the other hand, exacerbated the changes of metagenomic profiles in lupus-prone mice while worsening the disease, suggesting a possible correlation between gut microbiome and disease symptoms in lupus.

DISCUSSION

In this study, we reported the dynamics of gut microbiota in murine lupus. By using lupus-prone mice carrying the Fas^{lpr} mutation, we showed that the composition and diversity of gut microbiota differed between control and diseased mice (Fig. 1). Many differences were sex specific and varied over time during lupus progression (Fig. 2). In addition, we found that the use of different vitamin A treatments that increased abundance of *Lactobacillaceae* in the intestine correlated with improved lupus symptoms, whereas greater colonization of *Lachnospiraceae* correlated with disease deterioration (Fig. 3). Through prediction analysis of metagenomes (Fig. 4), we also showed that certain functions, such as bacterial motility, increased with lupus disease in mice. In addition, gut microbiome metabolic functions appeared to correlate with disease severity in lupus.

Members of the *Lactobacillaceae* family have been recom-

mended as probiotics with anti-inflammatory functions (60). *Lactobacillus rhamnosus* GG, for example, suppresses proinflammatory responses by increasing interleukin-10 and regulatory T (Treg) cells (61, 62). Several other members of *Lactobacillaceae*, including *L. acidophilus* and *L. reuteri*, were also found to induce anti-inflammatory Treg cells (63, 64). Although colonization of *Lactobacillaceae* in the guts of both humans and mice has been shown to suppress inflammation (65, 66), a direct relationship between intestinal *Lactobacillaceae* and autoimmune lupus has not been established. In the present study we show that lower colonization of lactobacilli in the gut is associated with disease state in lupus, an autoimmune disease characterized by chronic inflammation.

Several groups have recently reported that short-chain fatty acids produced by gut microbiota, especially butyrate produced by *Clostridia*, promote the differentiation of Treg cells to suppress inflammation (67–70). However, in the present study, we found that both young (5-week-old) and old (14-week-old) female MRL/lpr mice had higher levels of *Lachnospiraceae*, where butyrate-producing *Clostridium* XIVa belongs, than their MRL counterparts (Fig. 1D and Fig. 2A). In the B6/lpr model, the levels of *Lachnospiraceae*, as well as of another butyrate-producing family, *Clostridiaceae*, were also higher in lupus-prone mice compared to B6 controls (Fig. 2C). Moreover, we found strong positive correlations between the abundance of *Lachnospiraceae* and lupus disease parameters, including lymphadenopathy and renal pathology (Fig. 3C). All of these results suggest that *Lachnospiraceae*, or butyrate-producing bacteria in general, may not be able to suppress inflammation in the lupus-prone MRL/lpr and B6/lpr models. Indeed, a recent report has shown that in mice that are deficient in Fas signaling (e.g., *Fas^{lpr}* mutation), butyrate-induced apoptosis of T cells is abolished (71). Thus, even though *Lachnospiraceae* accumulated in MRL/lpr and B6/lpr mice, the butyrate that they produced would lack the mechanism required for T-cell apoptosis. This would likely lead to uncontrolled proinflammatory T-cell responses and thereby lupus progression. The notion is supported by our observation that further changes of the abundance of *Lachnospiraceae* through vitamin A intervention of MRL/lpr mice failed to alter the percentages of different T-cell populations in the periphery (data not shown).

Many autoimmune disorders are more prevalent in females (72). Sex-specific differences in gut microbiota have not been reported until recently, where microbiota was found to influence sex bias in type 1 diabetes (19, 20). In nonobese diabetic (NOD) mice, females were found to possess greater microbial diversity than male counterparts (19). In addition, it was found that the levels of *Alistipes* (family *Erysipelotrichaceae*) were higher in male NOD mice, although the difference was not statistically significant (20). Our data from the MRL/lpr model agreed with the above reports that female mice had significantly higher microbial diversity (see Fig. S2D in the supplemental material), and *Erysipelotrichaceae* was significantly enriched in the gut microbiota of male MRL/lpr mice (Fig. 2A). In addition to these differences, we found that in lupus-prone mice, females had significantly higher levels of *Lachnospiraceae*, whereas males had significantly higher levels of *Bifidobacterium* (Fig. 2A). *Bifidobacterium*, like lactobacilli, has been suggested to exert anti-inflammatory functions (73). Thus, a greater abundance of *Bifidobacterium* in the gut may be associated with attenuated lupus symptoms in males. It was also noted that the variation of the abundance of some bacteria appeared to be

greater in females than in males (see, for example, *Verrucomicrobiaceae* in Fig. 2A). This could be due to the lack of synchronization of the estrus cycle, since microbiota may change according to the female reproductive cycle (74).

Important roles of vitamin A and its derivatives, including RA, in regulating antiviral and/or antibacterial immune responses have been recognized since the 1920s (75). However, whether and how RA affects lupus is not clearly understood. We found here that treatment with RA attenuated lupus-like symptoms while increasing the *Lactobacillaceae* family of commensal bacteria in the colons of lupus-prone MRL/lpr mice (Fig. 3C). RA can sustain the stability and function of natural Treg cells (76) and promote the differentiation of inducible Treg cells in the periphery (77–79), which may in turn attenuate lupus. *Lactobacillus* spp. can also suppress proinflammatory responses by increasing the number of inducible Treg cells (61, 62). Thus, it is likely that RA and *Lactobacillaceae* that RA can enrich in the gut of lupus-prone mice (Fig. 3A and B) could work in concert to induce Treg cells and attenuate inflammatory flares in lupus.

Retinol is the primary ingredient in vitamin A supplements. In this report we found that retinol, represented by VARA treatment, increased the fraction of *Lachnospiraceae* in the gut of MRL/lpr mice (Fig. 3B) that was already higher than that in MRL mice (Fig. 1D). It did not attenuate lupus (Fig. 3C) or affect members of the *Lactobacillaceae* family (Fig. 3B). This suggests that unlike RA, retinol may promote intestinal colonization of *Lachnospiraceae* that does not attenuate lupus pathogenesis. In fact, the percentage of *Lachnospiraceae* in MRL/lpr mice negatively correlated with that of *Lactobacillaceae* and positively correlated with lymphadenopathy of the spleen and impaired renal function (Fig. 3C). Therefore, our results suggest that RA, given as a direct dose, may be better than retinol in suppressing inflammation in lupus. Metagenomic predication of microbial metabolic functions, where RA was shown to reverse, while retinol (VARA) exacerbated, lupus-associated changes (Fig. 4), supports this hypothesis.

The composition of gut microbiota can be greatly altered by the housing condition. In the present study, all mice were maintained in the same room under the same specific-pathogen-free housing condition. In addition, mice in each experimental group were randomly selected from different litters to eliminate the cage effect as a potential confounding factor. We kept control and lupus animals in different cages to avoid the possible effect of coprophagy on disease progression. It has been noted, however, after the completion of the study, that the combination of housing condition adaptation and dam cohousing between control and lupus strains prior to their respective breeding could have further equilibrated the baseline microbiota.

Altogether, our study showed several important lupus-associated changes in gut microbiota. Intestinal colonization of *Lactobacillaceae*, in particular, was found to negatively correlate with lupus activity. This suggests that probiotics containing lactobacilli may be able to decrease the occurrence and/or severity of inflammatory flares suffered by lupus patients, but future studies are required to address whether a causal relationship between lactobacilli and lupus exists. In addition, the potential therapeutic benefit of probiotics against lupus may be enhanced when combined with RA, a dietary supplement that can increase the levels of lactobacilli in the gut and attenuate lupus symptoms.

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