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Impact of dietary deviation on disease progression and gut microbiome composition in lupus-prone SNF_1 mice

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Summary

Environmental factors, including microbes and diet, play a key role in initiating autoimmunity in genetically predisposed individuals. However, the influence of gut microflora in the initiation and progression of systemic lupus erythematosus (SLE) is not well understood. In this study, we have examined the impact of drinking water pH on immune response, disease incidence and gut microbiome in a spontaneous mouse model of SLE. Our results show that (SWR × NZB) F_1 (SNF_1) mice that were given acidic pH water (AW) developed nephritis at a slower pace compared to those on neutral pH water (NW). Immunological analyses revealed that the NW-recipient mice carry relatively higher levels of circulating autoantibodies against nuclear antigen (nAg) as well as plasma cells. Importantly, 16S rRNA gene-targeted sequencing revealed that the composition of gut microbiome is significantly different between NW and AW groups of mice. In addition, analysis of cytokine and transcription factor expression revealed that immune response in the gut mucosa of NW recipient mice is dominated by T helper type 17 (Th17) and Th9-associated factors. Segmented filamentous bacteria (SFB) promote a Th17 response and autoimmunity in mouse models of arthritis and multiple sclerosis. Interestingly, however, not only was SFB colonization unaffected by the pH of drinking water, but also SFB failed to cause a profound increase in Th17 response and had no significant effect on lupus incidence. Overall, these observations show that simple dietary deviations such as the pH of drinking water can influence lupus incidence and affect the composition of gut microbiome.

Keywords: autoimmunity, microbiota, drinking water pH, immune modulation, segmented filamentous bacteria, systemic lupus erythematosus

Introduction

Systemic lupus erythematosus (SLE) is a complex autoimmune disorder that affects multiple organs, including the kidneys, skin, heart and lungs [1]. Disease arises when abnormally functioning B lymphocytes produce autoantibodies to DNA and nuclear proteins, resulting in immune complexes that cause damage to the tissue. While the triggers are not known, it is widely accepted that SLE is the consequence of a complex interaction between genetic and environmental factors. Dietary factors can also have a profound impact on the gut and systemic immune responses, and a recent study showing the effect of high sodium salt intake on experimental autoimmune encephalitis (EAE) progression [2,3] supports this notion. Studies using mouse models have demonstrated that different dietary factors can influence SLE pathogenesis [2]. Calorie restriction improved SLE survival by eliminating immunoglobulin (Ig)A and IgG2 autoantibodies as well as the increased secretion of interleukin (IL)-12 and interferon (IFN)-γ [2,4]. Moreover, vitamin D, as a dietary supplement, has shown immunomodulatory potential. It can inhibit the B cell activation and differentiation into plasmablast and subsequent immunoglobulin production, increase regulatory T cells (T_{regs}) and lower T helper type 1 (Th1) and Th17 cell frequencies [2,5–7]. In addition to direct effects on the systemic and gut immune cell

functions, dietary factors can change the composition of gut microbiota (dysbiosis), further affecting the immune homeostasis [8].

In this regard, a recent report from our laboratory demonstrated that the pH of drinking water influences type 1 diabetes (T1D) incidence in non-obese diabetic (NOD)/Ltj mice [9]. NOD mice that were maintained under specific pathogen-free (SPF) conditions and received acidified drinking water (AW) developed T1D more rapidly and showed higher disease incidence compared to mice that were given neutral pH drinking water (NW). This difference in disease progression was found to be associated with a change in the composition and diversity of gut microbiome when the pH of drinking water was switched from acidic to neutral. This report also showed that segmented filamentous bacteria (SFB)-associated protection of NOD mice from T1D can be influenced by the pH of drinking water [9].

Given the difference in the characteristics of autoimmune responses in T1D and SLE, we investigated the influence of drinking water pH on disease progression in the SLE prone (SWR × NZB) F_1 (SNF_1) mouse model. Here we show that, in contrast to T1D in NOD mice [9], SNF_1 mice maintained on AW showed slower lupus progression and delayed proteinuria compared to NW recipients. Examination of serum autoantibodies revealed that AW recipient mice have relatively lower overall levels of antibodies against anti-nuclear antigens. We also found that mucosal immune response in NW recipients is dominated by Th17- and Th9-associated cytokines. In addition, we show that NW- and AW-recipient mice acquire significantly diverse gut microbial communities, and the presence of SFB, unlike the other autoimmune disease models [9–12], has no significant effect on Th17 response or on the progression of lupus in SNF_1 mice.

Materials and methods

Mice

SWR and NZB mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and housed under SPF conditions at the animal facilities of Medical University of South Carolina (MUSC). SWR × NZB F_1 (SNF_1) hybrids were bred at the SPF facility of MUSC. C57BL/6 mice were purchased from Taconic Farms, Inc. (TAC, Hudson, NY, USA). Female mice were used throughout the study. SNF_1 mice were maintained on autoclaved neutral pH (7.0–7.2) water (NW) or acidic pH (3.0–3.2) water (AW), as described previously [9]. Parental breeding cages were also maintained on either NW or AW to ensure that SNF_1 mice used in this study are exposed to a specific type of drinking water throughout their lives. All animal experiments were performed according to ethical principles and guidelines of the Institutional Animal Care and Use Committee (IACUC) of MUSC. This study was approved by the IACUC of MUSC.

Proteinuria

Urine samples were tested weekly for proteinuria. Protein level in the urine was determined by Bradford assay (BioRad, Hercules, CA, USA) against bovine serum albumin standards. Proteinuria was scored as follows; 0: 0–1 mg/ml, 1: 1–2 mg/ml, 2: 2–5 mg/ml, 3: 5–10 mg/ml and 4: $\geq$10 mg/ml. Mice that showed proteinuria $>$ 5 mg/ml were considered to have severe nephritis in this study.

Enzyme-linked immunosorbent assay (ELISA)

Antibodies against nucleohistone and dsDNA in mouse sera were evaluated by ELISA. Briefly, 0·5 μg/well of nucleohistone (Sigma-Aldrich, St Louis, MO, USA) or 1 μg/well dsDNA from calf thymus (Sigma-Aldrich) was coated as antigen, overnight, onto ELISA plate wells in a carbonate buffer. Serial dilutions of the sera were made and IgG, IgG1, IgG2a, IgG3 or IgM were detected using horseradish peroxidase (HRP)-conjugated anti-mouse antibodies [Sigma-Aldrich, eBioscience (San Diego, CA, USA) and Invitrogen, Carlsbad, CA, USA)]. In addition, total IgA levels were determined in 72-h spent medium from the cells of mesenteric lymph nodes (MLN), Peyers' patches (PP) and small intestine (SI).

Quantitative polymerase chain reaction (PCR)

RNA was extracted from 2-cm pieces of the distal ileum using Isol-RNA Lysis Reagent (5 Prime), according to the manufacturer's instructions. cDNA was prepared from RNA using Moloney murine leukaemia virus (MVLV) reverse transcriptase (Promega, Madison, WI, USA) and PCR was performed using SYBR green master-mix (Bio-Rad) and target-specific custom-made primer sets (Supporting information, Table S1). A StepOne Plus (Applied Biosystems, Carlsbad, CA, USA) real-time PCR machine was used and relative expression of each factor was calculated by the 2-ΔCT cycle threshold method against β-actin control.

16S rRNA gene-targeted sequencing and bacterial community profiling

Total DNA was prepared from the caecum samples for bacterial community profiling. Briefly, faecal and crushed intestinal samples were processed using Isol-RNA Lysis Reagent (5 Prime), as recommended by the manufacturer, centrifuged at 16,200xg and DNA was precipitated from the interphase and organic phase using 0·1 M trisodium citrate in 10% ethanol. DNA was washed, dried and suspended in 8 mM NaOH, and the insoluble material was

removed by centrifugation at 16,200xg. DNA in the samples was amplified by PCR using 16S rRNA gene-targeted primer sets to assess the bacterial levels.

16S rRNA gene sequencing was performed using two different platforms. For 12-week-old SNF_1 mouse samples, the Illumina MiSeq (San Diego, CA, USA) platform was employed to sequence the V3–V4 region of the 16S rRNA gene at MUSC genomic centre. Sequencing reads were preprocessed using QIIME version 1.8.0 [13] to produce high-quality reads. We required Q20 over at least 95% of the read length for a read to be retained, and the reads containing ambiguous characters were removed. This step produced 2 039 988 high-quality reads, which then were chimera-checked using usearch61 [14]. Reads identified as chimeric sequences were removed and the resulting 1 854 135 were fed into QIIME open reference operational taxonomic units (OTU) picking pipeline using uclust and the 97_otus sequences of the GreenGenes (release of 13_8). We required a minimum of five reads in an OTU to be retained. This procedure produced a total of 6849 OTUs, using 1 812 290 reads (~98% of the input sequences). Then alpha and beta diversity measures were calculated after rarefying the BIOM file to 53 000 sequences per sample. The OTUs were normalized and used for metagenomes prediction of Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologues employing PICRUSt, as described previously [15–18]. The predictions were summarized to multiple levels and level 3 functional categories were compared among NW- and AW-recipient groups using the statistical Analysis of Metagenomic Profile Package (STAMP), as described previously [16].

For 50-week-old nephritic SNF_1 mouse samples, we used the Ion Torrent (Guilford, CT, USA) platform to sequence the V4–V5 region of the 16S rRNA gene. This sequencing and data analysis was performed by Molecular Research LP (MR-DNA, Shallowater, TX, USA). For the analysis, raw sequences were identified by their unique barcodes and analysed using QIIME as described above. OTUs were classified taxonomically using the Basic Local Alignment Search Tool (BLAST) against GreenGenes database and compiled into each taxonomic level [13,19,20]. The OTUs were compiled to different taxonomical levels based upon the percentage identity to reference sequences (i.e. $> 97\%$ identity) and the percentage values of sequences within each sample that map to respective levels were calculated.

Microbiota transfer

To transfer microbiota, content from the chilled caecum of AW-recipient mice were collected and suspended in cold saline and administered to NW-recipient mice by oral gavage. Content from one donor caecum was used for six recipient mice. This process was repeated for 3 consecutive days and the recipients, along with NW- and AW-recipient controls, were tested for proteinuria and autoantibody levels every week, as described above.

SFB-containing faecal transplant

SFB faecal transplant was carried out as described in our previous study [9]. Briefly, faecal pellets and bedding materials from cages of SFB^+ C57BL/6 mice of TAC (on NW) were transferred (faecal transplant; FT) into SNF_1 mouse cages that were on AW or NW every third day for a total of 9 days. On day 10, these FT-recipient mice were switched to fresh bedding materials and monitored for proteinuria. A quantitative PCR (qPCR) assay using SFB-specific 16S rRNA gene-targeted primers was conducted to confirm the transfer of SFB to SNF_1 mice.

Statistical analysis

Proteinuria curves were analysed using the log-rank method and the proteinuria scores were analysed using Fisher's exact test. Student's *t*-test and two-tailed χ^2 test were also employed to calculate *P*-values where indicated. A *P*-value $\leq 0{\cdot}05$ was considered statistically significant, as follows: $^*P \leq 0{\cdot}05$, $^{**}P \leq 0{\cdot}01$, $^{***}P \leq 0{\cdot}001$. GraphPad Prism or Microsoft Excel was used for calculating statistical significance for data from most experiments. All the statistical analyses for microbial sequences were performed employing the two-tailed *t*-test and the *P*-values were corrected from multiple tests using the Benjamini and Hochberg approach.

Results

AW recipients show slow disease progression

SNF_1 female mice develop SLE that is characterized with lethal glomerulonephritis within a mean of 29 weeks and mimic human SLE aetiology [21,22]. To evaluate the effect of drinking water pH on SLE disease progression, we monitored the female SNF_1 mice that were on NW or AW weekly for nephritis by measuring protein levels in the urine for up to 49 weeks of age. Mice that showed $\geq$ 5 mg/ml protein in the urine for 2 consecutive weeks were considered to have severe nephritis. We observed a slower rate of disease progression in mice receiving AW compared to those on NW (Fig. 1a). By 30 weeks of age, $>$ 70% of NW recipients showed severe nephritis compared to approximately 20% of AW recipients (Fig. 1b). A profound difference in the percentage of NW and AW groups of mice with severe nephritis was observed at the age of 34 weeks. While 80% of the NW-recipient SNF_1 mice showed high proteinuria, only approximately 22% of mice on AW were found to have high protein levels in the urine at this age. Although the overall disease incidence rates at $>$ 40 weeks of age were comparable in NW and AW groups of mice, larger numbers of NW-recipient

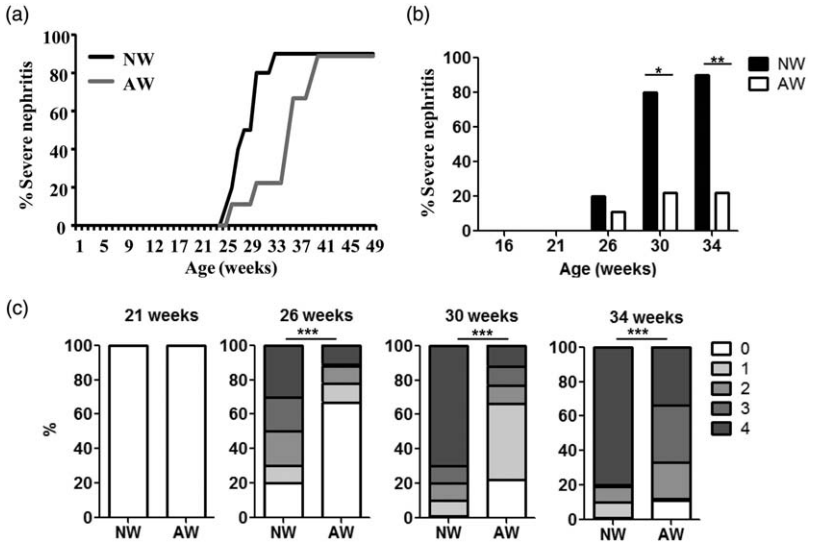

Fig. 1. pH of drinking water influences lupus-like disease progression in SWR × NZB F_1 (SNF_1) mice. Female SNF_1 mice (10/group) were maintained on either neutral pH drinking water (NW) or acidic pH drinking water (AW) and examined for nephritis. (a) Protein levels in urine samples were quantified by Bradford assay for up to 49 weeks of age. Mice showing $\geq$ 5 mg/ml protein in the urine for consecutive weeks were considered to have severe nephritis. (b) Bar graphs show the percentage of mice with severe nephritis for specified time-points. Fisher's exact test was used to calculate the significance. (c) Severity of proteinuria in female mice at different time-points is presented as a percentage for each score. Proteinuria score was given as follows: 0, 0–1 mg/ml; 1, 1–2 mg/ml; 2, 2–5 mg/ml; 3, 5–10 mg/ml; 4, > 10 mg/ml.

mice succumbed to severe disease by this age compared to AW recipients (not shown).

To assess further the nephritis severity in NW- and AW-recipients, proteinuria was graded based on the amount of protein detected in the urine sample. SNF_1 mice that showed protein levels of 0–1, 1–2, 2–5, 5–10 and > 10 mg/ml for 2 consecutive weeks were considered to have a nephritis severity of grades 0, 1, 2, 3 and 4, respectively. As observed in Fig. 1c, 80% of NW-recipient mice had nephritis severity varying from grades 1 to 4 by week 26 of age, while only approximately 30% of AW recipients had mild or severe nephritis. At the age of 30 weeks, 100% of NW recipients showed grades 2–4 nephritis compared to approximately 30% of AW recipients with similar disease severity. These results demonstrate that the pH of drinking water can indeed have a profound influence on disease progression in lupus-prone mice. Intriguingly, acidic pH of drinking water appears to have a protective effect in SLE, as opposed to its T1D-promoting effect that we observed in our recent report [9].

AW-recipient mice showed low anti-nucleohistone and -dsDNA antibody levels and immune cell infiltration in the kidney

SLE is characterized by the production of autoantibodies, mainly IgG and IgM, against chromatin and dsDNA which deposit in the kidney, causing immune cell infiltration and glomerular necrosis [22–25]. Therefore, the serum antibody levels against dsDNA and nucleohistone in NW- and AW-recipient mice were compared by ELISA beginning at 16 weeks of age. As observed in Fig. 2a, relatively lower serum total IgG anti-dsDNA antibody levels were observed in the AW-recipient mice compared to those on NW throughout the monitoring period, and a significant difference was observed at the age of 34 weeks. In addition, IgG2a and IgM levels against dsDNA were found to be significantly lower at the age of 34 and 26 weeks, respectively, in the AW group compared to their NW counterparts. Further, the serum levels of IgG2a and IgM antibodies against nucleohistone complex were lower in AW recipients compared to their NW counterparts at later time-points (26 weeks or 30 and 34 weeks) (Fig. 2a). However, anti-nucleohistone total IgG levels as well as IgG1 and IgG3 isotype antibodies against dsDNA and nucleohistone were comparable between NW and AW mice groups throughout the observation period (not shown).

To evaluate the renal pathology, sections of kidneys from 8-month-old SNF_1 mice that were receiving AW and NW were subjected to haematoxylin and eosin (H&E) staining and examined for immune cell infiltration and glomerular changes. As shown in Fig. 2b, kidney sections of NW recipients showed significantly higher immune cell-infiltrated areas compared to the AW recipients. Importantly, tertiary lymphoid-like structures covered more than 20% of kidney sections from NW recipients and fewer than 5% of the kidney sections had immune cell infiltrates in AW recipients. In addition, changes to the glomeruli were more profound in kidneys from NW recipients compared to that of AW recipients. These

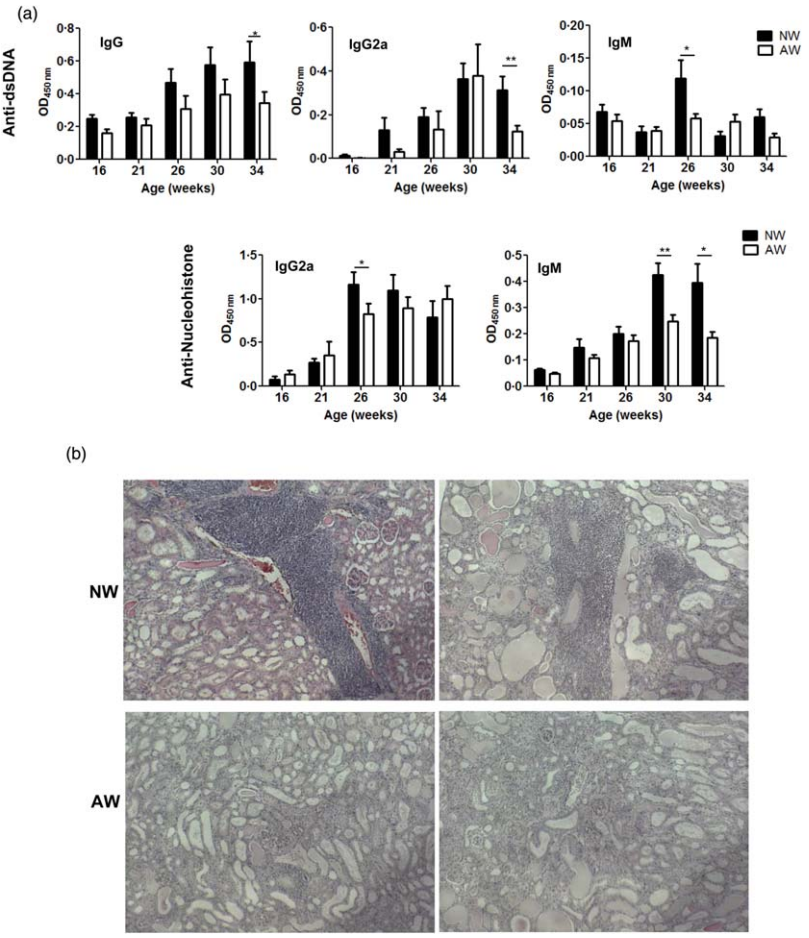

Fig. 2. Acidic pH drinking water (AW)- and neutral pH drinking water (NW)-recipient SWR × NZB F_1 (SNF_1) mice show differences in the serum autoantibody levels. (a) Female SNF_1 mice were maintained on either NW or AW and serum was collected every week. Serum levels of antibodies specific for dsDNA and nucleohistone were assessed by enzyme-linked immunosorbent assay (ELISA) for the indicated time-points. Relative levels of antibodies are presented as absorbance value [optical density (OD) 450 nm]. Each bar represents mean ± standard error of the mean from 10 mice/group for each time-point. (b) Sections of kidneys from 8-month-old NW and AW recipient mice (three/group) were stained using haematoxylin and eosin (H&E), imaged under a light microscope with a ×20 objective and two representative areas are shown. In addition to profoundly higher changes to the glomeruli in NW recipients, sections from NW recipients showed significantly higher immune cell-infiltrated areas compared to the AW recipients.

observations are in correlation with the high proteinuria and autoantibody levels that were observed in the NW recipients compared to their AW counterparts.

Small intestine of NW-recipient mice expresses high levels of proinflammatory cytokines

Because the primary effects of dietary deviations are expected to be on the gut mucosa, immune phenotype of small intestinal tissues from AW- and NW-recipient mice were examined. cDNA were prepared from the distal ileum of 8-month-old AW- and NW-recipient SNF_1 mice and the expression levels of cytokines and transcription factors were examined by qPCR. As shown in Fig. 3, the expression levels of *Il23, Il21*, *IL22*, *Il17*, *Il9*, *Ifnα*, *Ifnβ*, *Il4* and *Irf4* were profoundly higher in NW recipients compared to AW group of mice. Conversely, no considerable difference in the expression levels of *Tnfα*, *Il1β* and *Ifnγ* were detected in the small intestine of NW group of mice compared to AW recipients. Modestly higher expression levels of *Il6* and *Rorγt* were also observed in the NW group of mice. Overall, these results further support the notion that immune responses initiated in the gut of NW- and AW-recipient mice are different, and may be contributing to differences in the rate of disease progression.

AW- and NW-recipient SNF_1 mice at nephritic stage show differences in the composition of gut microbiome

Our previous study on T1D has demonstrated that the pH of drinking water can affect the acquisition of gut commensals as well as influence the overall composition of microbiome [9]. Importantly, recent studies have shown that changes in the composition of gut microbiome (dysbiosis) can have an effect on the immune homeostasis and disease outcome under autoimmune and inflammatory conditions [10,12,26–31]. Therefore, we examined whether microflora communities in the small intestine are different in NW- and AW-recipient SNF_1 mice described for Fig. 1 upon termination of the experiment. As observed in Fig. 4, 16S rRNA gene sequencing revealed that some of the microbial communities were present in the distal ileum of NW- and AW-recipient mice at significantly different levels. AW recipients showed higher levels of *Lactobacillus reuteri* and *Turicibacter* spp. (both belong to the phylum Firmicutes) compared to their NW recipient counterparts. The average firmicutes/bacteroidetes ratio was relatively higher, albeit not statistically significant, in AW recipients compared to the NW group (Fig. 4a,b). These results suggest that, similar to

Fig. 3. Small intestine of neutral pH drinking water (NW)-recipient mice expresses high T helper type 17 (Th17)/Th9-associated cytokines compared to acidic pH drinking water (AW) recipients. cDNA prepared from the distal ileum of 8-month-old NW- and AW-recipient female SWR × NZB F_1 (SNF_1) mice were subjected to real-time quantitative polymerase chain reaction (PCR) to assess the expression levels of immune cell-associated cytokines and other factors. Expression levels of individual factors were calculated against the value of β-actin. Mean of these values (four to five mice/group) was used for generating the heatmap using the GENE-E application. The lowest value of an individual factor among the two groups of mice was considered as 1 (row minimum) for calculating fold expression values for the other value.

our observations in the NOD mouse model of T1D [9], the pH of drinking water has an influence on the composition of gut microbiome in SNF_1 mice.

The composition of gut microbiome is significantly different in NW and AW recipient prenephritic mice

Because the autoimmune initiation and progression are influenced by immunological events at an early age, microbial communities of AW- and NW-recipient mice at 12 weeks of age (prenephritis) were profiled. Similar to the observations of Fig. 4, prenephritic 12-week-old SNF_1 mice that were receiving NW and AW showed a significant difference in the composition and diversity of gut microbiome (Fig. 5). Both Bray–Curtis (Fig. 5a), unweighted and weighted Unifrac (Supporting information, Fig. S1a) distance measures show significant differences in the gut microbial communities in NW- and AW-recipient mice. Further, the β-diversity measures (observed species; Choa1 and Shannon indices) in AW recipients were significantly low compared to their NW counterparts (Fig. 5b and Supporting information, Fig. S1b) suggesting that AW consumption restricts the acquisition of microbial communities. Compilation of OTUs to different taxonomical levels showed that, at prenephritic stage, the overall firmicutes/bacteroidetes ratio was not significantly different in NW and AW recipients (Supporting information, Fig. S2). However, this analysis revealed that the Rikenellaceae family of bacteria, which belong to the phylum Bacteroidetes, were profoundly higher in NW group of mice (Fig. 5c). In addition, AW recipients showed significantly higher levels of a minor family of bacteria belonging to the Christensenellaceae family (phylum: Firmicutes; order: Clostridiales) compared to AW-recipient mice. PICRUSt application was employed for OTU-based prediction of metabolic functions that may be associated with the lupus disease process. The PICRUSt analysis data of 12-week-old NW- and AW-recipient mice obtained from KEGG pathways helped us to predict processes potentially associated with lupus disease progression in NW recipients compared to the AW group. Predicted pathways that are over-represented in the NW group of mice, which showed rapid disease progression based on 16S rRNA gene identity, include the drug metabolism-related enzymes, flavonoid and phenylpropanoid biosynthesis pathways (Fig. 5d). Conversely, replication, recombination and other DNA repair and phosphotransferase system pathways were under-represented in the NW group of mice. Additional studies are needed to understand if these metabolic pathways contribute to modulation of immune response, disease progression or suppression in lupus. These observations further support the notion that functionally distinct microbial communities are established under NW and AW conditions.

Although approximately 70% of 16S rRNA gene sequences of this analysis were not assigned to any communities at the species level, a statistically significant difference in the richness of microbial communities at species level was observed with a large number of minor communities (Fig. 6). Among these, many members of firmicutes phyla (*Ruminococcus gnavus*, *Peptoniphilus coxii*, *Caloramator mitchellensis*, *Lactobacillus hayakitensis*, *L. intermedius*, *L. siliginis*, *L. equi* and *Peptoniphilus methioninivorax*), except Clostridum genera member (*C. histolyticum* and *C. thermosuccinogenes*), were present at relatively higher levels in AW recipients (Fig. 6). In addition, cyanobacteria member *Trichodesmium hildebrandti*, proteobacteria member *Hydrocarboniphaga daqingensis* and bacteroidetes member *Polaribacter butkevichii* were also present at higher levels in AW recipients. Conversely, relatively higher levels of bacteroidetes members *Pedobacter kwangyangensis* and *Flavobacterium antarcticum*

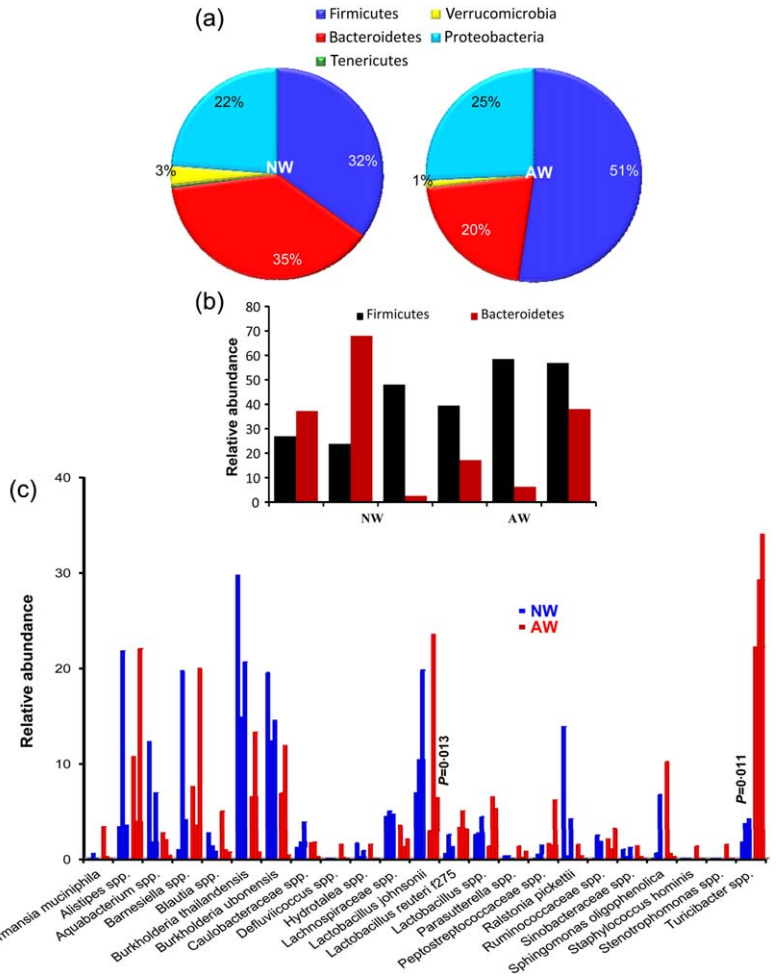

Fig. 4. Acidic pH drinking water (AW)- and neutral pH drinking water (NW)-recipient SWR × NZB F_1 (SNF_1) mice at nephritic stage show differences in the composition of gut microbiome. DNA prepared from the distal ileum of 50-week-old nephritic SNF_1 mice that were on NW or AW (three/group) was subjected to 16S rRNA gene (V4V5)-targeted sequencing using Ion Torrent and the sequences were analysed as described in the Methods. The operational taxonomic units (OTUs) that were compiled to the species level based upon the percentage identity to reference sequences (i.e. > 97% identity) and the percentage values of sequences within each sample that map to specific phylum and species were calculated. (a) Mean relative abundance at phyla level, (b) relative values of firmicutes and bacteroidetes and (c) relative abundance of sequences at species level. Values of individual animals are plotted for (b) and (c), and the *P*-values for the communities that showed statistically significant differences between the NW- and AW-recipient groups are shown. *P*-values were obtained by the two-tailed *t*-test.

were detected in NW recipients. The overall trend, in association with results presented in Fig. 4, suggests that AW consumption promotes gut colonization by firmicutes. These results also indicate that gut colonization by specific microbial communities are influenced by the pH of drinking water even at the prenephritic stage.

Small intestine of NW-recipient SNF_1 mice expresses high levels of IL-23 at prenephritic stage

To understand further the influence of gut microbial communities of NW- and AW-recipient mice on the mucosal immune response, cDNA was prepared from the distal ileum of 12-week-old (prenephritic) AW- and NW-recipient mice and subjected to qPCR to examine the expression levels of cytokines, transcription factors and chemokines. As shown in Fig. 7, the expression levels of *Il23*, *Il21* and *Irf4* were considerably higher in 12-week-old NW recipients compared to AW recipients. Importantly, *Il23* expression levels were profoundly higher in the small intestine of NW recipients compared to AW recipients. Conversely, expression levels of other cytokines, chemokines and transcription factors were comparable in NW- and AW-recipient mice. Importantly, differences in the expression levels of many of the proinflammatory cytokines in 12-week-old mice were not as significantly high as that of 8-month-old mice (Fig. 3), suggesting an age-dependent progressive increase in the expression of Th17- and Th9-associated cytokines in NW-recipient mice compared to their AW counterparts.

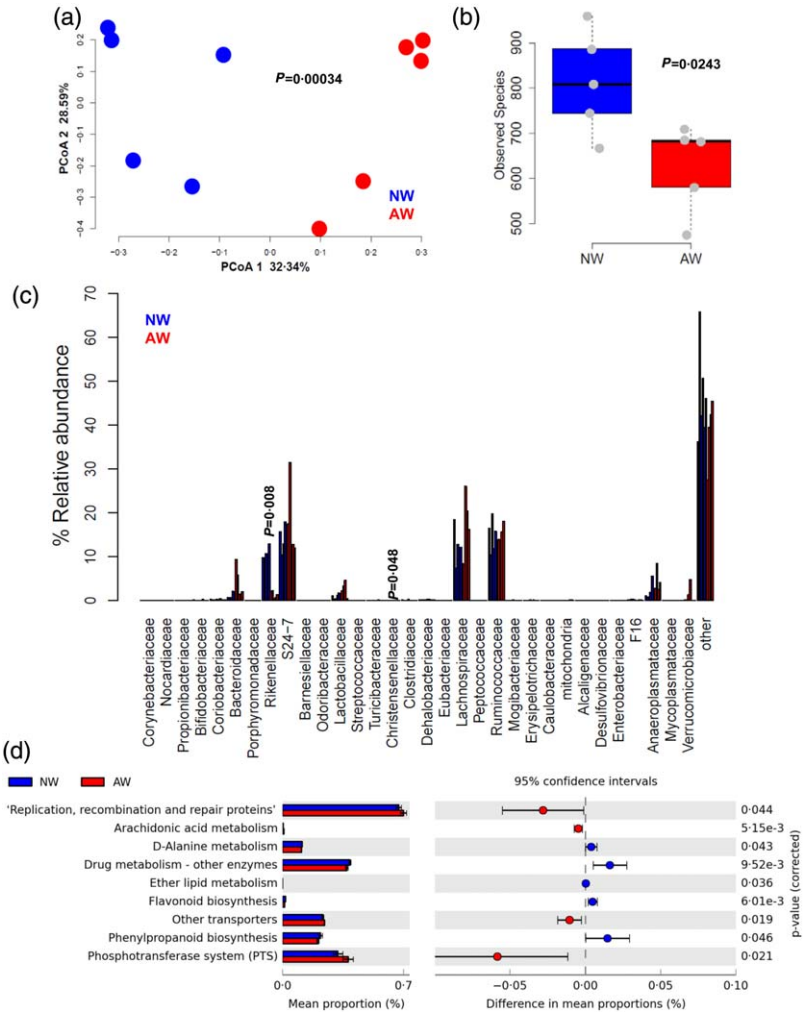

Fig. 5. The composition of gut microbiome in prenephritic stage of acidic pH drinking water (AW)- and neutral pH drinking water (NW)-recipient SWR × NZB F_1 (SNF_1) mice. DNA prepared from the caecum of 12-week-old SNF_1 mice that were on NW or AW (five/group) were subjected to 16S rRNA gene (V3V4 region)-targeted sequencing and the sequences were analysed as described under methods. Then alpha (a, Bray–Curtis distance) and beta diversity (b, species richness) measures were calculated after rarefying the BIOM file to 53 000 sequences per sample. Percentage values of sequences from individual animals that are assigned to family levels were plotted for (c). Sequences that are assigned to species level are shown in Supporting information, Fig. S3. (d) Normalized OTU-BIOM tables were used for predicting gene functional categories using PICRUSt software and the level 3 categories of the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway are shown. Plots were generated using Statistical Analysis of Metagenomic Profile Package (STAMP) software.

These results support the notion that immune responses initiated in the gut of NW- and AW-recipient mice are different.

Gut microbiota of AW-recipient mice suppresses autoimmunity in NW recipients

Because AW recipients showed slower lupus disease progression, caecum microbiota from this group of mice were introduced to NW recipients by oral gavage to determine if the disease progression rate is microbiota-associated. The microbiota-manipulated (AW → NW T) group of mice was monitored along with AW and NW recipients for disease progression. As observed in Fig. 8, the AW → NW T group of mice showed a significant suppression of disease progression, as indicated by low proteinuria and autoantibodies against dsDNA and nucleohistone. Interestingly, the AW → NW T group of mice showed relatively lower proteinuria and autoantibody levels compared to that of AW-recipient controls. Overall, these results suggest that slower disease progression in AW recipients compared to NW recipients could, in fact, be gut microbiota-dependent.

SFB does not appear to have an influence on disease progression in SLE

The mouse gut-resident bacterium, SFB, has been shown to have an influence on the disease outcome in various autoimmune models through induction of Th17 cells [10,11,29]. Our recent study demonstrated that SFB-associated modulation of disease outcome in T1D occurs primarily in AW- but not NW-recipient NOD mice [9]. Colonization of NOD mouse gut by SFB also caused significant increase in Th17 helper cell-associated factors [9,11]. Further, although the influence of SFB on the disease outcome of different autoimmune disorders such as T1D, EAE and arthritis has been studied [10–12], the effect of SFB on lupus incidence in mouse models is not known. Importantly, our results show that NW-recipient SNF_1 mice that develop lupus rapidly compared to AW recipients express higher levels of Th17-associated factors

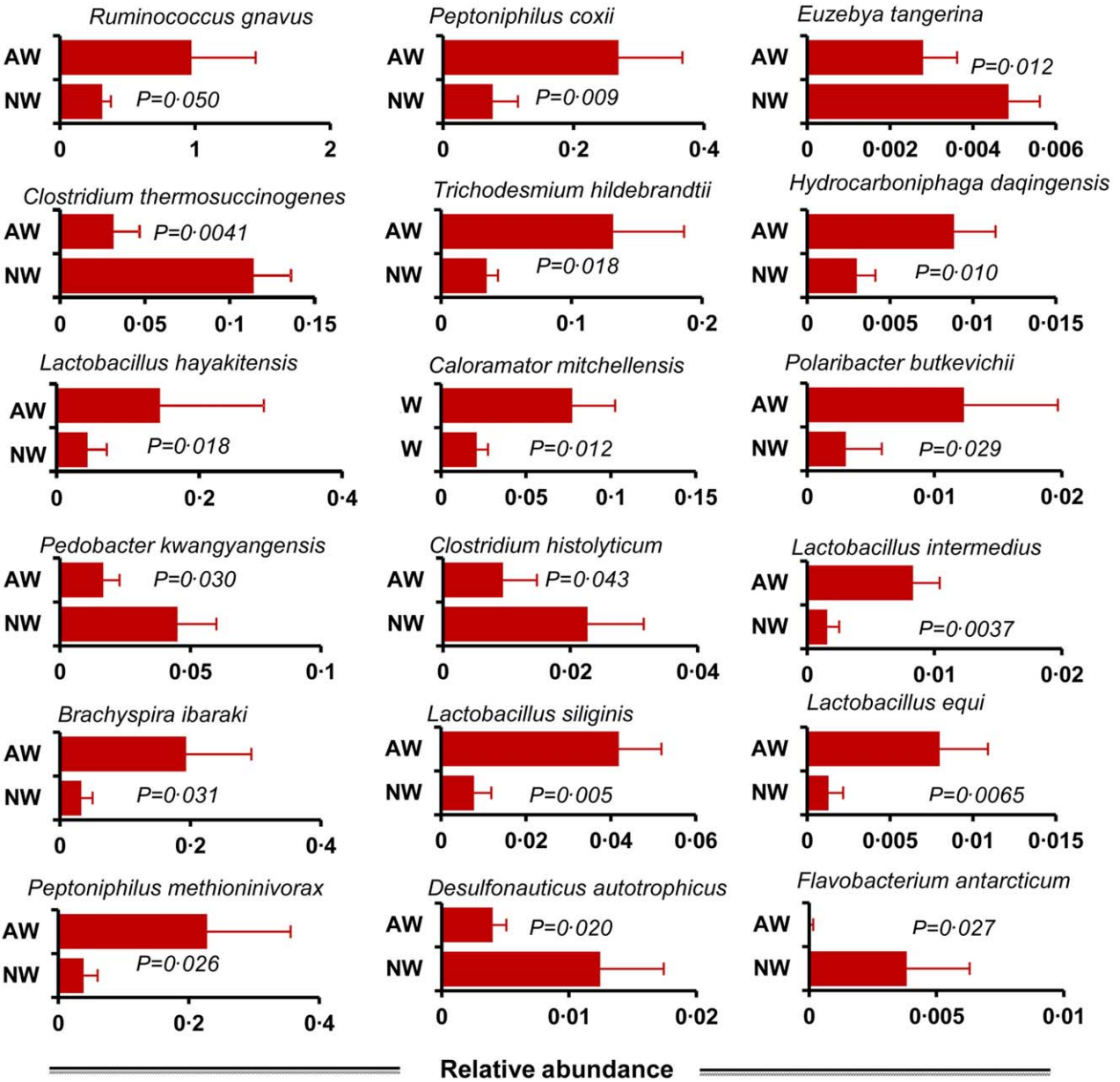

Fig. 6. Neutral pH drinking water (NW) and acidic pH drinking water (AW) recipients show different levels of specific microbial communities. 16s rRNA gene sequences described in Fig. 5 were assigned to the species level based upon the percentage identity to reference sequences (i.e. > 97% identity), as mentioned in the Materials and methods section. Mean relative abundance values of the communities that showed statistically significant differences between NW and AW groups are shown.

in the gut mucosa (Figs 3 and 7). Therefore, we determined if SFB colonization is affected by the pH of drinking water and SFB causes rapid progression of lupus in SNF_1 mice through up-regulating the Th17-type immune response. Faecal pellets from SFB^+ mice were transferred to SNF_1 mouse cages (FT) that were on NW and AW, as detailed in our recent report [9], and monitored for proteinuria. As shown in Fig. 9a, NW- and AW-recipient mice that were subjected to SFB-FT showed the presence of SFB-specific 16S rRNA gene in faecal pellets several months post-FT, indicating effective transfer of the bacteria. SFB^+ NW and AW recipients showed disease progression, as indicated by the proteinuria, comparable to that of their control counterparts (Fig. 9b). Interestingly, the NW control group of mice showed relatively higher expression of Th17-associated factors, including *Il17*, *Il21* and *Il22*, compared to that of the SFB^- NW (NW-FT) group of mice (Fig. 9c). Conversely, SFB^+ AW recipients (NW-FT group) showed a relatively higher expression of Th17-associated factors (*Il17*, *Il21*, *Il22*, *Il23*, *Irf4* and

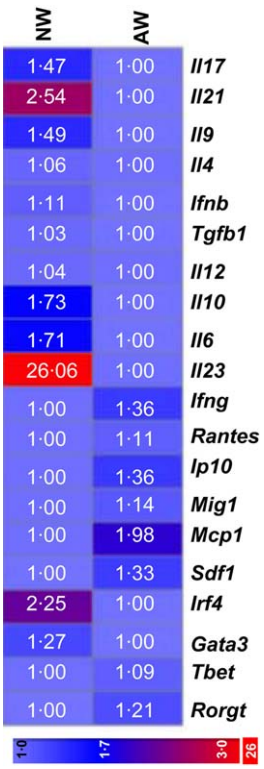

Fig. 7. Expression profile of small intestine from 12-week-old prenephritic SWR × NZB F_1 (SNF_1) mice. cDNA prepared from the distal ileum of 12-week-old neutral pH drinking water (NW)- and acidic pH drinking water (AW)-recipient female SNF_1 mice were subjected to real-time quantitative polymerase chain reaction (PCR) to assess the expression levels of immune-cell associated cytokines, transcription factors and chemokines. Expression levels of individual factors were calculated against the value of β-actin. Mean of these values (four to five mice/group) was used for generating the heatmap using the GENE-E application. The lowest value of an individual factor among the two groups of mice was considered as 1 (row minimum) for calculating fold expression values for the other value.

Rorgt) compared to the AW control groups. These results not only show that SFB-associated gut immune response has no influence on the disease outcome in SNF_1 mice, but also suggest that differences in the mucosal immune response of NW- and AW-recipient mice with and without SFB are due to the differences in the overall composition of microbiota, but not SFB alone.

Overall, our observations demonstrate how dietary deviations such as the pH of drinking water affect the gut microbiome composition as well as the gut immune responses, and suggest that the altered microbiota and proinflammatory immune response initiated in the gut mucosa contributes to the disease progression in SLE. This study, in association with our recent report on T1D, also shows that drinking water pH has contrasting effects on autoimmune diseases that involve different types of immune responses.

Discussion

In SLE, pathogenic antibody-producing autoreactive B cells are the major contributors to disease development. Both environmental triggers and genetic susceptibility combine to initiate autoreactive B and T cell responses in SLE. Environmental factors that can contribute to the disease initiation and progression or modulate the disease outcome include viral infections, exposure to ultraviolet (UV) and toxic substances, dietary factors and gut commensals [2,5,26,32–34]. In this report, we show that simple dietary deviations such as a change in the pH of drinking water from neutral to acidic, which is achieved by minute amounts of acid, can have a significant impact on disease progression in the spontaneous mouse model of SLE. Our study shows that the rate of disease progression in SNF_1 mice that received AW is slower compared to mice that were given NW. In addition, both anti-nucleohistone and anti-dsDNA antibody levels were found to be significantly lower at some nephritic ages in the AW group of mice compared to NW recipients. Further analyses revealed considerable differences in the composition of gut microflora at both nephritic and prenephritic stages as well as gut immune responses in SNF_1 mice that were on NW and AW.

Our recent report showed that T1D incidence and gut microbiota composition in NOD mice can be influenced by the pH of drinking water [9]. Considering the difference in the immune responses between SLE and T1D [35–37], we investigated the influence of drinking water pH on disease progression in SLE, especially the composition of gut microbiome and the gut immune response in SNF_1 mice. As observed in this study, the pH of drinking water produced opposite effects in SNF_1 and NOD mice, i.e. slower disease progression by AW in lupus recipients, while NOD mice under the same conditions showed rapid insulitis and higher T1D incidence [9]. Our observations showing that SNF_1 mice that were on AW produced relatively lower amounts of autoantibodies against nuclear antigens compared to NW mice group indicate that autoantibody production is impacted negatively by AW consumption. However, whether the minute amounts of HCl used for acidifying drinking water have a direct effect on the autoimmune process *in vivo* is not known. Importantly, as described in our previous report [9], AW that is produced using a miniscule amount of HCl is neutralized by physiological processes even before it reaches the stomach, which is naturally acidic. As the intestinal content is neutral in both AW and NW recipients [9], we believe that the pH of drinking water does not affect the gut immune response directly. The difference in gut microbial communities in SNF_1 mice that received NW and AW indicates that the drinking water pH affects the acquisition of gut microbiota, and this pH-influenced gut microflora may be

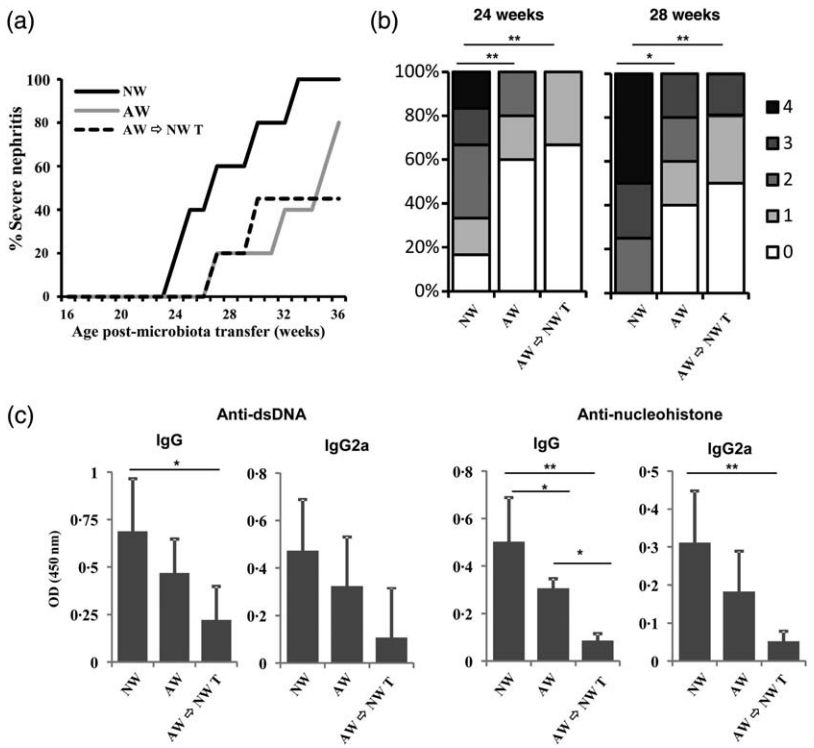

Fig. 8. Microbiota manipulation suppresses lupus progression in neutral pH drinking water (NW) recipients. NW-recipient mice (16 weeks old) were given caecum content from acidic pH drinking water (AW)-recipients by oral gavage on 3 consecutive days as described in the Methods. (a,b) Protein levels in urine from these mice were determined along with age-matched AW and NW control groups every week and % severe nephritis and the disease severity was calculated as described for Fig. 1. Five to six mice were included in each group. (c) Serum samples from these mice were collected at 28 weeks of age and tested for anti-dsDNA and -nucleohistone levels by enzyme-linked immunosorbent assay (ELISA). Mean ± standard deviation values are shown.

contributing to differences in autoantibody production and disease progression. Slow disease progression in NW-recipient mice that received microbiota from the AW group support this notion. However, additional studies involving transfer of gut microbial communities between AW and NW groups of SNF_1 mice and transfer of microbiota from these groups to germ-free SNF_1 mice are needed in the future to prove conclusively the involvement of gut microbiota in modulating the disease in NW and AW recipients.

Two recent reports have shown a potential association between gut microbiota and SLE. One of these two studies, which compared gut microbiota of SLE patients and healthy subjects, show that intestinal dysbiosis is associated with SLE [17]. This report not only showed that lupus patients have a significantly lower firmicutes/bacteroidetes ratio compared to healthy controls, but also predicts an over-representation of oxidative phosphorylation and glycan utilization pathways in SLE patient microbiota. However, whether this dysbiosis is caused by the disease process or gut microbial communities contributes to lupus is not known. Our study, which was focused on assessing the effect of drinking water pH and changes in the composition of microbiota on lupus, did not show such an over-representation of oxidative phosphorylation and glycan utilization pathways in NW recipients. However, drug metabolism-related enzymes, flavonoid and phenylpropanoid biosynthesis pathways were over-represented in the gut microbiota of this group compared to that in AW recipients. Phenylpropanoids and flavonoids are common plant natural products, and the associated pathways in bacteria and fungi are considered as plant-like biosynthesis pathways [32]. The second study determined the dynamics of gut microbiota in lupus-susceptible mice and the influence of dietary intervention on the gut microbiota [18]. This study showed under-representation of replication, recombination and DNA repair pathways in the lupus-susceptible strain of MRL/lpr mice, similar to our observations in NW-recipient mice. The phosphoenolpyruvate (PEP)-dependent phosphotransferase system, which is a major carbohydrate metabolic pathway used by bacteria as a source of energy [38], is also under-represented in NW mice. However, additional studies are needed to understand if these metabolic pathways contribute to modulation of immune response and its effect on disease progression or suppression in lupus.

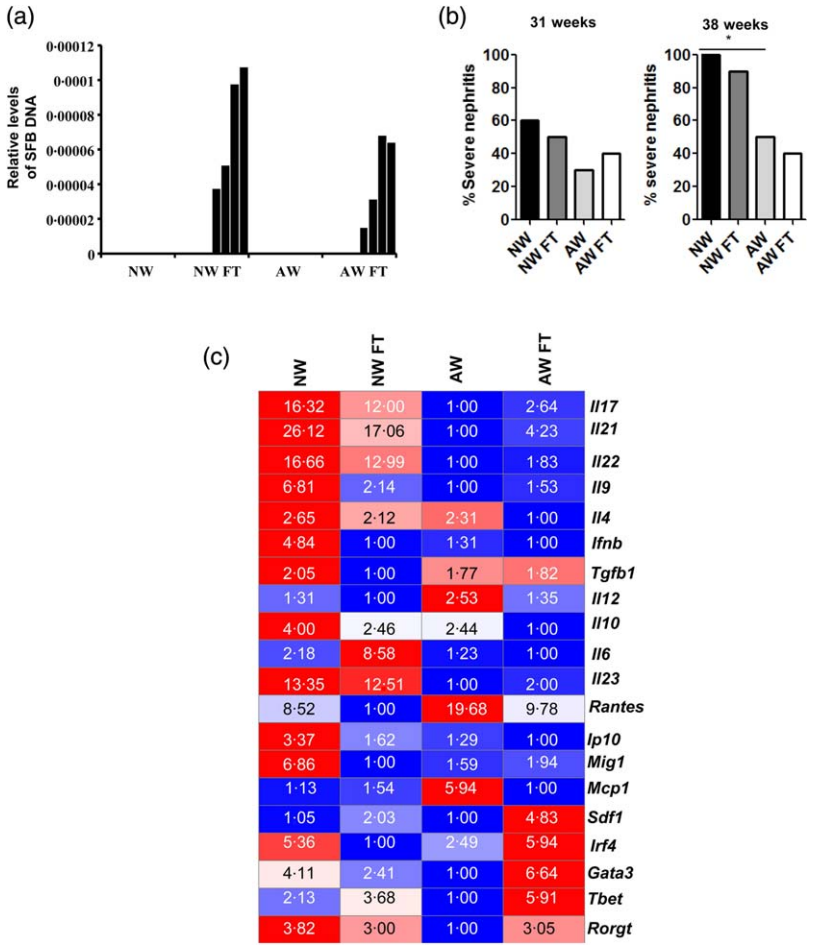

Fig. 9. Segmented filamentous bacteria (SFB) do not influence lupus-like disease progression in SWR × NZB F_1 (SNF_1) mice. Female SNF_1 mice were maintained on either neutral pH drinking water (NW) or acidic pH drinking water (AW) and cages of one set of each group (eight to nine mice/set) of mice were transplanted with faecal pellets [faecal transplant (FT) groups] of SFB^+ C57BL/6 mice from Taconic Farms, Inc. (TAC), as described in the Materials and methods. (a) Faecal pellets collected from each mouse were tested for SFB-specific 16S rRNA gene levels by quantitative polymerase chain reaction (qPCR) analysis at 40 weeks of age. Relative values were calculated against universal 16S rRNA gene and plotted as a bar-diagram. Faecal pellets from C57BL/6 mice of Jackson Laboratory origin were used as SFB-negative (background) controls. Values of two representative pellets from each cage (total two cages/group) are shown. (b) Proteinuria was assessed by Bradford assay for up to 40 weeks of age. Bar graphs show the percentage of mice with severe nephritis as indicated by high proteinuria ($\geq$ 5 mg/ml) for specified time-points. Each bar represents mean ± standard error of the mean of samples from eight to nine mice/group for each time-point. (c) cDNA prepared from the distal ileum of all four groups of mice (three/group) were subjected to real-time qPCR and the heatmap was generated as described for Fig. 3. Expression levels of individual factors were calculated against the value of β-actin. The lowest value of an individual factor among four groups of mice was considered as 1 (row minimum) for calculating fold expression values for the other value.

The differences observed in the gut bacterial communities in NW- and AW-recipient mice suggest that the drinking water pH-associated effect on intestinal mucosa may be linked to this difference in the gut microbiota. Importantly, gut microbes and their metabolites can also significantly influence the phenotype and functionality of systemic and gut-associated immune cells [28,30,39]. Our observation that the overall gut immune profile in NW-recipient mice has a Th17 and Th9 bias indicates the potential role for associated cytokines in rapid disease progression in SNF_1 mice. In fact, recent reports have suggested a dominant role for the Th17 pathway in the pathogenesis of rheumatoid diseases, including SLE [40–42]. While patients with SLE showed higher number of IL-17- and IL-22-producing T cells in the peripheral blood, studies using preclinical models have demonstrated the pathogenic role of IL-17 in lupus [40,42,43]. IL-9 is known to promote high IgE production and lung eosinophilia and causes airway hyperresponsiveness [44–47]. In addition, cells that produce this cytokine have been implicated in skin inflammation [48]. Importantly, a recent study has shown higher frequency of IL-9-producing T

cells in the peripheral blood of SLE patients compared to controls [49]. Of note, previous reports showing a protective role for IL17-producing cells in T1D [11] explain the drinking water pH-associated discrepancy in the outcome of disease in the SLE model used in this study and the T1D model employed in our previous report [9].

Incidentally, gut colonization by SFB promotes EAE and arthritis but protects NOD mice from T1D through promoting a Th17 response [10,12,29]. Our previous report showed that SFB-associated protection of NOD mice from T1D was evident only when the mice were given AW, but not NW [9]. However, SNF_1 mice that received SFB^+ faecal transplant did not show a significant difference in the rate of disease progression under either NW or AW condition. Importantly, NW recipient SFB^+ SNF_1 mice produced relatively lower levels of Th17-associated factors in the gut mucosa than SFB^- controls. Conversely, AW-recipient SFB^+ mice showed higher expression of Th17-related factors, albeit slower disease progression, compared to NW recipients, suggesting that the gut immune response and disease progression could be determined by the overall composition of microbiota, but not dominated by SFB. Additional studies are needed in the future to understand whether lupus-susceptible strains of mice produce a similar type of gut immune responses as other strains upon gut colonization by SFB. Importantly, gut commensal–mucosa interactions mediated by pathogen-associated molecular patterns (PAMPs) and pattern recognition receptors (PRRs), in addition to the secreted metabolites that are absorbed into circulation, are the major contributors of gut immune cell activation and differentiation. Hence, we believe that NW- and AW-influenced gut microbial communities may be inducing different types of innate and adaptive immune responses and indirectly influencing the rate of disease progression in SNF_1 mice. We conclude that simple dietary deviations such as changes in the pH of drinking water can influence the acquisition and composition of gut microbiota and these microbial communities, in turn, influence the disease outcome by inducing changes in the gut immune response. While a series of microbiota manipulation studies are needed in the future to identify specific microbial communities that are responsible for promoting or suppressing SLE, our study, for the first time to our knowledge, shows that mere change in the pH of drinking water can affect the composition of gut microbiome and disease progression in lupus-prone mice. The simplicity of the treatment in this study, which is changing the pH of drinking water from neutral to acidic, by adding miniscule amounts of acid, suggests the profound effects of environmental factors in modulating autoimmunity and the disease outcome in genetically predisposed individuals.

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Author contributions

M. C. G. performed the experiments and wrote the paper, B. M. J. performed the experiments and reviewed the paper, M. M. A. performed the experiments, R. G. performed experiments and reviewed the paper, and C.V. designed the study and wrote the paper. C. V. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity and accuracy of the data and analysis.

Disclosure

The authors have no conflicts of interest to disclose.

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Supporting information

Additional Supporting information may be found in the online version of this article at the publisher's Web site:

Fig. S1. Analysis of gut microbial composition in neutral pH drinking water (NW) and acidic pH drinking water (AW)-recipient SWR × NZB F_1 (SNF_1) mice. 16s rRNA gene sequences of 12-week-old NW and AW recipients were analysed as described in Fig. 5. Detailed analyses for these sequences are shown in Figs 5 and 6. This supplementary figure shows additional alpha (a) and beta diversity (b) indices, further demonstrating that gut microbiome is different in NW and AW recipients.

Fig. S2. Analysis of gut microbial composition in neutral pH drinking water (NW) and acidic pH drinking water (AW)-recipient SWR × NZB F_1 (SNF_1) mice. 16s rRNA gene sequences of 12-week-old NW and AW recipients were analysed as described in Fig. 5. (a) Relative abundance of bacterial communities at phyla level in individual mice. (b) Relative values of firmicutes and bacteroidetes in individual mice.

Table S1. Quantitative polymerase chain reaction (qPCR) primers used in this study.