Article

Discovery Medicine 2024; 36(181): 424–436
https://doi.org/10.24976/Discov.Med.202436181.40

Profiling the Gut Microbiome Unraveled Signature Bacterial Groups in Autoimmune Diabetes, which Remain Unperturbed by the Low Ionic Strength of the Drinking Water in NOD mice

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Published: 20 February 2024

Background: Non-obese diabetic (NOD) mice develop type 1 diabetes (T1D) spontaneously and serve as a good model for investigating the underlying pathological mechanisms and devising novel treatment procedures. Although acid water consumption has been reported to exaggerate or reduce diabetes incidence in female NOD mice by two groups, the causative bacteria responsible for these contrasting changes remain unclear. On the contrary, we and others failed to observe the effect of acid water consumption on diabetes incidence. This study aimed to determine whether the consumption of low-pH drinking water could alter the frequencies of prominent bacterial groups independent of diabetes manifestation.

Methods: Six-week-old female NOD mice maintained on acidified drinking water at the Jackson Laboratories were transferred to neutral pH water or continuously provided with low pH drinking water at our facility. Diabetes was monitored weekly using a glucometer. Using the 454-pyrosequencing methodology, we profiled the gut microbiome of mice transferred to neutral water and developed diabetes. Further, we performed quantitative real-time polymerase chain reactions (qRT-PCR) using primers specific for prominent 16S rRNA genes on the fecal DNA of mice provided with low pH or neutral water and displayed diabetes similarly.

Results: Consistent with our earlier report, the incidence of T1D was robust (80–100%) regardless of whether female NOD mice consumed acid (~pH 2.9) or neutral water. The 454-pyrosequencing of fecal DNA indicated no substantial influence of transferring mice to neutral pH drinking water on the gut microbiome. To validate these findings, we conducted qRT-PCR on the fecal DNA of mice longitudinally from six weeks of age to adulthood that consumed acidic or neutral pH water and developed diabetes similarly. Among the 15 selected bacterial groups examined, the frequency of *Lactobacillus* sp. remained consistently lower ($p < 0.05$) throughout the life of NOD mice compared to that found in young (6-week-old) mice, regardless of the pH of the drinking water. The relative frequencies of the *Firmicutes Ruminococcaceae* and the *Bactereoidetes* members *Anaerophaga* sp. and *Paludibacter* sp. increased significantly ($p < 0.05$) during the transition to the overtly diabetic stage irrespective of the ionic strength of the drinking water. Interestingly, the *Firmicutes* members *Clostridium coccoides*, *C. leptum*, and *Lachnospiraceae* and the *Bacteroidetes* members *Bacteroides* sp. and *Prevottella* sp. remained unchanged throughout the analysis irrespective of the pH of the drinking water. Paradoxically, the representations of *Akkermansia muciniphila* and the segmented filamentous bacteria implicated in diabetes protection did not differ regardless of the age or the ionic strength of the drinking water.

Conclusions: The data presented herein validate the lack of influence of acidic drinking water on T1D development in female NOD mice. Diabetes was associated with the lower representation of *Lactobacillus* sp. throughout life, which was not influenced by the differing pH of the drinking water. Significantly, segmented filamentous bacteria and *A. muciniphila*, previously implicated in protection against T1D, were not perturbed by the varying pH of the water consumed. These data indicate that although acidified water consumption was reported previously to diminish specific gastrointestinal pathogens, it failed to perturb gut commensals that influence diabetes development.

Keywords: 16S rRNA genes; acid water; Jax; microbiome; NOD mice; qRT-PCR; segmented filamentous bacteria; specific pathogen-free; type 1 diabetes

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Introduction

Type 1 diabetes (T1D) is an autoimmune disease characterized by the loss of insulin-producing beta-cells and metabolic complications [1]. Several immunosuppressive therapies, including indiscriminate ablation of T lymphocytes, have been proposed to manage T1D. Much interest has been shown recently in understanding the purported link between the enteric microbiota and T1D [2]. Earlier attempts to study the impact of acidic drinking water (pH 6.2–6.9) in T1D patients, possibly via modifications of the intestinal microbiota, remained uninformative and inconclusive [3,4].

Many colonic lumen factors, such as host secretions, micronutrients, antimicrobial compounds, and pH, influence the composition of the gut resident microbiota [5]. Historically, several studies indicated the benefits of low pH water consumption, including inhibition of gastrointestinal infections in broilers, pigs, and mice, prevention of gut-derived sepsis, and reduced bacterial numbers in the terminal ileum of mice [6–8]. Lower pH resulting from substrate fermentation in the colon enhanced butyrate production and butyrate-producing *Firmicutes* phylum group *Roseburia* and negatively impacted the abundance of *Bacteroides* sp. [9]. Thus, the simple dietary variable, acidified drinking water, is not innocuous and influences the survival of the enteric microbiota variably in animals. Since almost all alcoholic and non-alcoholic beverages, citrus fruits, and berries consumed on a regular basis are acidic (pH 2.0–3.5) in nature, it remains a formidable task to conclusively assess the value of drinking water acidification in treating diseases, including T1D.

For unexplained reasons, female non-obese diabetic (NOD) mice develop more robust T1D than male mice and serve as a reliable model for studying the underlying pathological mechanisms and exploring novel therapeutic strategies for this disease [10,11]. NOD mice spontaneously developing T1D were derived while selecting for cataracts in outbred Swiss mice in Japan, and their subsequent transit to Australia, Europe, and the US has tremendously facilitated the study of T1D [11]. Spontaneous T1D varies remarkably among different centers worldwide, which remains problematic even with the implementation of specific pathogen-free (SPF) monitoring systems in recent years [12]. Variables, including cleanliness of the housing facility, untreated subclinical infections, breeding conditions, and diet, have been attributed to variations in diabetes incidence in NOD mice at various locations. The incidence of T1D in female NOD mice provided with acidified drinking water (pH 2.8–3.1) and maintained under strict hygienic conditions over many generations at the Jackson Laboratory (Jax) remained remarkably high (80–100%) [12]. We observed a similar high incidence (80–100%) of diabetes in female NOD mice procured at 6 weeks of age from Jax and housed under SPF conditions in our facility and provided with neutral pH drinking water [13–18]. Similarly, mice bred and maintained on neutral pH water at the National Institutes of Health (NIH) displayed higher diabetes incidence [19]. By contrast, it was observed that 3 to 4-week-old weanlings, but not 6 to 8-week-old mice procured from Jax and provided with acidic drinking water, displayed increased T1D incidence [20]. Paradoxically, female NOD mice born to breeding pairs obtained from Jax and maintained with acidified water showed decreased diabetes occurrence [21]. However, neither of these contradictory studies identified the causative bacteria responsible for the apparent increase or decrease in T1D incidence when mice consumed low-pH water. These data suggested that the acidity of the drinking water is not a reliable parameter of diabetes manipulation.

We determined whether the discrepancies in diabetes incidence observed in our [18] and other studies [19–21] could be due to the modulation of different enteric bacteria consequent to acidic water consumption. To this end, we profiled the intestinal microbiome of female NOD mice maintained on acidic drinking water at Jax and switched to neutral pH water at our facility using the same pyrosequencing platform as others [20,21]. For the first time, we longitudinally quantified the abundance of selected enteric bacterial groups implicated in diabetes induction/protection in young (6 weeks), prediabetic (12 weeks), and overtly diabetic (28–30 weeks) mice maintained on acidic water or switched to neutral drinking water at 6-weeks of age. We used the quantitative real-time polymerase chain reactions (qRT-PCR) and primers specific for 16S rRNA genes described elsewhere and those designed and validated by us (see **Supplementary Table 1**). Our data demonstrated the low abundance of *Lactobacillus* sp. and increased frequencies of *Ruminococcaceae*, *Anaerophaga* sp., and *Paludibacter* sp. in the gut of the mice throughout their lives. Notably, the pH of the drinking water was not linked to diabetes incidence nor the abundance of these commensals, as well as the segmented filamentous bacteria (SFB) [22,23] and *A. muciniphila* [24] implicated previously in diabetes protection.

Materials and Methods

Animals and Diabetes Monitoring

Six-week-old female NOD/ShiLtj and C57BL/6J mice were purchased from The Jackson Laboratory (Jax, Bar Harbor, ME, USA), housed under SPF conditions in filter-top cages, and subjected to a 12-hour dark-light cycle. They were provided with irradiated Lab chow and neutral (pH 7.0–7.3) or acidic (pH 2.8–3.2) autoclaved tap water, as described previously [18]. Experiments were conducted following the NIH guidelines for the care and use of laboratory animals. Blood glucose levels were monitored weekly using a glucometer, as described [18]. Glucose levels exceeding 250 mg/dL were confirmed a few days after the first

determination and considered diabetic. Fresh fecal samples were collected aseptically from individual mice immediately after diabetes confirmation.

Pyrosequencing of the Gut Microbiome

Fecal DNA was isolated individually from 5 mice using the automated Maxwell 16 Research System with a Blood LEV kit (Promega, WI, USA). The 600-bp region of 16S rRNA genes was amplified using the bTEFAP method at the Molecular Research DNA Lab (MR DNA, Shallowater, TX, USA) [25]. This was accomplished by using 100 ng of DNA and barcoded Eubacterial primers 530F (5′-GTG CCA GCM GCN GCG G-3′) and 1100R (5′-GGG TTN CGN TCG TTG-3′). The Roche-454 FLX Titanium instrument (F. Hoffman-La Roche AG, Basel, Switzerland) was used for pyrosequencing, and the data were processed using a proprietary analysis pipeline at MR DNA. Final operational taxonomic units were taxonomically classified by MR DNA using the BLASTn against a curated Green Gene database [26].

Analysis of 16S rRNA Genes by qRT-PCR and Standard PCR

For qRT-PCR, fecal DNA was isolated from individual mice using the QIAamp PowerFecal DNA kit (Qiagen, Valencia, CA, USA). The bacterial 16S rRNA genes were quantified using 100 ng of fecal DNA, forward and reverse primers at 400 nM final concentrations, and 5 µL of 2× SYBR Green Master mix in a total volume of 10 µL, and the Applied Biosystems ViiA7 Real-Time PCR system (Life Technologies, Carlsbad, CA, USA). The cycling conditions for the amplification of *Eubacteria*, *Bacteroides*, *Ruminococcus*, *Lachnospiraceae*, *Paludibacter*, *Helicobacter*, *Turicibacter*, and *Anerophaga* included an initial denaturation step at 95 °C for 20 s and 40 cycles of denaturation at 95 °C for 1 s and annealing at 60 °C for 20 s followed by melt curve analysis. An annealing temperature of 55 °C for 30 s was used to probe *Enterobacteriaceae*, *A. muciniphila*, *Clostridium coccoides*, *C. leptum*, *Lactobacillus*, *Prevotella*, and SFB using the SFB-1 primer set [27]. Amplification using the SFB-2 primers [28] consisted of an annealing temperature of 62 °C for 30 s with an extension at 72 °C for 7 min at the end of 40 cycles.

The primers for *Ruminococcaceae*, *Lachnospiraceae*, *C. leptum*, *Turicibacter* sp., *Anaerophaga* sp., and *A. muciniphila* were designed based on gene information available in RDP-release-II (rdpstaff@msu.edu) [29] and Gen Bank (http://ncbi.nlm.nih.gov). All primers used in this study were synthesized at the Integrated DNA Technologies (Coralville, IA, USA) and listed in **Supplementary Table 1**. Whereas the SFB-1 primer set was designed using the SFB sequence isolated from mice (accession number X77814) [27], Dalby *et al*. [28] designed SFB-2 primers based on the SFB sequence derived from rats (accession number X87244). The specificities of both primers were verified by sequencing the clone libraries of PCR amplicons and alignment with the database of rRNA genes. Preliminary analysis revealed a comparable abundance of *Eubacteria* (all bacteria) in 10 mice that consumed neutral or acidic drinking water. Therefore, equal amounts of DNA from individual mice in each group were pooled and assayed in triplicate. The experiment was repeated with two or three groups of 5 mice each. The data from a representative experiment are presented as the mean ± SD of triplicate determinations. Melting curve analysis was performed for the lack of primer dimers at the end of the qRT-PCR. Amplicons were analyzed for the expected sizes by running on a 4% low melt agarose gel, stained with ethidium iodide, and imaged. The relative gene expression was determined using *Eubacteria* (all bacteria) [28] as the normalizer and the $2^{-\Delta\Delta CT}$ method [13].

A standard PCR was performed using 100 ng of DNA, forward and reverse primers (final 400 nM concentrations), 2.5 µL of 300 GC Enhancer, 3 µL of water, and 12.5 µL of AmpliTaq Gold 360 Master Mix (Applied Biosystems, Carlsbad, CA, USA). The amplicons were analyzed on a 4% low melt agarose gel stained with ethidium bromide and imaged. Genomic DNA extracted from the human T cell leukemia Jurkat clone E6-1 and water served as negative controls.

Statistics

Data are presented as mean ± SD, and group comparisons were performed using one-way analysis of variance (ANOVA). Comparisons were made between mice of the same age provided with acid or neutral water. As indicated in the figures, comparisons were also made between 6-week-old mice that consumed acid water and other age groups provided with acidic or neutral water. $p < 0.05$ was considered significant (GraphPad Prism 6.0, San Diego, CA, USA).

Results

16S rRNA Targeted Sequencing of the Intestinal Microbiome of Diabetic Mice

Previously, we reported that switching 6-week-old female NOD mice maintained on acidic water from conception at Jax to neutral water at our facility did not alter the frequency or rate of diabetes [18]. The data shown in Fig. 1 validated this observation and those reported in pups born to breeding pairs continuously maintained on neutral or acidic drinking water at the NIH [19].

Although acidified drinking was reported to either increase or decrease diabetes incidence, 454-pyrosequencing of the enteric microbiome did not reveal changes in the bacterial taxa accounting for altered diabetes frequency [20,21]. We used the same 454-pyrosequencing methodology to profile the 16S rRNA genes in the fecal DNA of five female mice transferred to neutral tap water and developed

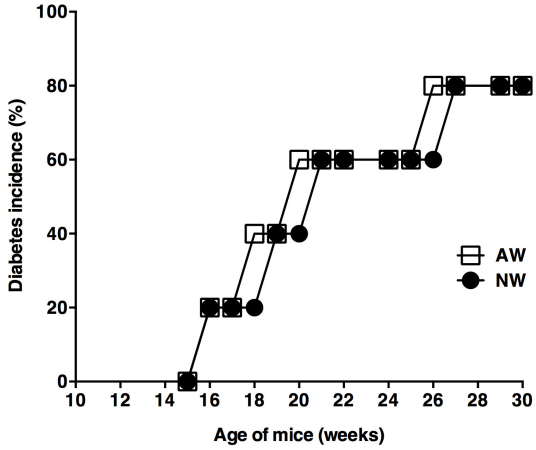

Fig. 1. The pH of the drinking water did not impact type 1 diabetes development. Littermates of female non-obese diabetic (NOD) mice were provided with acidified (empty squares, AW) (n = 20) or neutral pH (filled circles, NW) (n = 20) drinking water. Diabetes was monitored weekly using a glucometer. Data from two independent experiments were pooled.

diabetes. Bacterial strain richness (α-diversity) and the relative abundance of bacteria (β-diversity index) remained unremarkable. The 3:1 ratio of *Firmicutes:Bacteroidetes* was maintained in these mice (Fig. 2, **Supplementary Table 2**), in contrast to a 1.9 ratio reported in mice maintained on neutral pH water by another group [21]. Importantly, our 454-pyrosequencing analysis revealed that the *Firmicutes* phylum member Gram-positive *Lachnospiraceae* constituted 67% of the gut microbiome of neutral water-consumed mice. The *Firmicute Lactobacillus* sp. represented only a small fraction (1.8%) of the enteric microbiome in these mice, like another study [21], but in contrast to ~30% of the total gut microbiome reported by others [20].

Interestingly, at the phylum level, *Bacteroidetes* (Gram-negative) represented 27%, like a previous report [21], while a slightly diminished frequency, 21%, was observed in another study [20]. *Actinobacteria* and *Proteobacteria* (Gram-negative) represented less than 0.1% of the total gut microbiome in our samples, whereas 10% was reported in another study [21]. Thus, analyzing the intestinal microbiome using the same 454-pyrosequencing platform as others revealed significant differences and few similarities between these investigations [20,21] and ours. Importantly, our analysis did not uncover marked changes in the microbiome, termed dysbiosis, mentioned in a previous study [20]. One possible reason for these inconsistencies in the gut microbiome of diabetic mice could be the bias introduced during data analysis. Whereas the Molecular Research DNA Lab analyzed our microbiome data, in-house analysis was undertaken in other studies [20,21], likely contributing to the discrepancy.

Lack of Influence of the pH of Drinking Water on Prominent Firmicutes Phylum Members

It is difficult to ascertain whether the changes observed in microbial taxa due to switching young (6-week-old) female NOD mice to neutral water can directly impact diabetes incidence based only on the sequence reads without further validation since most of them represented less than 1% of the microbiome (**Supplementary Table 2**, Fig. 2). The qRT-PCR is a precise, sensitive, and quantitative method routinely used for gene expression analysis. The qRT-PCR was previously employed to determine the abundance of *Lactobacillus*, *Bacteroides*, and *C. coccoides* in prediabetic (5–20-week-old) mice [21]. Unfortunately, a similar investigation was not conducted in hyperglycemic (25–35-week-old) mice. Besides, it will be impossible to compare the microflora of mice sacrificed at weaning and that manifested diabetes in a distinct group of mice later at 25–35 weeks of age [21]. Thus, the correlation between changes in microflora and diabetes incidence due to switching from acidic to neutral water remains to be assessed using qRT-PCR sequentially during varying stages of diabetes development.

We have systematically analyzed the relative abundance of selected bacterial groups in the fecal DNA of young (6-week-old) mice, prediabetic mice (12 weeks of age), and adults (28–30-week-old) and correlated with diabetes development. The data shown in Fig. 3 indicated that the representation of the *Firmicutes* phylum member *Ruminococcaceae* significantly increased throughout the lifetime of the NOD mouse regardless of the pH of the drinking water compared to 6-week-old mice receiving acid drinking water ($p < 0.05$). Interestingly, the *Turicibacter* sp. transiently increased in frequency during the prediabetic stage (12 weeks) regardless of the quality of the drinking water ($p < 0.05$) but subsided in diabetic adults provided with either acidic or neutral drinking water. Only in diabetic adults (28–30-week-old) was the representation of *C. coccoides* lower ($p < 0.05$) in the acid-water-fed group compared to the 6-week-old mice and adults provided with neutral water. However, the frequencies of *Lachnospiraceae* and *C. leptum* did not change throughout the life of the mice, irrespective of the pH of the drinking water. Notably, the abundance of *Eubacterium rectale* was somewhat lower in diabetic adults compared to 6-week-old mice, which was not perturbed by the drinking water quality. Importantly, *Lactobacillus* sp. was consistently lower at all stages of life compared to young (6-week-old) mice ($p < 0.05$), especially when prediabetic (12-week-old) and diabetic (28–30-week-old) mice were provided with neutral drinking water. Overall, the representation of the analyzed *Firmicutes* members was barely influenced by the lower pH of the drinking water. The exception was *C. coccoides*, which diminished in diabetic adults provided with acidified water.

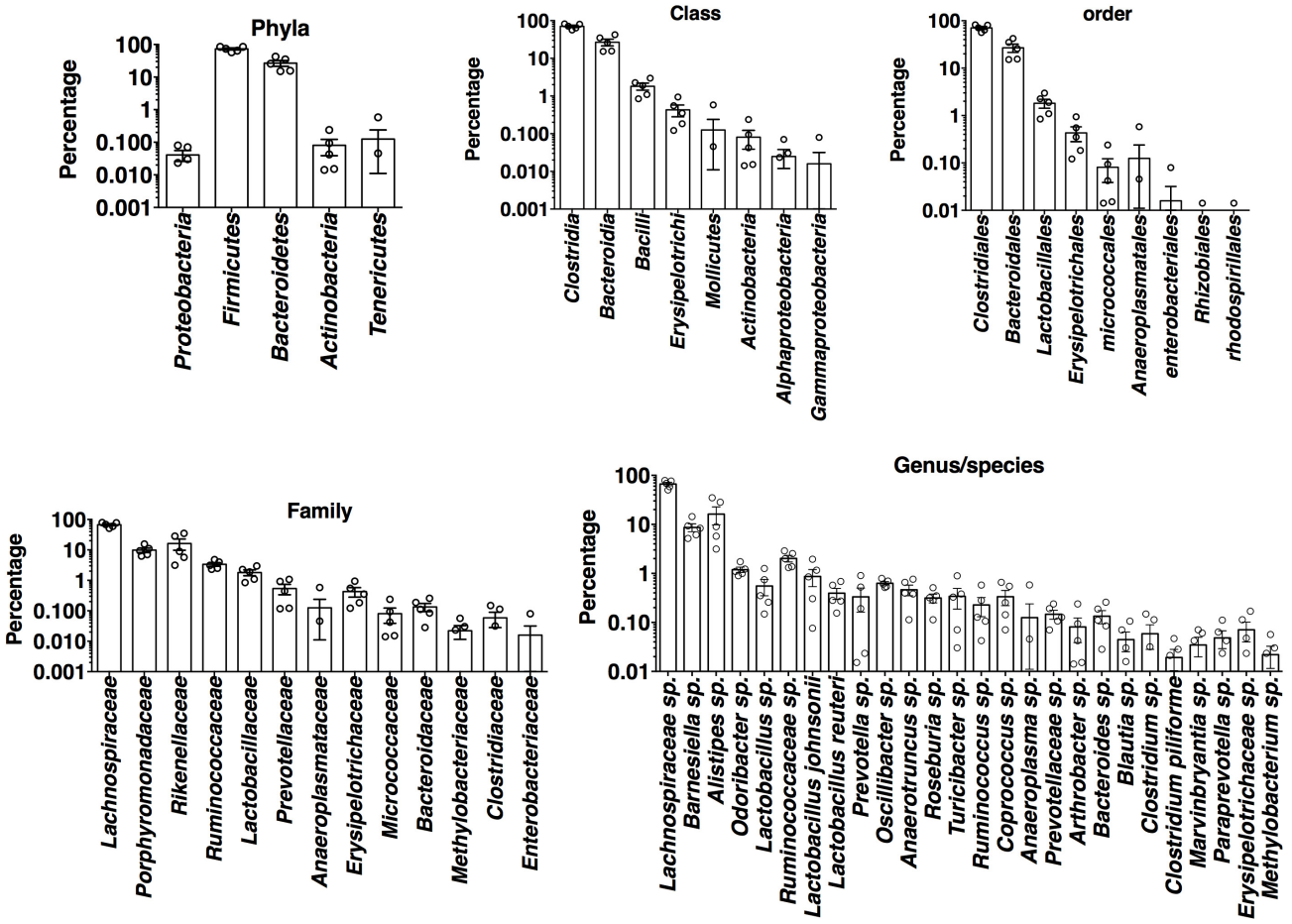

Fig. 2. 16S rRNA targeted sequencing of the intestinal microbiota of diabetic NOD mice. DNA was isolated from the feces of five (28–30-week-old) female NOD mice that were transferred to neutral pH water at six weeks of age. All these mice developed diabetes. The DNA samples were pyrosequenced individually. The data shown are depicted as scatter plots (empty circles). Mean ± SD of 5 mice is shown.

Lack of Influence of the pH of Drinking Water on Other Bacterial Taxa

The *Bactereoidetes* phylum members, *Anaerophaga* sp. and *Paludibacter* sp., were significantly ($p < 0.05$) more abundant in the prediabetic (12 weeks old) and diabetic (28–30-week-old) mice compared to young mice (6-week-old) (Fig. 4). No difference was noted in the representation of these bacterial groups between mice of the same age group provided with varying pH of the drinking water. The high copy numbers of *Bacteroides* sp. and *Prevotella* sp. remained unaltered throughout the life of the mouse, irrespective of the ionic strength of the drinking water. The frequency of *Helicobacter pylori*, a human pathogen, was barely detectable, irrespective of the variables. The *Proteobacteria* phylum member, *Enterobacteriaceae*, transiently increased in abundance during the prediabetic stage regardless of the pH of the drinking water ($p < 0.05$). However, the *Enterobacteriacea* diminished later in adult life regardless of the quality of the drinking water. Finally, the only representative of the *Verrucomicrobia* phylum in mice, *A. muciniphila*, remained low in abundance throughout the life, which decreased significantly ($p < 0.05$) in acidic water consumed diabetic (28–30-week) mice compared to those provided with neutral water and other age groups of mice. The qRT-PCR analysis of these 14 enteric bacterial taxa unraveled that diabetes was associated with the lower frequency of the *Firmicute Lactobacillus* sp. and increased abundance of *Bactereoidetes* phylum members *Anaerophaga* sp. and *Paludibacter* sp. in female NOD mice without a consistent impact of acid water consumption.

Differences in Bacterial Members in Sensitivity to Low pH Water in Non-Diabetic C57BL/6 Mice

Since the microbiota of diabetes-prone NOD mice were minimally sensitive to acidic drinking water, we next examined whether the enteric bacteria of C57BL/6 mice that do not develop T1D display differential sensitivity to low pH drinking water. The overall frequencies of intestinal bacteria in five C57BL/6 mice procured from Jax (Fig. 5) were comparable to those observed in diabetic NOD mice

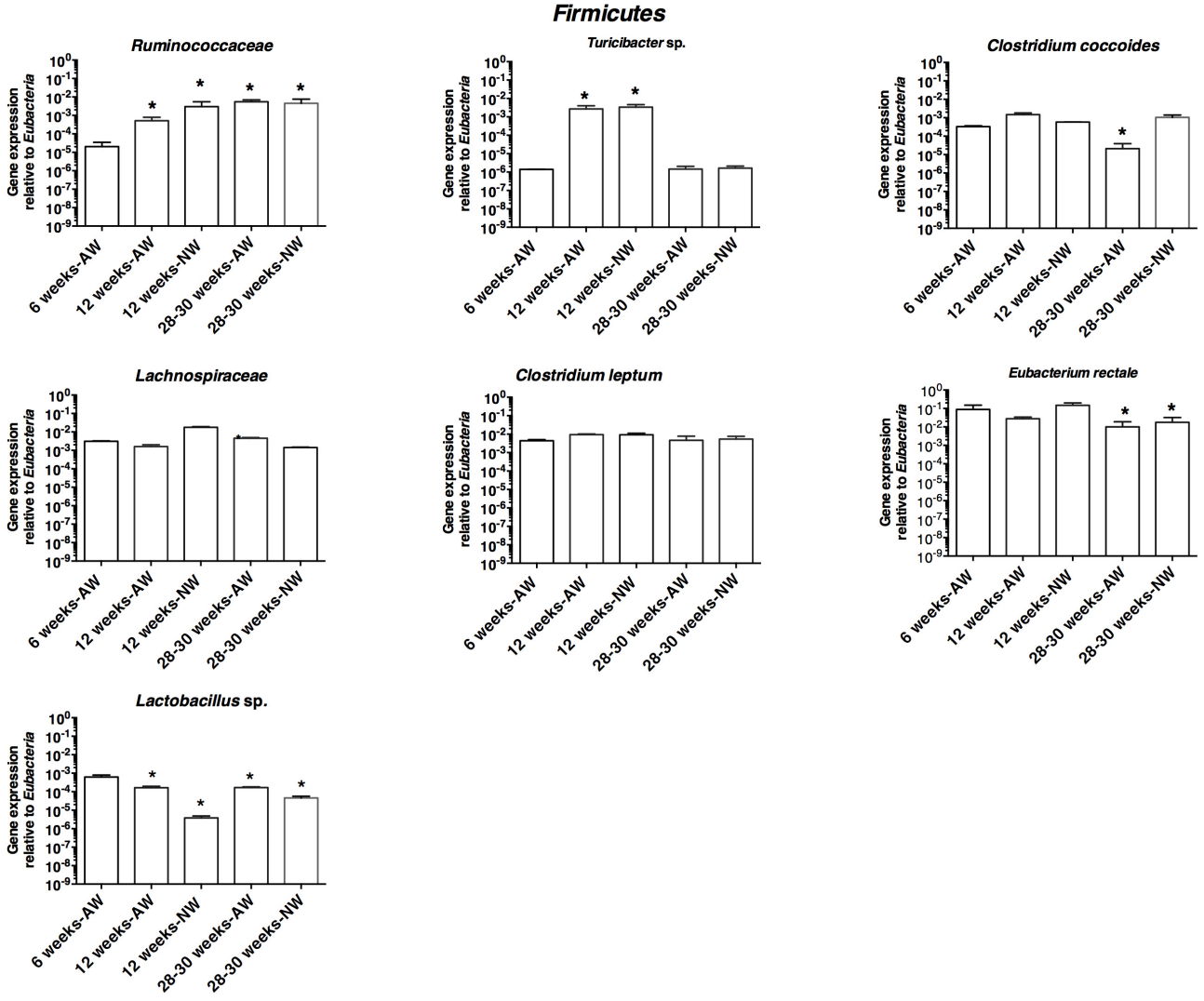

Fig. 3. Selected influence of the pH of drinking water on the members of the *Firmicutes* phylum. The DNA was isolated from the stool of 5 young 6-week-old female NOD mice individually within 24 hours of arrival from Jackson Laboratory (Jax). Fecal DNA was also isolated from the stool of five 12-week-old prediabetic mice and 28–30-week-old diabetic mice continuously provided with acidic or neutral pH drinking water starting at 6 weeks of age in our facility upon receipt from Jax. An equal amount of DNA was pooled from 10 mice per group in three different experiments, and quantitative real-time polymerase chain reaction (qRT-PCR) was analyzed in triplicate per experiment. Representative data are presented as the mean ± SD of 3 determinations. Gene-specific amplification was performed using specific oligonucleotide primers and quantified compared to the level of all bacteria, *Eubacterium*. Statistical significance of values compared to 6-week-old mice is indicated by asterisks ($p < 0.05$). The frequency of *C. Coccoides* in diabetic mice was also statistically lower ($p < 0.05$) compared to those consuming neutral pH water. The abundance of *E. rectale* was lower ($p < 0.05$) in both groups of mice receiving different drinking water than that of 6-week-old mice provided with acidic drinking water. The frequency of *Lactobacillus* sp. was consistently lower ($p < 0.05$) in prediabetic 12-week-old and 28–30-week-old mice that consumed neutral water. Acidified drinking water decreased the abundance of *Lactobacillus* sp. ($p < 0.05$) in comparison to 6-weeks old mice receiving acidic drinking water.

(*vide supra*). Interestingly, long-term acid water consumption significantly reduced the abundance of the members of *Firmicutes* (*Turicibacter* sp., *C. leptum*, *C. coccoides*), *Bacteroidetes* (*Anaerophaga*, *Paulidibacter*, *Prevotella* sp., and *Bacteroides* sp.), *Proteobacteria* (*Enterobacteriaceae*), and *Verrucomicrobia* (*A. muciniphila*). Interestingly, SFB initially reported to be absent in C57BL/6 mice procured from Jax [30] were abundant, like in another study [31], and acid water consumption did not affect their abundance. These data indicate that the intestinal microbiota of non-diabetic C57BL/6 mice displayed increased susceptibility to depletion by acidic drinking water. Thus, the sensitivity of bacterial groups to acidic drinking water is mouse strain-dependent and unrelated to the propensity to develop T1D.

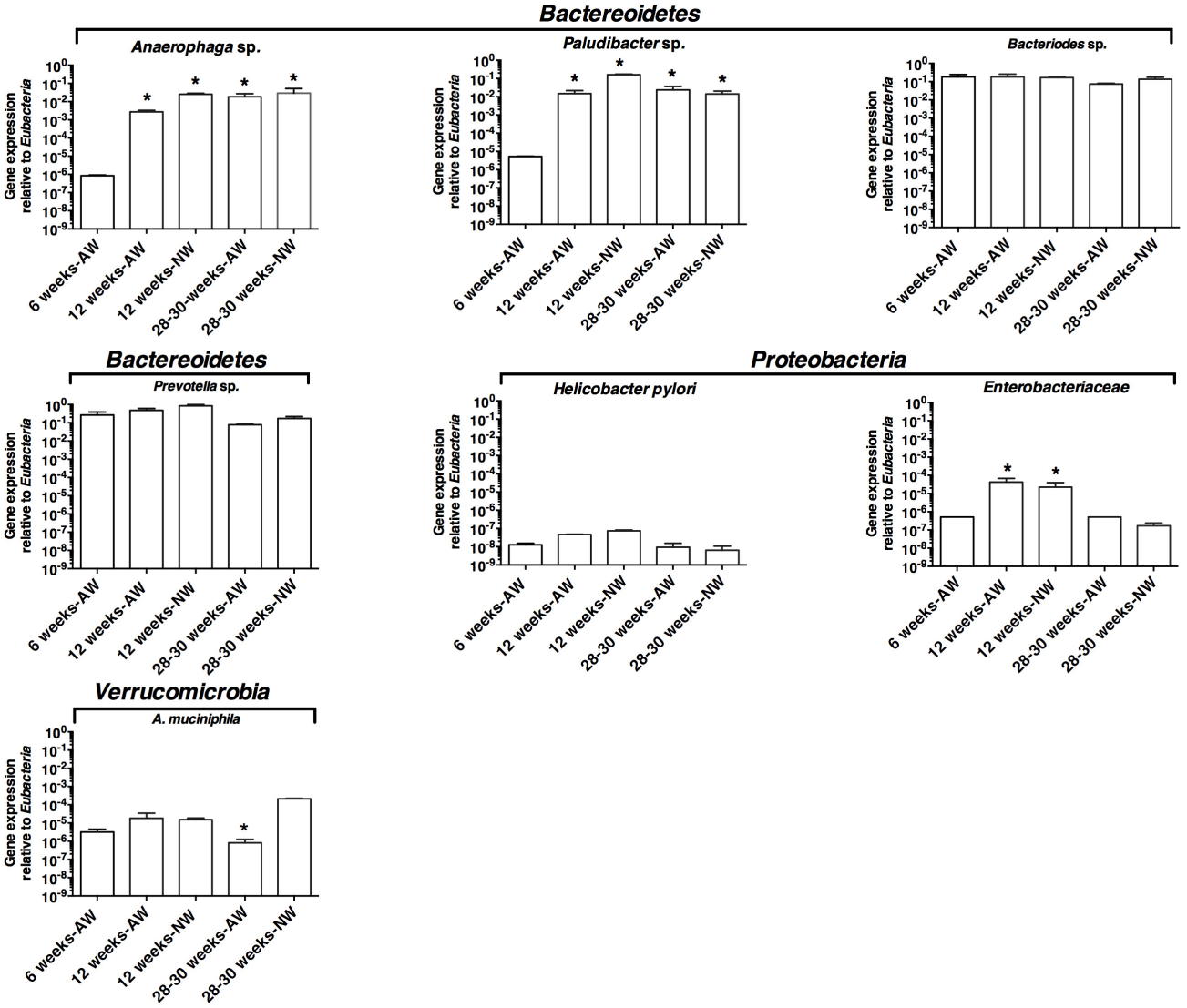

Fig. 4. Impact of the pH of drinking water on *Bacteroidetes*, *Proteobacteria*, and *Verrucomicrobia* phyla members. Fecal DNA was isolated from 5 individual 6-week-old female NOD mice within 24 h of arrival from Jax. Fecal DNA was also isolated from the stool of five 12-week-old prediabetic mice and 28–30-week-old diabetic mice continuously provided with acidic water or switched to neutral pH water starting at 6 weeks of age in our facility. An equal amount of DNA was pooled from 10 mice per group. Gene-specific amplification was performed by qRT-PCR using specific oligonucleotide primers and quantified compared to the level of all bacteria, *Eubacterium*. The data are presented as scatter plots and mean ± SD of 3 determinations. Asterisks indicate statistical significance ($p < 0.05$) between specified groups and 6-week-old mice. Although the frequency of *Enterobacteriaceae* in prediabetic 12-week-old mice was higher than in the 6-week-old mice ($p < 0.05$), there was no difference between mice of the same age group, irrespective of the water source. The abundance of *A. muciniphila* was lower in acid-water-consumed diabetic mice ($p < 0.05$).

SFB Colonization does not Correlate with Resistance to T1D

The SFB-*specific* primer set 1 (SFB-1) designed based on the SFB isolate derived from the mouse [27] was reported to be suboptimal for detecting SFB in NOD [22,23], CB7BL/6 mice procured from Jax [30], and FvB mice purchased from The Taconic Farms [27]. Similarly, using the same SFB-1 primer set, we could amplify a very low level of SFB in the fecal DNA of all five six-week-old female NOD mice within 24 h of their arrival from Jax (Fig. 6A). However, higher SFB gene copy numbers were detected in C57BL/6 mice ($p < 0.05$) also procured from Jax. The use of SFB-2 primers designed based on the SFB isolate obtained from rats [28] resulted in considerably higher amplification of SFB in the same fecal DNA samples of NOD mice probed with the SFB-1 primer set ($p < 0.05$). Amplicons derived from the qRT-PCR reactions were pooled, resolved on an agarose gel, and stained with ethidium bromide. The SFB-1 and SFB-2 primers generated single amplicons of expected sizes (Fig. 6B). Standard PCR per-

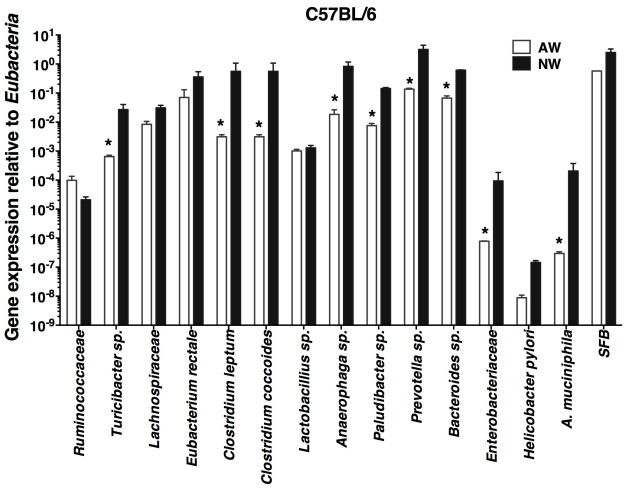

Fig. 5. Long-term effects of low pH water consumption on the intestinal microbiota of non-autoimmune prone C57BL/6 mice. Female C57BL/6 (n = 5) mice were provided with acidified drinking water (AW, empty bars) from birth and neutral (NW, filled bars) pH drinking water starting from 6 weeks of age. Fecal DNA was extracted from 28–30-week-old mice. An equal amount of DNA was pooled from the same group to determine the abundance of the indicated bacterial taxa by qRT-PCR in triplicate. The data are depicted as mean $\pm$ SD. The experiment was repeated with 5 more mice per group with similar results. Asterisks indicate statistical significance between the groups compared ($p < 0.05$). Segmented filamentous bacteria (SFB) was analyzed using the SFB-2 primer set (see Materials and Methods for details).

formed on the pooled DNA from neutral pH water-treated 28–30-week-old diabetic mice confirmed the generation of different sizes of amplicons with two distinct sets of primers (Fig. 6C). Notably, these SFB primers failed to result in amplifications when the template derived from human acute T cell leukemia, Jurkat was used, indicating the absence of spurious amplification by these bacterial primers.

The qRT-PCR was conducted on the pooled fecal DNA of non-autoimmune prone 26 to 28 weeks old C57BL/6 mice, older non-diabetic NOD mice (22 to 24 weeks of age), and overtly diabetic, 28 to 30-week-old NOD mice using the SFB-2 primer set. It resulted in the uniform amplification of the expected size of the amplicon from all fecal DNA samples analyzed (Fig. 6D). Importantly, the nature of the drinking water did not affect the abundance of SFB in either NOD or C57BL/6 mice (Fig. 6D). Longitudinal qRT-PCR analysis of NOD mice revealed higher levels of SFB in prediabetic six-week and 12-week-old mice and 28 to 30-week-old diabetic mice regardless of the source of the drinking water (Fig. 6E). These data demonstrate that the abundance of SFB was not proportional to diabetes resistance in NOD mice. This contrasts the notion that diabetes was inversely proportional to SFB abundance detected by using the suboptimal SFB-1 primer set [22,23]. Notably, others reported that the representation of SFB was lower but not absent under diabetic conditions in female NOD mice [31,32]. Taken together, these data are congruent with the notion that SFB representation and T1D induction are unrelated events.

Discussion

The NOD mouse model has been widely used to study the intrinsic (genetic) and extrinsic (environmental) factors involved in the generation of autoimmune diabetes [10,11]. The development of diabetes in female NOD mice under germ-free conditions attests that genes are essential and sufficient for T1D induction, and bacteria are not critical for triggering diabetes [33–35]. However, commensal bacteria have been implicated in modulating autoimmune responses against insulin-producing β cells and, hence, diabetes [20–22]. Broad-spectrum antibiotics mostly worsened diabetes [23,24,36–38], except for the marginal protection reported in one study [24]. Interestingly, a mixture of broad-spectrum antibiotics, ampicillin, metronidazole, and neomycin administered to adult NOD mice failed to inhibit diabetes development despite decreasing the gut microbiome [38]. Providing probiotics also yielded mixed results on protection against diabetes [39–44]. Thus, identifying the bacterial taxa crucial for T1D remains a formidable task.

The consumption of acidified drinking water has been shown to increase [20], decrease [21], or without influence [18,19] on diabetes incidence in female NOD mice. Pyrosequencing of the fecal DNA did not reveal the causative bacterial taxa responsible for the pro- or anti-diabetic effects of drinking water with differing pH [20,21]. The data presented in the current report provide a bacterial taxa signature of autoimmune diabetes that differs from NOD mice that have increased [20] or lowered [21] diabetes incidence but shares certain similarities with NOD mice maintained on neutral pH water and displayed robust diabetes [31,45].

Interestingly, 454-pyrosequencing, as employed before [20,21,31,44], revealed that the frequency of *Lactobacilliaceae* represented less than 2% of the intestinal microbiome of NOD mice switched to neutral drinking water. The employment of qRT-PCR indicated that the copy number of *Lactobacillus* sp. remained consistently lower throughout the lifetime of the NOD mice compared to young (6-week-old) mice, regardless of the pH of the drinking water consumed. These data suggest an inverse relationship between the abundance of *Lactobacillus* sp. and diabetes prevalence. This is consistent with the presence of *Lactobacillus* sp. in diabetes-resistant mice but less abundant in diabetes-prone NOD mice [31]. Interestingly, *Lactobacillus* and *Bifidobacteria* failed to colonize either the ileum or the colon of NOD mice when VSL#3 containing these commensals was administered [31]. This indicates that the ‘protective’ bacteria in VSL#3 failed to overcome

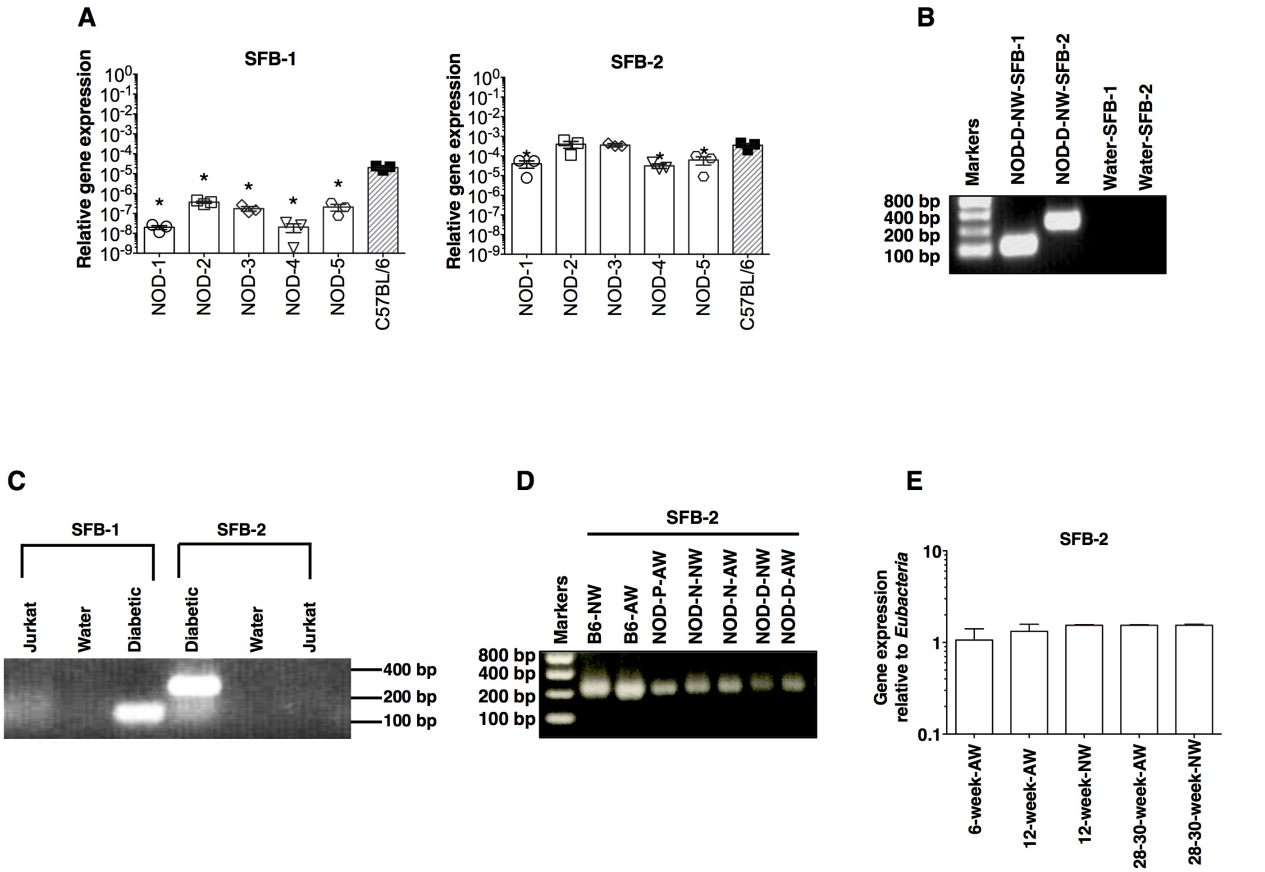

Fig. 6. Lack of impact of SFB on type 1 diabetes development. (A) Fecal DNA was individually isolated from 5 freshly procured 6-week-old female NOD and 5 C57BL/6 mice and probed with two oligonucleotide primers for SFB using qRT-PCR. These mice were provided with acidic drinking water at Jax. Scatter plots with mean ± SD are depicted. Asterisks indicate statistically lower amplification in individual NOD mice than in C57BL/6 mice using the SFB-1 primer set ($p < 0.05$). The same DNA samples were then probed using the SFB-2 primer set, which resulted in higher SFB amplification, which was comparable to that found in C57BL/6 mice. (B) Using two primer sets, the standard PCR was performed on the fecal DNA pooled from ten 6-week-old mice analyzed in (A). Controls included the omission of the template. Amplicons were analyzed on an agarose gel, stained with ethidium bromide, and imaged. Different sizes of amplicons were obtained using the SFB-1 (~110-bp) and SFB-2 (~300-bp) primer sets. (C) Pooled DNA from 10 diabetic NOD mice was amplified by qRT-PCR using SFB-1 and SFB-2 probe sets. The amplicons were analyzed on an agarose gel. Water and DNA from Jurkat cells served as negative controls. (D) The amplicons of qRT-PCR performed on fecal DNA using the SFB-2 primer set were analyzed on an agarose gel. Fecal DNA was obtained from 28–30-week-old C57BL/6 (B6) mice (n = 10), 12-week-old prediabetic (P) NOD mice (n = 10), 22-week-old nondiabetic (N) NOD mice (n = 6) or 28–30-week-old diabetic (D) NOD mice (n = 12). Mice were provided with either acidic (AW) or neutral pH (NW) drinking water. (E) The abundance of SFB was determined by qRT-PCR using the SFB-2 primer set in pooled DNA derived from the stool of 6-week-old (n = 10), 12-week-old prediabetic mice (n = 10) and 28–30-week-old diabetic NOD mice (n = 20). They were maintained on acidic (AW) or neutral pH (NW) drinking water. Data are presented as mean ± SD.

the effects of a diabetogenic microbiome due to colony resistance. Interestingly, oral administration of heat-killed *L. casei* reduced diabetes incidence in NOD mice [39], indicating that colonization of the gut with live bacteria was unnecessary to bestow protection against T1D. Similarly, protection against diabetes was afforded by the administration of complete Freund's adjuvant containing heat-killed, dried *Mycobacterium tuberculosis* (H37Ra) [46–48]. However, for unknown reasons, complete Freund's adjuvant was more effective in reducing T1D incidence when administered during the pre-diabetic period rather than later in life [18]. Although the oral transfer of *L. Johnsonii 6.2* strain derived from Bio-Breeding diabetes-resistant to Bio-Breeding diabetes-prone rats afforded protection against diabetes, *L. reuteri* strain failed to do so [40], indicating a strain-specific effect. Interestingly, oral gavage of VSL#3 containing lyophilized bacteria (*Streptococcus thermophilus*, *Bifidobacterium breve*, *B. longum*, and *B. infantis*) and several *Lactobacillus* spp. (*L. acidophilus*, *L. casei*, *L. plantarum*, *L. bulgaricus*, *L. paracasei*, and *L. delbrueckii*) reduced diabetes incidence in NOD mice [41], perhaps partly attributed to the presence of *Lactobacillus*

sp. in VSL#3. Vancomycin, a glycopeptide antibiotic administered from birth, increased diabetes frequency in female NOD mice, which was associated with decreased *S24-7*, *Ruminococcus*, *Clostridia*, and *Lachnospiraceae* [31]. In contrast, another report indicated that vancomycin treatment increased diabetes in male but not female NOD mice and was accompanied by increases in *Lactobacillus* sp., *Escherichia*, and *Sutterrela* genera [36]. A mixture of streptomycin, colistin, and ampicillin decreased the *S24-7* family, *Escherichia*, and *Lactobacillus* despite similar diabetes increment [36], reflecting the differences in the efficacy of antibiotics on eradication of *Lactobacillus*. The decreased prevalence of *Lactobacillus* sp. throughout the life of the NOD mouse, regardless of the pH of the drinking water, is consistent with the notion that the low frequency of this bacterium, along with other unknown bacterial taxa, is associated with diabetes induction.

Our data indicate that *Firmicute Lachnospiraceae* sp. was the most prominent bacterial taxon found in diabetic mice at the microbiome level, which remained similar irrespective of the drinking water pH. However, it is unclear whether *Lachnospiraceae* sp. could play a role in diabetogenesis. An increase in *Lachnospiraceae* sp. has been attributed to the T1D pathogenesis of NOD mice maintained on neutral drinking water [45]. In contrast, maternal treatment with a mixture of neomycin, polymyxin B, and streptomycin protected the offspring from diabetes accompanied by an increased abundance of *Lachnospiraceae* [49]. Thus, the role of *Lachnospiraceae* in diabetogenesis remains controversial. We found that the *Firmicutes* phylum member *Ruminococcaceae* steadily increased through maturation, similar to a previous study [45] but in contrast to a lower representation of *Ruminococcaceae* reported [31]. Among the *Bacteroidetes* phylum members investigated, *Anaerophaga* sp. and *Paludibacter* sp. increased strikingly through the diabetic adult stage. On the contrary, *Prevotella* was reported to be more abundant in non-diabetic mice provided with neutral water [45]. Interestingly, *Bacteroides* and *Prevotella* diminished in mice switched to neutral water, which displayed decreased diabetes incidence [21]. A decrease in *S24-7*, *Bacteriodales*, and *Prevotella* and an increase in *Lachnospiraceae*, *Ruminococcus*, and *Oscillospira* have been attributed to T1D pathogenesis in mice drinking neutral water [45]. Our data agree with an increased representation of *Lachnospiraceae* and *Ruminococcaceae* without altering the abundance of *Bacteriodales* and *Prevotella* in diabetic mice. Importantly, our data demonstrate decreased *Lactobacillus* sp., with increases in *Anaerophaga* sp., and *Paludibater* sp. in these mice. Since most of them (80–100%) developed robust diabetes, regardless of the ionic strength of the drinking water, these bacterial groups likely represent a signature microbiome of autoimmune NOD mice.

Notably, the data presented in this study demystify the role of SFB in T1D. The *Firmicutes* member SFB or '*Candidatus Arthromitus*' consists of heterogeneous, unculturable, *non-pathogenic*, spore-forming, Gram-positive filamentous bacteria ubiquitously found in invertebrates and vertebrates [50,51]. Several lines of evidence are against a role for SFB in T1D, unlike proposed [22,23]. Only 16% of female NOD mice colonized by SFB developed T1D, but 90% of SFB-negative mice were reported to develop T1D [22]. It should be noted that the SFB negativity was based on the lack of qRT-PCR amplification using the suboptimal SFB-1 primer set [22,23]. Paradoxically, SFB abundance was not attributed to the low diabetes incidence in male NOD mice [22]. It has been reported that the SFB, a commensal but not a *pathogen*, was also detected in female NOD mice with robust diabetes [24,31]. Furthermore, neomycin treatment reduced diabetes marginally without depleting SFB [24]. Notably, SFB colonization could not be uniformly detected using the SFB-1 primer set [22,23,37]. Interestingly, the original group that failed to detect SFB in C57BL/6 mice obtained from Jax [30] later found that NOD mice from different locations of the animal facility were not uniformly SFB-negative [52]. Therefore, the lack of SFB detection could be attributed to the poor sensitivity of SFB-1 primers and the variable abundance of SFB. We could determine detectable levels of SFB by qRT-PCR in fecal DNA using two different SFB primer sets [27,28] in all 6-week-old NOD and C57BL/6 mice within 24 hours of arrival from Jax. The data indicate that these mice hoarded SFB at the vendor's facility despite the claim that they were eradicated by ampicillin treatment of NOD mice in 2014 [23]. We observed that NOD mice procured later than 2014 had substantial colonization of SFB and robust diabetes incidence. Although SFB-1 primers poorly amplified SFB, it was nevertheless concluded that diabetes in males treated therapeutically with pulse antibiotics was independent of SFB [37]. The broad-spectrum antibiotic ampicillin administration for four weeks in adults slightly increased diabetes incidence from 65 to 85% and was associated with reduced SFB abundance, as assessed using the SFB-1 primer set and qRT-PCR [23]. Although ampicillin is a broad-spectrum antibiotic [53], these investigators did not determine alterations of bacteria sensitive to ampicillin other than the SFB [23]. Hence, the bacteria depleted by ampicillin accompanying increased diabetes incidence remain unidentified. The SFB commensals are ubiquitously present, including in NOD mice [52], and therefore cannot be attributed to protection against diabetes, as proposed [22,23]. Interestingly, the level of SFB DNA decreased in mice aged 7 to 13 weeks, identically in non-diabetic C57BL/6 mice and diabetes-prone NOD mice [32]. Unfortunately, NOD mice were not followed beyond 13 weeks until they became diabetic, typically >18 weeks of age. Hence, the conclusion that the abundance of SFB decreased due to T-cell-mediated pathological changes is not plausible [21]. In contrast, we found a high abundance of SFB as detected, especially using the SFB-2 primer set in NOD mice

freshly procured from Jax and during maintenance in our facility under SPF conditions. Importantly, the SFB abundance was not impacted by the pH of the drinking water. Thus, our data allow us to conclude that the abundance of ubiquitously present SFB is unlinked to diabetogenesis.

Previously, SFB-positivity was associated with interleukin-17 (IL-17) production in the lamina propria of the small intestine [30], suggesting a role for $CD4^+$ T-cells producing IL-17 in diabetes protection [22]. However, the link between the generation of Th17 cells by SFB and diabetes induction has been challenged by many observations. IL-17 silencing by RNA interference did not protect NOD mice from T1D [54], indicating the lack of a role for IL-17 in diabetogenesis. Importantly, we observed that Interferon (IFN)-γ-producing Th1 cells and IL-17A-expressing Th17 cells polarized from prediabetic NOD mice maintained on neutral drinking water could independently transfer diabetes to immunodeficient NOD.*scid* mice [17]. Consistently, the levels of mRNA encoding IL-17A and IFN-γ were higher in diabetic mice than in prediabetic (12-week-old) mice, which were concurrently downregulated by a histone deacetylase modifier that afforded protection against diabetes [16]. It was reported that the acquisition of diabetes resistance in weanling upon switching from acidic to neutral water was associated with lower IFN-γ and IL-23 expression without affecting IL-17 in the small intestine [20]. On the contrary, decreased diabetes incidence in two-week-old mice provided with acidic water was accompanied by higher numbers of IL-17-producing cells and fewer IFN-γ^+ cells in the small intestine lamina propria [21]. Thus, controversy exists about the role of Th17 cells in diabetes protection. We showed that treatment with the histone deacetylase inhibitor Trichostatin A, concurrently repressed the expression of IL-17A and IFN-γ and afforded robust protection against diabetes [16], indicating that both lymphokines can independently contribute to T1D and their downregulation can negatively impact diabetes occurrence. Interestingly, the numbers of $CD4^+FoxP3^+$ T regulatory cells did not differ in NOD mice that consumed neutral or acidic water [14,21,22], indicating these cells lack a regulatory role in diabetes. Diabetes protection by the histone modifier treatment was associated with increased $CD4^+CD62L^+$ but not $CD4^+FoxP3^+$ T regulatory cells [14]. Further work is necessary to decipher the role of immunomodulation in diabetes.

The extremely low representation of *H. pylori* in the intestinal microbiota of diabetic mice provided with acidic or neutral water suggests the lack of a role for this bacterium in T1D of NOD mice, unlike its involvement in human gastroenteric diseases such as chronic gastritis, peptic ulcers, cancers, and insulin resistance [55]. Interestingly, the frequency of *A. muciniphila*, the mucin-degrading bacterium and the only member of the *Verrucomicrobia* phylum represented in mice, and *H. pylori* remained at a lower level in diabetic mice than most other bacteria investigated. However, treatment of adult NOD mice with vancomycin, selective against Gram-positive bacteria, decreased diabetes incidence associated with a dramatic increase (~86% of the microbiome) of *A. muciniphila* [24]. Moreover, it was shown that the transfer of *A. muciniphila* promoted mucus production and delayed diabetes incidence, suggesting an inverse relationship between this bacterium and diabetes [43]. Given the diversity and complexity of the gut microbiota, it is unlikely that a single bacterium can have a profound pathogenic or protective role in T1D. It should be pointed out that all of these bacterial groups represented less than 1% of the microbiome, and the qRT-PCR allowed the estimation over a dynamic range. It should be acknowledged that acid water consumption depleted several gastrointestinal pathogens in many species [6–8]. Yet, it did not modify most of the commensal bacteria investigated except for a reduction in the abundance of *C. coccoides* and a transient decrease in *A. muciniphila* in diabetic NOD mice. However, pyrosequencing of the fecal DNA from mice provided with acidic water that increased [20], reduced [21], or did not affect (this study) diabetes failed to identify putative pathogens in T1D. This is similar to treatment with wide-spectrum antibiotics, probiotics, and bacterial products, which have not unraveled the specific pathobionts involved in diabetes.

Conclusions

The lack of influence of acidified drinking water on the spontaneous development of T1D in female NOD mice reported herein is consistent with our previous observation. Pyrosequencing and validation by qRT-PCR through discrete stages of life of NOD mice using primers specific for 16S rRNA genes indicated a consistently lower representation of the *Firmicutes Lactobacillus* sp. with a concurrent increase in the copy numbers of *Ruminococcaceae* as well as the *Bacteroidetes* phylum members *Anaerophaga* sp. and *Paludibacter* sp. regardless of the pH of the drinking water. Importantly, the bacterial groups implicated in diabetes protection, such as SFB and *A. muciniphila* remained abundant in diabetic mice and unperturbed by acidified drinking water. Thus, perturbation of gut-resident commensals by acidified drinking water is unlikely to alter T1D incidence in NOD mice. Further studies elucidating the signature microbiome of NOD mice fully protected from diabetes, such as by histone deacetylase inhibitor treatment, may help understand the critical role of microbiota in this autoimmune disease.

Availability of Data and Materials

The 454-pyrosequencing raw data have been deposited in the NCBI SRA (Sequence Read Archive) database, and the BioProject SRA accession number is PRJNA608025.

Author Contributions

SJ, MB, TAS, and AKJ conducted research. SJ designed the study, acquired funding for research, analyzed the data, and wrote the paper. MB, TAS, and AKJ were also involved in revising the manuscript critically for important intellectual content. All authors read and approved the manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all study aspects.

Ethics Approval and Consent to Participate

The Office of Animal Care and Institutional Biosafety of the University of Illinois at Chicago approved the animal protocol (ACC Protocol No: 15-200).

Acknowledgment

The Core DNA Facility staff at the University of Illinois at Chicago is acknowledged for facilitating the pyrosequencing studies performed at the Molecular Research DNA Lab.

Funding

This research received no external funding.

Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.24976/Discov.Med.202436181.40.

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