

RESEARCH ARTICLE

High levels of fucosylation and sialylation of milk N-glycans from mothers with gestational diabetes mellitus alter the offspring gut microbiome and immune balance in mice

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Abstract

Gestational diabetes mellitus (GDM) is significantly associated with allergen sensitization in early childhood, and this may influence the gut microbiome and immune system of the children. In addition to mother-to-child transmission of microbes, milk glycans play a pivotal role in shaping the gut microbiome of infants. A previous study has demonstrated alterations in the major milk N-glycans of mothers with GDM. However, the impact of these changes on the gut microbiome and immune response of the neonates has yet to be studied. Here, we aimed to compare the glycosylation levels of various milk glycans between normal and GDM mice, and to characterize the intestinal microbiome and immune responses of the offspring after weaning. We found that GDM mouse milk contained significantly higher concentrations of fucosylated and sialylated N-glycans than control mice, but there was no difference in the concentration of milk oligosaccharides between the groups. The differences in milk N-glycans had direct effects on the intestinal microbiome of the offspring, which in turn affected their immune response upon challenge with ovalbumin (OVA), with disruptions in the Th1/Th2 and Th17/Treg cell balances. This study lays the foundation for further research and development of specific nutritional care for the offspring of GDM mothers.

KEYWORDS

Bacteroides, gestational diabetes mellitus, gut microbiome, immune balance, milk N-glycans

Abbreviations: AAL, aleuria aurantia lectin; ATCC, American Type Culture Collection; CBB, coomassie brilliant blue; CON group, control group; GDM, gestational diabetes mellitus; GDM group, gestational diabetes mellitus group; HMOs, human milk oligosaccharides; IADPSG, International Association of Diabetes and Pregnancy Study Groups; ICR, Institute of Cancer Research; IFN- α , interferon- α ; IgG, immunoglobulin G; IL-4, interleukin-4; IL-10, interleukin-10; IL-17, interleukin-17; LCA, lens culinaris lectin; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time of flight mass spectrometry; OVA sIgE, ovalbumin specific immunoglobulin E; PBS, phosphate-buffered saline; PHA-E, phaseolus vulgaris erythroagglutinin; PPs, Peyer's patches; sIgA, secretory immunoglobulin A; SNA, sambucus nigra lectin; Treg, regulatory T cell.

Jiaorui Zhou and Yue Wang contributed equally to this work.

1 | INTRODUCTION

Maternal milk is essential for the healthy growth of human infants, and specifically for the establishment of the intestinal microbiome in early infancy.^{1,2} Breast milk glycans—mainly consisting of human milk oligosaccharides (HMOs) and glycoconjugates (glycoproteins and glycolipids)—have been shown to selectively enrich the infant gut microbiome with beneficial bacteria, such as *Bifidobacterium* spp. and *Lactobacillus* spp.^{3,4} The establishment of a healthy gut microbiome in this way significantly contributes to the growth and development of infants by providing essential nutrients, improving the defense against pathogens, and promoting the development of the immune system.⁵

Gestational diabetes mellitus (GDM) refers to abnormal glucose metabolism that occurs for the first time during pregnancy.⁶ Using the International Association of Diabetes and Pregnancy Study Groups (IADPSG) criteria, the prevalence of GDM has been reported to be 17.5% in a retrospective study by Zhu *et al*, which recorded data from 17,186 pregnant women in 13 hospitals in China between 2010 and 2011,⁷ and the incidence has been increasing in recent years.⁸ GDM has various adverse effects on mothers and their infants during gestation, delivery, and lactation, such as greater risks of obesity, diabetes mellitus, and other metabolic disorders.^{9,10} In term births, GDM is significantly associated with atopic dermatitis and allergen sensitization in early childhood.¹¹ Alterations in the gut microbiome of GDM offspring were also found in a study of more than 200 neonatal samples in China,¹² in which lower diversity and depletion of Kyoto Encyclopedia of Genes and Genomes orthologues, and greater abundance of some viral genes, were detected in the meconium microbiome of neonates from mothers with GDM. Although direct mother-to-child bacterial transmission significantly contributes to the establishment of the neonatal microbiome,¹³ feeding pattern also has a major influence.² Because gut microorganisms have differing abilities to utilize different types of glycans as carbon sources for growth and metabolism,^{14,15} differences in milk glycans may also affect the composition of the gut microbiome in the offspring. It has been shown that the milk N-glycans of secretory immunoglobulin A (sIgA) and lactoferrin are affected by GDM, but not the HMOs.¹⁶ However, the impact of these differences in GDM milk glycans on the gut microbiome of neonates has yet to be studied in detail.

More importantly, there is a close relationship between intestinal dysbiosis and the abnormal development of immune tolerance in early childhood.¹⁷ Alterations in the intestinal microbiota are associated with over-activation of the immune response, which underlies the development of allergic and autoimmune diseases.^{18,19} Regulatory T (Treg) cells are a subset of T cells that regulate autoimmune responses *in vivo* and are closely related to both the intestinal microbiome and allergic

diseases in neonates.²⁰ Previous studies have shown that one of the most important factors in the development of allergic diseases is the deficiency or inhibition of Treg cell function, which results in a reduction in the immune tolerance of allergens, thus promoting the differentiation of immature CD4⁺ T cells into Th2 cells, which mediate allergies.²¹ In addition, Th17 cells have recently been shown to be a subset of CD4⁺ T cells, and to secrete the pro-inflammatory cytokine interleukin-17 (IL-17). This breakdown in Treg/Th17 balance is an important factor in the development of both allergic and autoimmune diseases.^{22–24} The intestinal microbiome plays an important role in the regulation of the differentiation, development, and function of Treg cells.²⁵ Colonization of the gut with *Bifidobacterium*, *Lactobacillus*, and some *Clostridium* spp. in germ-free mice was found to be associated with Treg cell proliferation.^{26,27} Conversely, gut dysbiosis, involving a reduction in the abundance of these microorganisms, may lead to a reduction in the number of Treg cells or impaired function, which in turn affects the development of immune tolerance. Given that GDM is associated with a high risk of allergic disease in the offspring,¹¹ we hypothesized that dysbiosis of the gut microbiome of the offspring of GDM mothers might contribute to the disruption of normal immune balance and impaired development of immune tolerance.

In this study, we used a mouse model of GDM to compare the glycosylation level of milk glycans between normal mothers and mothers with GDM, characterize the intestinal microbiome of their offspring after weaning, and investigate the impact of alterations in the gut microbiome on the immune response of the offspring. Our results show that changes in milk N-glycans in GDM mouse mothers have direct effects on the intestinal microbiome of the offspring, which in turn disrupts the Th1/Th2 and Th17/Treg cell balance. Thus, this study lays a foundation for further research and the development of specific nutritional care regimens for the offspring of mothers with GDM.

2 | MATERIALS AND METHODS

2.1 | Animals study

About 30 female Institute of Cancer Research (ICR) mice aged 6 weeks were obtained from the specific pathogen free (SPF) animal laboratory of Dalian Medical University, Dalian, China. After 1 week of adaptation, the mice were randomly divided into two groups ($n = 15$ in each group), the control (CON) group of mice were fed with standard diet, the GDM group of mice were exposed to high-fat diet (20% protein, 20% carbohydrate, 60% fat) for 4 weeks. After that these female mice were mated with age-matched males. Mating pairs were housed in individual cages, and males were separated from the females following confirmation of

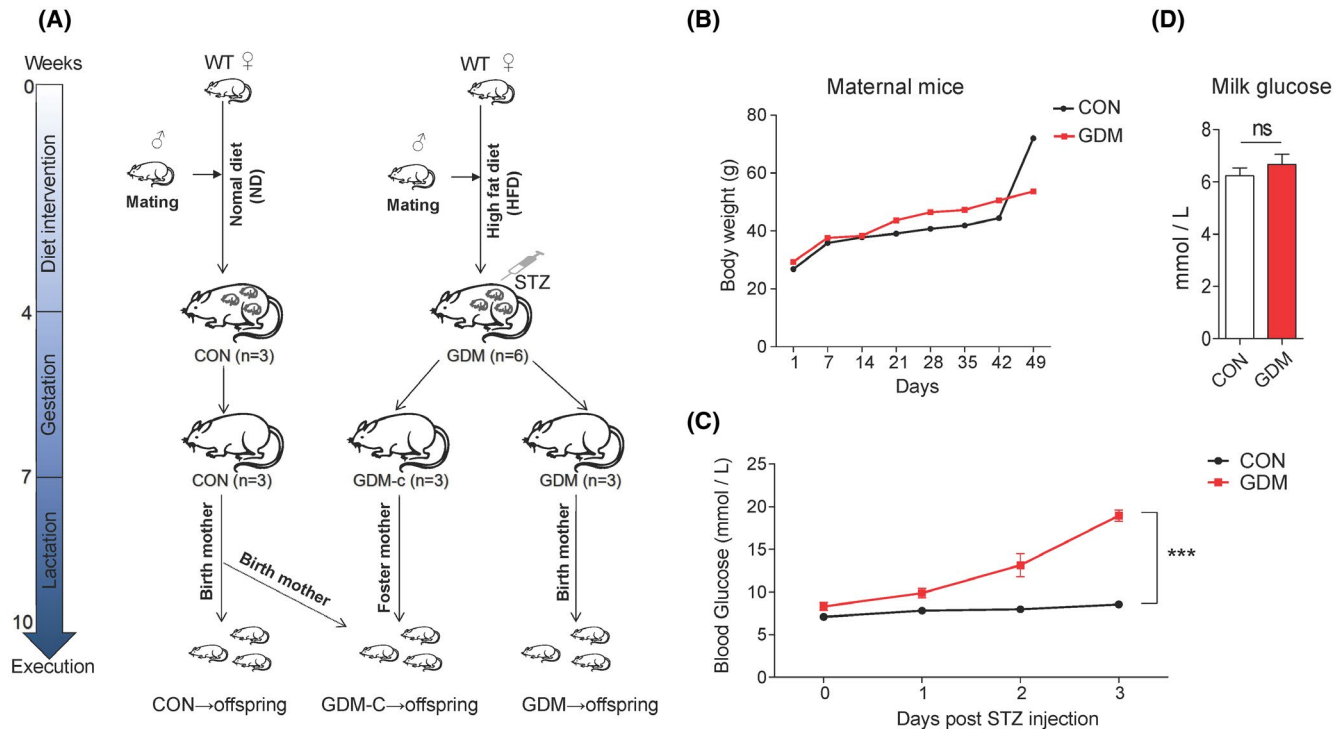


FIGURE 1 Animals study and basic information. A, CON group were fed with standard diet, GDM group were exposed to high-fat diet (HFD) for 4 weeks. After that these female mice were mated with age-matched males. During pregnancy, the GDM group of mice were intraperitoneal injected with 30 mg/kg/d STZ for continuous 3 days, the CON group was injected with equal dose of NS for 3 days. At birth, CON offspring were cross-fostered by different mothers for the period of lactation to produce three experimental groups: CON → offspring (n = 3), GDM → offspring (n = 3), GDM-C → offspring (n = 3). During lactation, GDM mothers were continued on HFD. After 3 weeks of lactation, three offspring mice in each group were executed. B, There were no differences in weight between the CON and GDM group before day 42 (6 weeks), but during the 7th week, the CON mice gained significantly more weight than GDM group of mice, in contrast, no significant weight gain was found in GDM group during this week. C, The fasting blood glucose level was significantly higher in GDM mice than in the CON mice 3 days post STZ injection. D, Post delivery, the glucose level in milk samples of maternal mice had no difference between the two groups. Data are shown as mean values $\pm$ SD (* P < .05; ** P < .01)

pregnancy. During pregnancy, the gestational diabetes mellitus group (GDM group) of mice were intraperitoneal injected with 30 mg/kg/d streptozotocin (STZ) for continuous 3 days, the control group (CON group) was injected with equal dose of normal saline (NS) for 3 days. Next, fasting blood glucose test was employed to confirm the occurrence of GDM (fasting blood glucose level > 11.1 mmol/L). Because of the success rate of pregnancy and modeling, three maternal mice in the CON group and six maternal mice in the GDM group were maintained. Fasting blood samples were obtained via tail venipuncture and quantified by glucometer (Roche Diagnostics, RischRotkreuz, Switzerland). Body weight was measured weekly using an electrical balance.

After parturition, the cross-fostering procedure (Figure 1A) was carried through in order to generate offspring of three distinct experimental groups as follows: CON → offspring, the CON maternal mice (biological mother)-fed neonates; GDM → offspring, the GDM maternal mice (biological mother)-fed neonates; GDM-C → offspring, the GDM maternal mice cross-fostered neonates (the CON mice were their biological mothers). After 21 days of lactation, three mice

were executed in each group and others were remained for further OVA injection experiments. The stool of each mouse after 21-day lactation was collected and stored at -80°C until analysis. After sacrifice, the entire mammary gland of the maternal mouse was gently peeled off with a scalpel, and then immersed in 1 ml of phosphate-buffered saline (PBS) until the white milk was precipitated and the milk-PBS solution was immediately frozen at -80°C . Serum of mice was obtained by centrifugation of blood at 3000 r.p.m for 15 minutes and stored at -80°C until further analysis. All animal experiments were approved by the Ethics Committee at the Dalian Medical University (approval No. AEE17013), and were performed in accordance with the 1996 National Institutes of Health Guide for the Care and use of Laboratory Animals.

2.2 | Milk protein extraction and lectin blotting

The mouse milk samples (500 μL) were mixed with 500 μL of deionized water and centrifuged at 8000 g at 4°C for

30 minutes twice. Removing the lipids and quantitating the concentration of whey protein (300 μ g) with bicinchoninic acid (BCA) protein assay. The protein concentration of mouse milk was determined with a BCA protein assay kit (Takara Bio, Otsu, Japan). After that, the equal amount (4 μ g) of milk proteins from each maternal mouse were separated by 10% of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). One gel was stained with Coomassie Brilliant Blue (CBB) R-250, while proteins on another gel were transferred to a 0.45 μ m of polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA) that were fixed according to our recently developed method.²⁸ Briefly, the electroblotted PVDF membranes were immersed in acetone at room temperature for 30 minutes with gentle shaking, and followed by sample heating at 100°C for 30 minutes. The fixed membranes were briefly immersed in methanol and then in TBS-T (Tris-buffered saline containing 0.05% Tween-20) for several minutes before their blocking for 1 hour with 5% bovine serum albumin (BSA) in TBS-T. After washing with TBS-T (5 minutes, three times), the membranes were incubated with biotinylated lectins Aleuria aurantia lectin (AAL), Lens culinaris lectin (LCA), Phaseolus vulgaris Erythroagglutinin (PHA-E), and Sambucus nigra lectin (SNA), all diluted 1:20 000 in TBS-T, then at 4°C overnight. After an additional washing with TBS-T (5 minutes, three times), the membranes were incubated with streptavidin-horseradish peroxidase (HRP) diluted with TBS-T for 1 hour at room temperature. The binding was visualized using the ECL (enhanced chemiluminescence) Plus reagents (Beyotime Institute of Biotechnology, Shanghai, China). The biotinylated lectins, AAL, LCA, PHA-E, SNA, were purchased from Vector Laboratories Inc and all lectins primary sugar specificity were showed as follows: AAL: broad specificity to fucosylated glycans, LCA: fuc α 1-6GlcNAc and α -Man, α -Glc, PHA-E: bi-antennary complex-type N-glycan with outer Gal and bisecting GlcNAc, SNA: sia2-6Gal/GalNAc (Burlingame, CA, USA). Streptavidin conjugated with HRP was purchased from Beyotime (Shanghai, China).

2.3 | N-glycan purification

The mouse milk samples (500 μ L) were mixed with 500 μ L of deionized water and centrifuged at 8000 *g* at 4°C for 30 minutes twice. Removing the lipids and quantitating the concentration of whey protein (300 μ g) with BCA protein assay. The aliquots were precipitated with 3-5 folds ethanol and centrifuged at 16 000 *g* at 4°C for 20 minutes. Then the whey proteins were collected and lyophilized. The protein pellet was resuspended in 200 μ L of 200 mM ammonium bicarbonate and 200 μ L deionized water, which contains 10 mM of DL-Dithiothreitol (DTT) to solubilize fully, and at

100°C boiled 5 minutes. Then, 2.2 μ L of 250 mM iodoacetamide was added to the mixtures, and incubated at room temperature for 60 minutes in the dark. The mixtures were then treated with 0.05 mg/mL trypsin (Sigma-Aldrich) at 37°C for 24 hours, followed by heat-inactivation of the trypsin at 95°C for 5 minutes. Finally, 3 mU of peptide N-glycanase F (PNGase F) (Takara Bio Inc, Dalian, China) was added, and the mixtures were incubated at 37°C for 18 hours. The mixtures were diluted in deionized water (500 μ L) and passed through cation exchange cartridge OASIS MCX (1 mL, Waters), and then the cartridge was washed with 1 mL of deionized water. The pass-through fraction and washings were combined and lyophilized.

2.4 | Permethylation of N-glycan and analysis by matrix-assisted laser desorption/ionization time of flight mass spectrometry

To enhance the sensitivity of N-glycan analyzed by mass spectrometry, permethylation of N-glycans were performed in a super-clean bench of the temperature-controlled (20°C) laboratory according to the method described previously.²⁸ Dimethyl sulfoxide (DMSO) containing 1% v/v of distilled water (50 μ L) was added to the lyophilized glycan sample under alkaline conditions with powdered sodium hydroxide. Methyl iodide (50 μ L) was then added to the mixture, and the reaction was performed at room temperature for 30 minutes with vigorous shaking. The methyl iodide reagent is stored in a -20°C refrigerator and used under a steam of argon. Distilled water (1 mL) was slowly added to the reaction mixture, and then the mixture was applied to a Sep-Pak Vac 18 cartridge (50 mg/mL; Waters) to purify the permethylated glycans. Then the permethylated glycans were eluted with 80% of acetonitrile and lyophilized. Obtained permethylated glycan was analyzed using Bruker Ultraflex matrix-assisted laser desorption ionization (MALDI-TOF) mass spectrometry.

Spectra were acquired using a MALDI-TOF/TOF mass spectrometry (New ultrafleXtreme; Bruker Daltonik, Bremen, Germany). Ions were generated with a pulsed 337-nm nitrogen laser and accelerated to 25 kV. All spectra were obtained in the reflectron mode with delayed extraction of 200 ns. The measurements were carried out in the positive ion mode and were repeated at least three times. The permethylated glycan were dissolved in 50% of acetonitrile and spotted on a target plate (MTP 384 target plate ground steel, Bruker Daltonik) with 2,5-dihydroxybenzoic acid (DHB) in 30% of ethanol. The intensity of the isotopic peaks of each glycan was normalized to an internal standard (maltoheptaose) with a known concentration. Each sample was done in triplicate and analyzed on MS. Although permethylation reactions of N-glycans were

performed under a stream of argon, there was still a phenomenon of under-methylation in our results. In order to accurately reflect the glycoforms of glycoproteins, each sample was done in triplicate to determine relative changes in the abundances of N-glycans between the two milks. For MS analysis, each spot was measured using 3000 laser shots in groups of 500 shots per sampling area of the spot by manually. MS was calibrated before being run, using a Peptide Calibration Standard II (external standard) from Bruker Daltonics (Part NO. 222570).

2.5 | Fecal DNA extraction, polymerase chain reaction (PCR), and 16S rRNA amplicon data processing

Microbial genome DNA was extracted from fecal samples of neonatal mice post weaning using a stool DNA kit (Omega Bio-tek, Inc) according to the manufacturer's instructions. The V3 variable regions of bacterial 16S rRNA genes were PCR amplified from genomic DNA using the primers 338F (5'-ACTCCTACGGGAGGCAGCA-3') and 518R (5'-ATTACCGCGGCTGCTGG-3'). The 50- μ L PCR mixture contained the following components: 5 μ L DNA template, 25 μ L PCR 2 $\times$ Easy Taq Super mix (HotStarTaq Plus DNA polymerase, dNTPs, MgCl₂, and reaction buffer), 1 μ L of each primer, and 18 μ L deionized water. The PCR program consisted of an initial step at 95°C for 5 minutes; 30 cycles of 94°C for 45 seconds, 55°C for 45 seconds, and 72°C for 60 seconds; and a final extension at 72°C for 8 minutes. PCR amplicons were sequenced and the data analyzed by Illumina HiSeq (Novogene Bioinformatics Technology Co., Ltd., Beijing, China).²⁹

2.6 | Flow cytometric analysis

After the neonatal mice were executed, their spleen and intestinal Peyer's patches (PPs) were picked up and grinded in PBS buffer, 1×10^6 cells were used for Flow Cytometry (FACS). Cells were incubated with anti-mouse CD16/CD32 mAb to block Fc γ receptors for 60 minutes and then stained on ice for 60 minutes with combinations of mAbs, as indicated in the Figure legends. The monoclonal-antibodies (mAbs) used in this study are anti-mouse CD16/CD32 (2.4G2), PE-labeled anti-mouse CD3 (2C11), PE-Cy5-anti-mouse CD19 (6D5), FITC-anti-mouse-Gr-1 (8C5), PE-labeled anti-mouse CD11c (N418), FITC-anti-mouse pan-NK cells (DX5), PE-labeled anti-mouse F4/80 (BM8), FITC-anti-mouse Foxp3 (MF23), APC-labeled anti-mouse CD4 (53-6.7), FITC-labeled anti-mouse CD69 (H1.2F3), all purchased from BD Biosciences. Flow cytometry assay was performed on a FACS Calibur (Becton Dickinson, Mountain View, CA, USA) and analyzed using FlowJo software (Tree Star).

2.7 | Immunization of the neonatal mice

Neonatal mice (4 week old) were immunized by subcutaneous injection with 200 mg of ovalbumin (OVA) mixed with an equal volume of Freund's complete adjuvant (CFA). Two weeks later, mice were immunized with 200 mg OVA again by intraperitoneal injection. Sera were collected at 28 days post-immunization.

2.8 | Measurement of serum cytokines

Blood samples of mice were collected and centrifuged at 3000 r.p.m for 15 minutes to obtain serum. sIgA, IL-17, interleukin-10 (IL-10), immunoglobulin G (IgG), OVA specific immunoglobulin E (OVA sIgE), interferon- α (IFN- α), and interleukin-4 (IL-4) were measured using enzyme-linked immuno sorbent assay (ELISA) (Shanghai Langton Biological Technology Co., Ltd., China). The absorbance was measured at 450 nm using a computer-interfaced microplate reader (BioRad, Houston, TX).

2.9 | Culture of *Bacteroidetes acidifaciens* ATCC800799 strain

The standard strain *Bacteroidetes acidifaciens* ATCC800799 used in this study was obtained from American Type Culture Collection (ATCC). It was routinely grown at 37°C on Brain-Heart Infusion (BHI) medium under anaerobic conditions. Growth was monitored by measuring the optical density (OD) at 546 nm.

2.10 | Culture of cells in vitro

To detect the effects of *B acidifaciens* on proliferation of B lymphocytes and T lymphocytes in the PPs of mice, we isolated total lymphocytes from the PPs of the neonatal mice and incubated them with different concentrations of *B acidifaciens* ATCC800799 (*B acidifaciens* ATCC800799) supernatant and cell lysate, respectively. Briefly, 1 mL of the *B acidifaciens* ATCC800799 culture broth (OD₅₄₆ = 1.5) was centrifuged at 3000 r.p.m for 15 minutes, the supernatant is regarded as bacterial secretion, the pellet was resuspended in BHI for ultrasonic crushing to release the cellular components, after that, the mixture was centrifuged at 3000 r.p.m for 15 minutes, and different volumes of the supernatant were added to the single-cell suspension after filtration by a 0.2 μ m-filter and regarded as *B acidifaciens* ATCC800799 lysate. The BHI medium was used as control.

2.11 | Statistical analysis

All data were evaluated as mean $\pm$ SD ($n = 3$). Statistical analysis of the quantitative multiple group comparisons was performed using one-way ANOVA followed by Tukey's test (compare all pairs of columns); when two groups were compared, the nonparametric t test was performed with the assistance of GraphPad Prism 5 (Graph Pad Software, La Jolla, CA, USA). P values $< .05$ were considered statistically significant. * $P < .05$, ** $P < .01$, *** $P < .001$.

3 | RESULTS

3.1 | Experimental design and basic information

To determine the effect of GDM maternal milk on the gut microbiome of their offspring and avoid the effect of genetic factors in maternal mice with GDM, we used a cross-fostering protocol (Figure 1A). There were no differences in body mass change between the CON and GDM groups before day 42 (6 weeks), but during the 7th week, the CON mice gained significantly more mass than the GDM group (Figure 1B); the GDM group gained no significant mass during this week. After STZ injection, an increasing trend of fasting blood glucose level was detected in serum of the GDM mice, and a significant difference was found 3 days post STZ injection ($P < .001$, Figure 1C). After delivery, the milk glucose concentrations of the maternal mice were also measured, but no difference was found between the two groups (Figure 1D).

3.2 | The N-glycan patterns of whey glycoproteins are altered in the milk of GDM mice

To characterize the features of the milk glycans in GDM maternal mice, we analyzed the major oligosaccharides and N-glycans in mouse milk. Sialylated milk oligosaccharides, such as 6'SL and 3'SL, were most abundant in the mouse milk, but there were no significant differences in the abundance of the identified oligosaccharides between the two groups (Figure S1). Next, to determine whether the profile of N-glycans is different in the milk of mice with GDM, we carried out a series of lectin blot analyses of whey glycoproteins from CON and GDM mouse milk and analyzed the signal intensities (Figure 2A). AAL ($P = .0472$, $n = 3$) and SNA ($P = .0232$, $n = 3$) lectin, which preferentially recognize fucosylation and $\alpha 2,6$ - sialylation, respectively, were present in large quantities in GDM mouse milk, and both LCA and E-PHA were present in higher concentrations in GDM than

CON milk; however, this difference was not statistically significant due to the small number of samples collected (Figure 2B). Thus, fucosylation and sialylation are markedly upregulated in GDM mouse milk.

3.3 | Higher levels of fucosylation and sialylation of milk N-glycans are present in GDM mice

To prepare a detailed profile of the altered N-glycans in the milk of GDM mice, the N-glycans were released from pooled mouse milk using PNGase F, then permethylated and analyzed by MALDI-TOF MS/MS (Figure 3A). All the glycan signals in the spectra were assigned and are summarized in Figure S2. The MS signals were examined (Figure 3B) to ascertain the monosaccharide composition. In total, 45 possible N-glycan structures were detected in CON and GDM mouse milk, of which five were detected only in GDM milk (peaks 24, 31, 35, 44, 45), which were all fucosylated or sialylated glycans. A total of 27 signals (peaks 2, 5, 7, 9, 12, 14, 15, 17, 19, 21, 23-25, 27, 28, 31-34, 36, 38, and 40-45) had 1-3 fucose units; four of them (peaks 12, 28, 36, 40) were di-fucosylated and signal 28 was stronger in GDM milk; signals 32 and 44 were tri-fucosylated and signal 44 was markedly upregulated in GDM milk. Twenty-one of them (peaks 2, 5, 7, 9, 14, 15, 17, 19, 21, 23-25, 27, 31, 33, 34, 38, 41-43, and 45) had a core fucose unit, and 13 of these (peaks 2, 14, 15, 17, 19, 21, 23-25, 27, 31, 33, and 34) were upregulated in GDM milk. In addition, 18 glycan signals (peaks 19, 22, 24, 25, 27, 29, 30, 33-39, 41-43, and 45) had one or two sialic acid groups, including N-acetyl neuraminic acid (NeuAc) and/or N-glycolylneuraminic acid (NeuGc), and nine of these (peaks 19, 24, 25, 27, 29, 33-35, and 45) signals were upregulated in GDM milk. These data indicate that the number of N-glycans, especially the levels of fucosylation and sialylation, are significantly higher in GDM milk than CON milk.

To determine the structures of the N-glycans identified in the MS analyses, we analyzed the CON and GDM milk samples using MALDI-TOF/TOF MS. The MS/MS spectra of precursor ions of m/z 2593.1, 2780.2, 2853.3, and 2997.4 are shown in Figure S3. In accordance with the above findings, we analyzed the relative intensities of mono-, di-, tri-, and total fucosylated N-glycans (Figure 3A,B), as well as of the mono-NeuAc/NeuGc, di-NeuAc/NeuGc, and total sialylated glycans in CON and GDM milk. The levels of mono-fucosylation ($P < .0001$, $n = 3$) and total fucosylation ($P = .0017$, $n = 3$) were measured in GDM and CON milk, and both tended to be higher in GDM milk with statistically significant differences. Similar to the level of fucosylation, mono-NeuAc ($P = .0129$, $n = 3$), mono-NeuGc ($P = .0029$, $n = 3$), and total sialylation ($P = .0329$, $n = 3$) were much higher in the GDM than the CON milk, but there were no significant differences

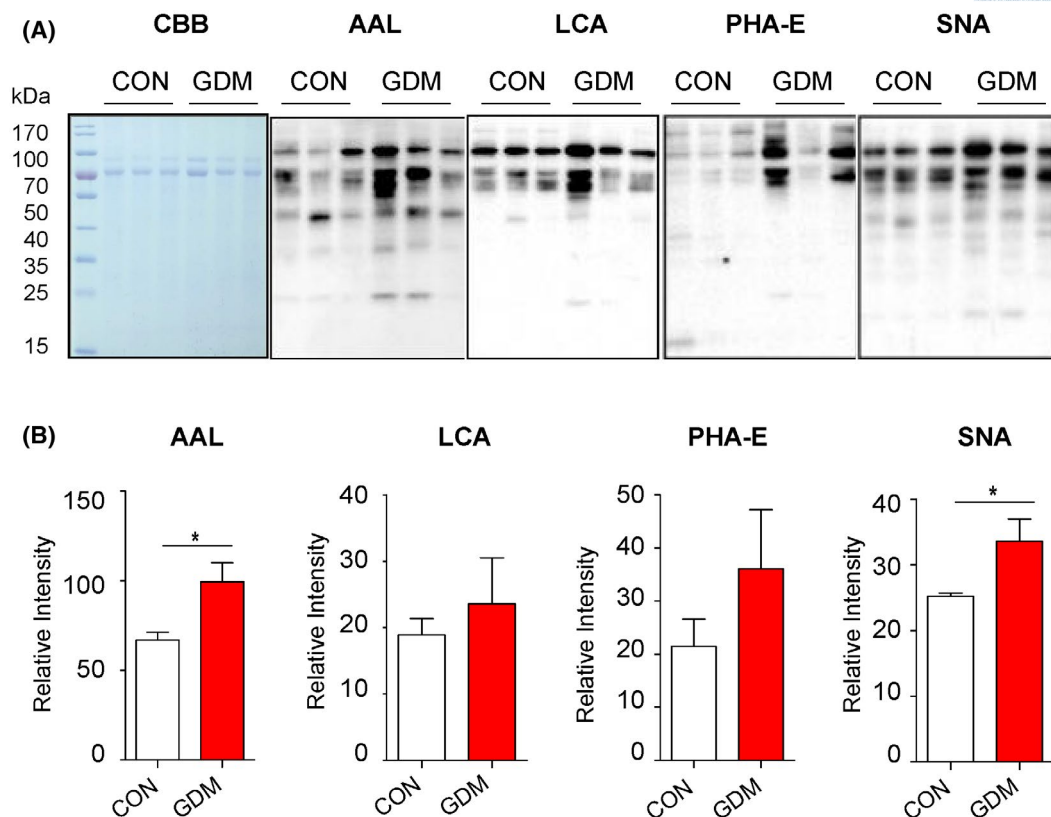


FIGURE 2 The N-glycan patterns of milk glycoproteins were altered in GDM mice detected by lectin blotting. A, Milk (6 μ g of proteins) from CON (lane 1-3, $n = 3$) and GDM (lane 4-6, $n = 3$) mice were subjected to 10% of SDS-PAGE and stained with Coomassie Brilliant Blue (CBB), and other electroblotted membranes were stained with AAL, LCA, PHA-E, and SNA lectins. AAL bind to 1-2, 1-4, and 1-6-linked fucose residues. LCA bind to 1-6-linked fucose residue. PHA-E bind with bisecting GlcNAc N-glycans. SNA bind with sia2 – 6Gal/GalNAc. B, The relative intensities of lectin blotting were analyzed by Image Lab and Graphed Prism (right). * $P < .05$

in the levels of di-NeuAc and di-NeuGc between CON and GDM milk (Figure 3C).

3.4 | *Bacteroides* were more abundant and *Lactobacillus* less abundant in the gut of offspring fed by GDM mothers

Because the fucosylation and sialylation of milk N-glycan were higher in GDM maternal mice than in CON mice, we aimed to determine whether these differences affected the gut microbiome composition of their offspring. We analyzed the intestinal microbiome of neonates by high-throughput sequencing of 16S rRNA genes in fecal samples after they were weaned. This obtained a total of 712,975 high-quality filtered reads, which were assembled into 7,219 effective tags per pup. The alpha diversity of the offspring gut microbiome, reflected in the Shannon index in GDM mouse-fed offspring, in both GDM $\rightarrow$ offspring ($P = .0343$, $n = 3$) and GDM-C $\rightarrow$ offspring ($P = .0180$, $n = 3$), was much lower than that in pups fed by healthy

mothers (CON $\rightarrow$ offspring) (Figure 4A). We then used principal coordinate analysis to categorize the Operational Taxonomic Units (OTU) data into two main factors that explained 56.6% of the variance (Figure 4B), which revealed a significant separation between the CON $\rightarrow$ offspring group and the other groups, suggesting altered beta diversity of the gut microbiome in offspring fed by GDM mothers. At the genus level (Figure 4C), GDM $\rightarrow$ offspring harbored more abundant *Bacteroides* ($41.4\% \pm 0.12$), whereas the relative abundance of *Lactobacillus* ($3.9\% \pm 0.01$) was lower in the gut of these mice than in CON $\rightarrow$ offspring (*Bacteroides*, $2.58\% \pm 0.01$; *Lactobacillus*, $11.1\% \pm 0.02$). Interestingly, in the gut of GDM-C $\rightarrow$ offspring, *Bacteroides* was also a major component of the microbiome, comprising $> 43.5\% \pm 0.04$ of the total, whereas *Lactobacillus* comprised $\sim 2.1\% \pm 0.01$ of the total, which was also a lower proportion than in CON $\rightarrow$ offspring ($P = .0740$). In the gut of mice, *Bifidobacterium* spp. is not a major genus, but a decreasing pattern was also found in GDM-C $\rightarrow$ offspring when compared with the CON group (Figure S4). The linear discriminant analysis effect size was also used to identify

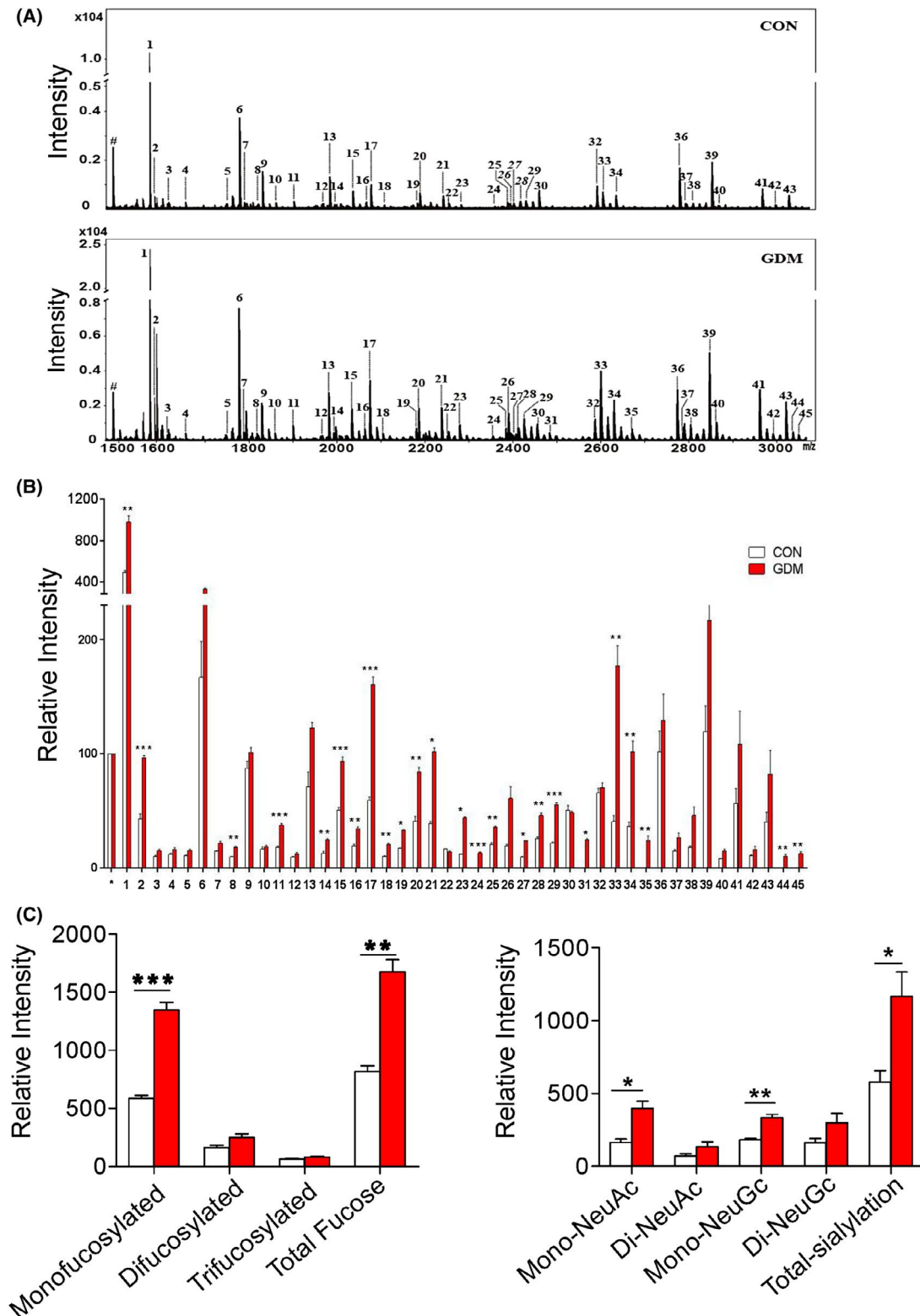


FIGURE 3 Comparison of milk N-glycans between CON and GDM maternal mice analyzed by MS. A, MALDI-TOF MS spectra of N-glycans of pooled milks (n = 3) from CON (upper) and GDM (lower) mice. B, Histograms of relative glycan intensities from panels A. The relative intensity of each glycan was calculated by normalizing the signal intensity of an individual glycan to the intensity of internal standard glycan. # indicates internal standard glycan. The relative intensities of released glycan from milks of CON and GDM mice were indicated by white and red columns, respectively. Glycan signals were shown by numerals and summarized in Figure S2. * $P < .05$, ** $P < .01$, *** $P < .001$. Each column represents the mean $\pm$ SD for three different experiments. C, Relative intensity of mono-, di-, tri-, and total fucosylated N-glycans were statistically analyzed. D, Relative intensity of mono-NeuAc/NeuGc, di-NeuAc/NeuGc, and total sialylated glycans were statistically analyzed. * $P < .05$, ** $P < .01$, *** $P < .001$. Each column represents the mean $\pm$ SD for three different experiments

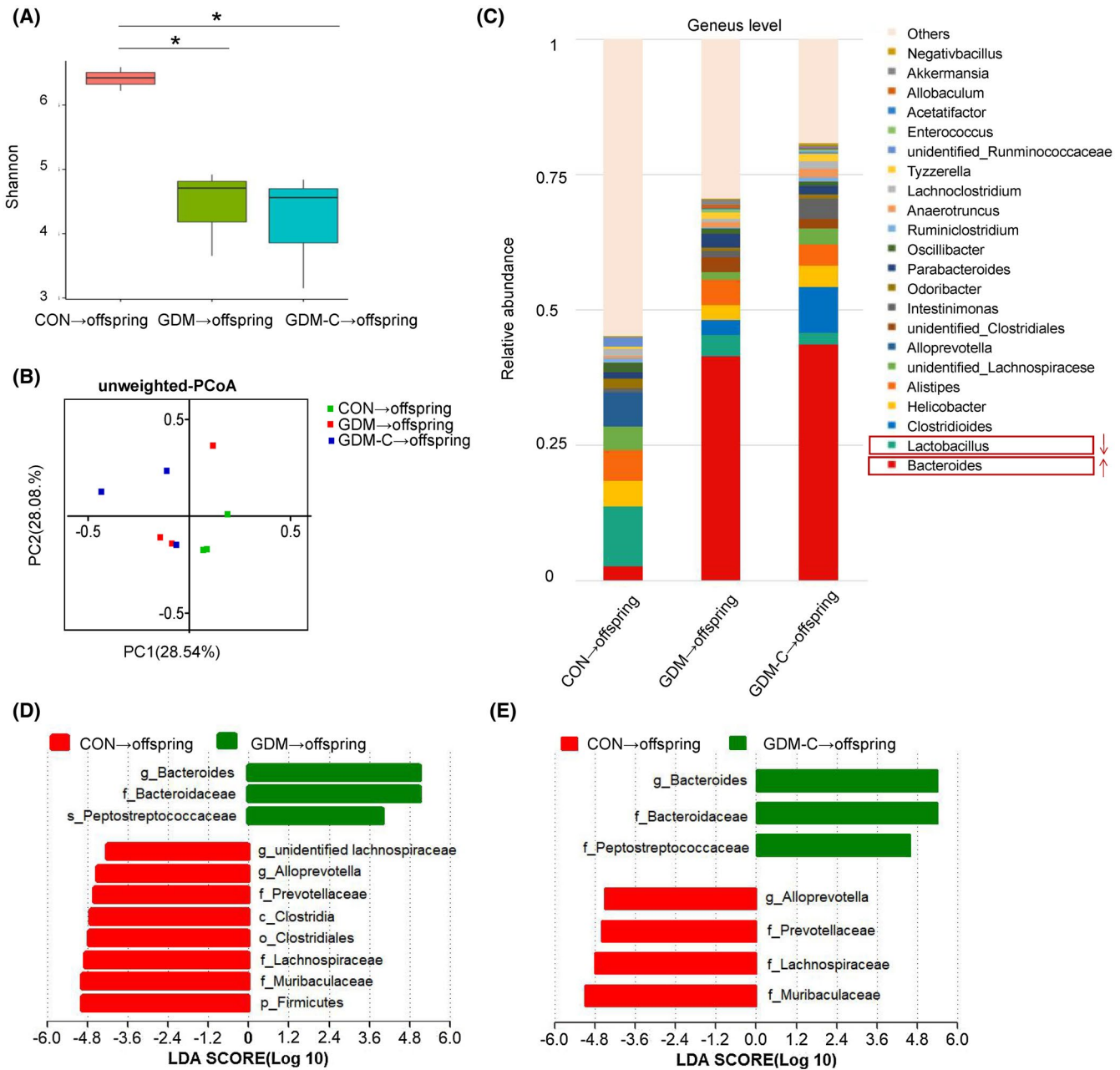


FIGURE 4 The gut microbiota of the offspring mice fed by CON or GDM maternal mice. A, The alpha diversity (indicated by Shannon index) in each group. B, The relative abundance of fecal microbial groups of CON → offspring, GDM → offspring and GDM-C → offspring at the genus level. C, The beta diversity of gut microbiota analyzed by principal-coordinate analysis (PCoA). D, The bacterial groups that showed significant differences between GON → offspring and GDM → offspring infants analyzed by the LefSe (linear discriminant analysis effect size) method. E, The bacterial groups that showed significant differences between GON → offspring and GDM → offspring infants analyzed by the LefSe method. * $P < .05$

the bacterial groups that were more or less abundant in each group (all $P < .05$). As shown in Figure 4D,E, the *Bacteroidaceae*, mainly comprising the *Bacteroides* genus, in the GDM → offspring and GDM-C → offspring groups, was significantly more abundant than in the CON → offspring group, whereas the *Prevotellaceae* and *Lachnospiraceae* families, and the *Alloprevotella* genus, were more abundant in CON → offspring.

3.5 | The offspring of GDM mice have higher proportions of splenic B and T lymphocytes

To determine whether the alteration in the gut microbiome of the GDM offspring affects their immune development and responses, we analyzed the splenic lymphocytes of the offspring after weaning by flow cytometry. This

revealed that the numbers of B cells ($CD19^+$) were higher in both GDM $\rightarrow$ offspring and GDM-C $\rightarrow$ offspring than CON $\rightarrow$ offspring (Figure 5A, $39.7 \pm 0.03\%$

vs $28.4 \pm 0.01\%$, $P = .0208$, $n = 3$; $43.9 \pm 0.05\%$ vs. $28.4 \pm 0.01\%$, $P = .0186$, $n = 3$). The proportion of T cells ($CD3^+$) was significantly higher in GDM-C $\rightarrow$ offspring

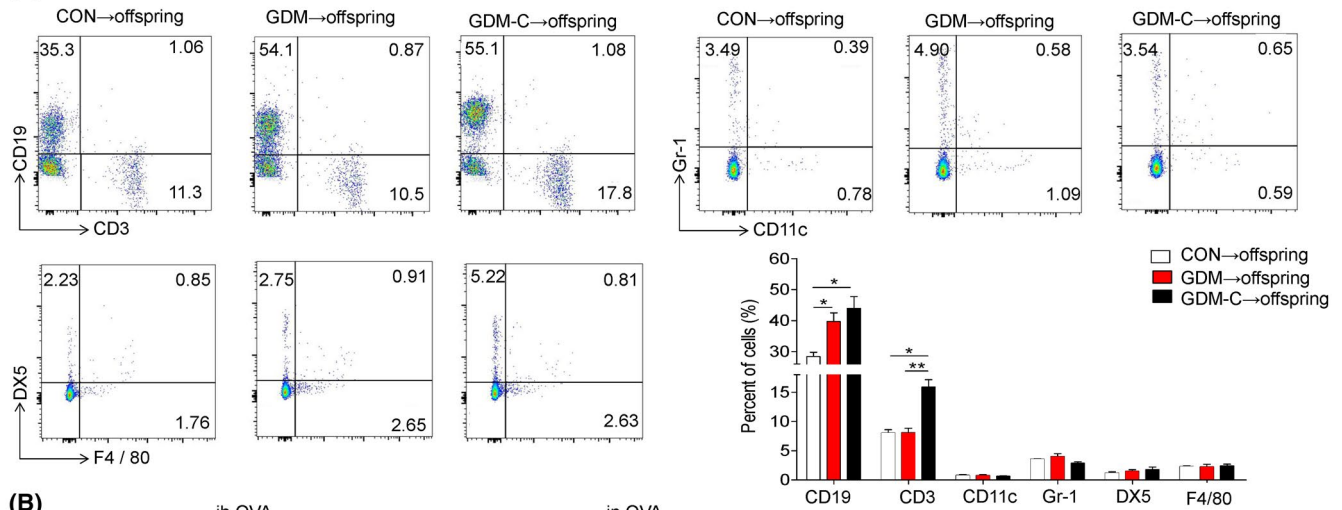
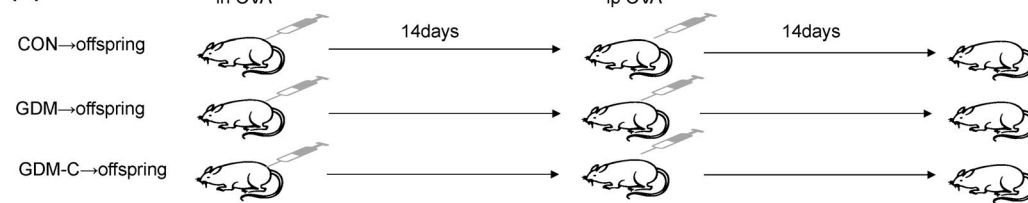
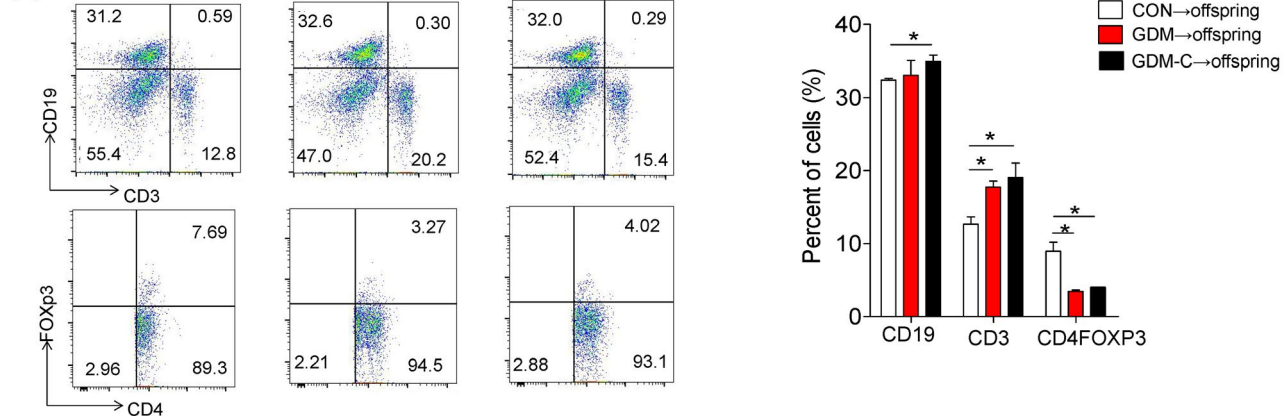
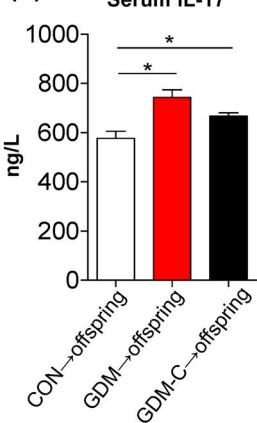
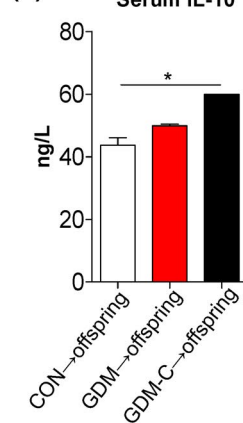
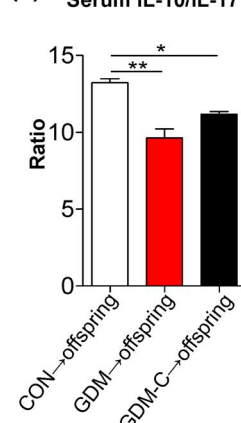
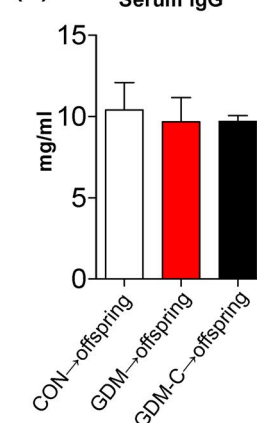
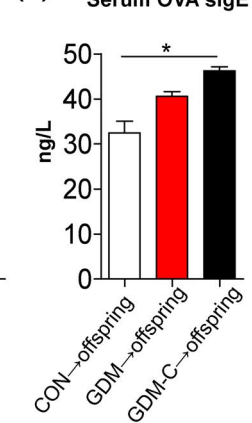
(A)**(B)****(C)****(D)****(E)****(F)****(G)****(H)**

FIGURE 5 The offspring fed by GDM mice showed higher proportion of splenic B and T lymphocytes. A, The flow cytometry analysis of the proportion of splenic cell subsets in CON → offspring, GDM → offspring, and GDM-C → offspring mice. Numbers indicate the percentage of the indicated cell groups in total splenic cells. 10,000 events were acquired for each analysis. Data are shown as mean values $\pm$ SD (* $P < .05$; ** $P < .01$). B, Experimental design: CON → offspring, GDM → offspring, and GDM-C → offspring mice were immunized by abdominal cavity injection with 50 μ g of OVA mixed with an equal volume of complete Freund's adjuvant (CFA). Two weeks later, mice were immunized with 50 μ g of OVA by subcutaneous injection. Mice sera were collected before and 14 days post immunization. C, Flow cytometry analysis of the proportion of spleen cell in CON → offspring, GDM → offspring, and GDM-C → offspring mice after immunization. Data are shown as mean values $\pm$ SD (* $P < .05$). D, IL-17 in serum of neonatal mice. E, The ratio of IL-10/IL-17. F, IgG in serum of neonatal mice. G) OVA sIgE in serum of neonatal mice. Values are means $\pm$ SD (* $P < .05$; ** $P < .01$)

($P = .0047$, $n = 3$), but the proportions of Gr-1⁺, CD11c⁺, and DX5⁺-positive cells, which are neutrophils (Gr-1), dendritic cells, and natural killer cells, respectively, did not differ among the groups.

Because the GDM-offspring had a significantly higher proportion of B/T lymphocytes in their spleen, we next determined whether this might affect the immune responses to OVA challenge. Significantly higher proportions of T cells were found in GDM → offspring and GDM-C → offspring (Figure 5B,C, $P = .0171$, and $P = .0457$, respectively; $n = 3$) than in CON → offspring after OVA-immunization. The number of B cells was significantly higher in GDM-C → offspring ($P = .0445$, $n = 3$), which was consistent with the smaller number of Treg cells (CD4⁺Foxp3⁺) found in these neonates ($P = .0496$ and $P = .0163$, respectively; $n = 3$). These results prompted us to determine whether these differences affect the concentrations of circulating cytokines. Using ELISA, we found that the serum concentrations of IL-17 and IL-10 were significantly higher in GDM → offspring and GDM-C → offspring than in CON → offspring ($P = .0164$ and $P = .0452$, respectively; $n = 3$), and the IL-10/IL-17 ratio was also significantly lower in these mice (Figure 5D–F). The serum concentration of IgG was similar among the groups, but the serum OVA sIgE was much higher in the GDM-C → offspring than controls ($P = .0081$, $n = 3$).

3.6 | Metabolites of *B acidifaciens* stimulate the activation of B and T cells obtained from PPs in vitro

To further analyze the interaction between the changes in the gut microbiome and the immune response of the GDM-fed neonates, we evaluated the relative abundance of *Bacteroides* species in the neonatal mice of each group. This showed that *B acidifaciens* was the major *Bacteroides* species in mouse intestine, and it was present in a significantly higher abundance in GDM → offspring and GDM-C → offspring than controls (Figure 6A, $P = .0395$ and $P = .0405$, respectively; $n = 3$). To determine whether *B acidifaciens* has direct effects on B and T lymphocytes, we isolated lymphocytes from the PPs of mice and incubated them with a number of concentrations of supernatant and lysate of a standard strain, *B acidifaciens* ATCC800799. This showed that the numbers

of B cells, T cells, and activated B cells (CD69⁺CD19⁺) and T cells (CD69⁺CD3⁺)³⁰ in PPs were much higher following stimulation with *B acidifaciens* ATCC800799 supernatant (Figure 6B–C; all $P < .05$, $n = 3$), whereas *B acidifaciens* ATCC800799 lysates had no effect (Figure S5). This suggests that *B acidifaciens* metabolites may hyper-activate mouse B and T lymphocytes. In addition, we measured cytokine concentrations in the culture media and found that IFN- γ concentrations (all $P < .005$, $n = 3$) were lower and IL-4 concentrations (all $P < .05$, $n = 3$) were much higher when PP lymphocytes were stimulated using *B acidifaciens* supernatant (Figure 6D).

4 | DISCUSSION

Human milk glycans provide a broad range of carbon sources for the gut microbiome of infants,^{31–33} in particular HMOs and proteins, of which more than 70% are glycosylated in humans.³⁴ Studies from our group and others have shown relationships between milk N-glycans and the gut microbiome of breast-fed infants, which can affect their immune responses.^{35–37} GDM is associated with a high risk of adverse consequences, including the inhibition of immune tolerance formation in infants.^{38–40} It has been suggested that GDM does not affect the abundance of HMOs but alters the glycosylation patterns of N-glycans in human milk.¹⁶ However, there is little understanding of the effects of altered glycosylation of GDM milk N-glycans on the gut microbiome of the offspring. In the present study, we have for the first time systematically characterized the features of the major N-glycans in the milk of a GDM mouse model and their impact on the gut microbiome and immune responses of the offspring.

Human milk oligosaccharides are the third largest component of human milk and encompass a collection of structurally complex glycans displaying an array of molecular linkages.^{41,42} We therefore first evaluated the abundance of the major oligosaccharide groups in the milk of GDM mice. A previous study had shown that there were no differences in HMOs between milk samples obtained from women with or without GDM,¹⁶ but the present data showed that instead of the largely fucosylated milk oligosaccharides found in human milk, the mouse milk instead principally contained mainly

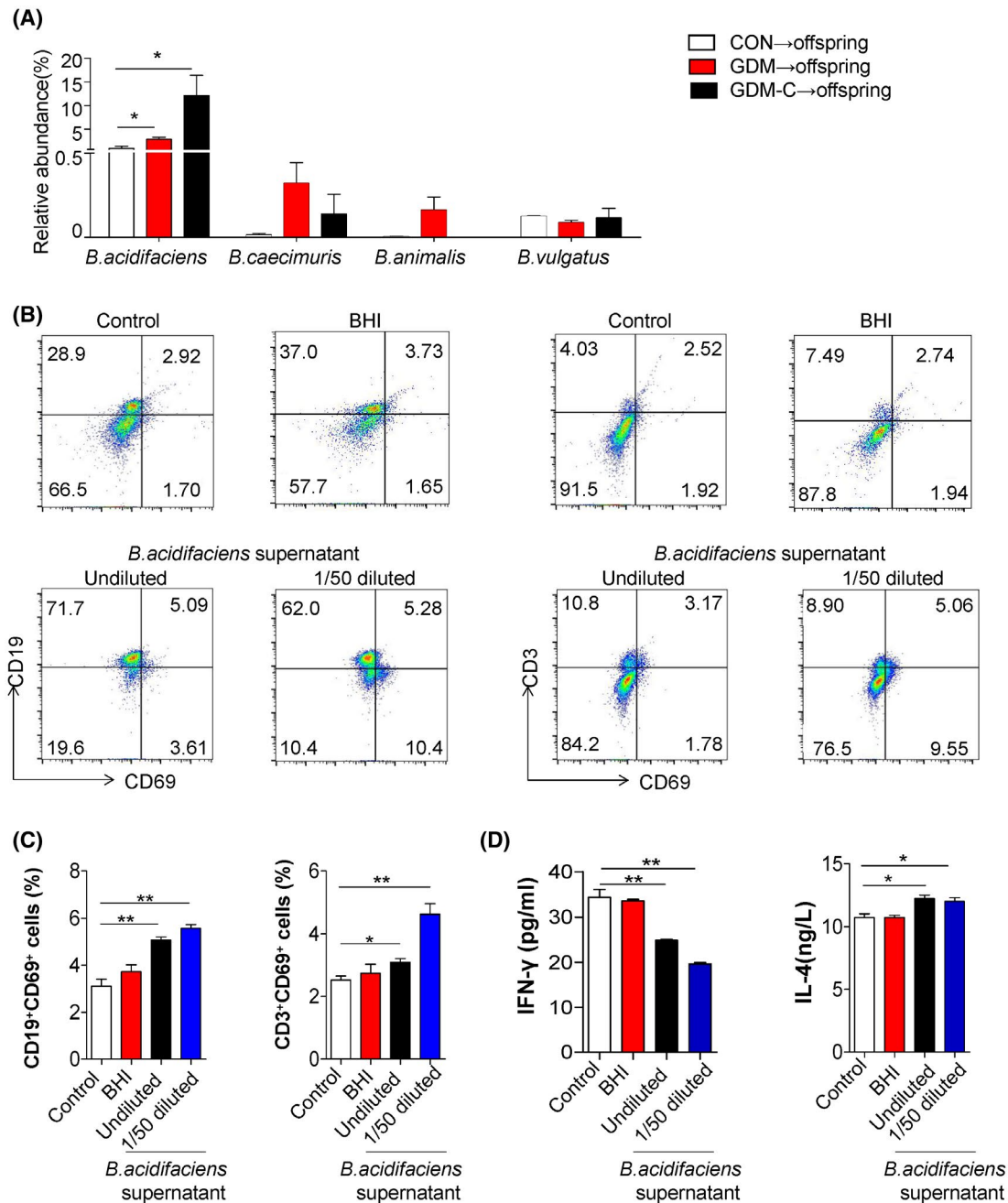


FIGURE 6 Metabolites of *B. acidifaciens* ATCC800799 stimulated B cell and T cell activation in vitro. A, The comparison of the relative abundances of *B. acidifaciens*, *B. caecimuris*, *B. animalis*, and *B. vulgatus* between groups. Data are shown as mean values ± SD (**P* < .05). B and C, Flow cytometry analysis of the proportion of activated B cells (CD19⁺CD69⁺) and T cells (CD3⁺CD69⁺) in Payer's patches (PPs) of mice post stimulation of *B. acidifaciens* ATCC800799 supernatant. The BHI medium was used as control. D, The concentration of IFN-γ in culture media. E) The concentration of IL-4 in culture media

sialylated milk oligosaccharides, such as sialyllactose and LST type oligosaccharides. Nevertheless, no significant differences in the abundance of milk oligosaccharides between the CON and GDM mice were identified, which is in accordance with the findings in human milk. However, due to the lack of large-scale human studies in this field, it is uncertain whether this finding has translational value. However, by means of MALDI-TOF-MS analysis, we have provided strong

evidence for alterations in milk N-glycans in GDM mice: the levels of fucosylation and sialylation of milk N-glycans were significantly higher in GDM mouse milk. To our knowledge, this is the first comparative quantitative study of whole milk N-glycosylation in a GDM model. We also quantitatively analyzed the mono or di-fucosylated and sialylated N-glycans in mouse milk, and found that the mono-fucosylated and sialylated N-glycans were the principal glycan groups that

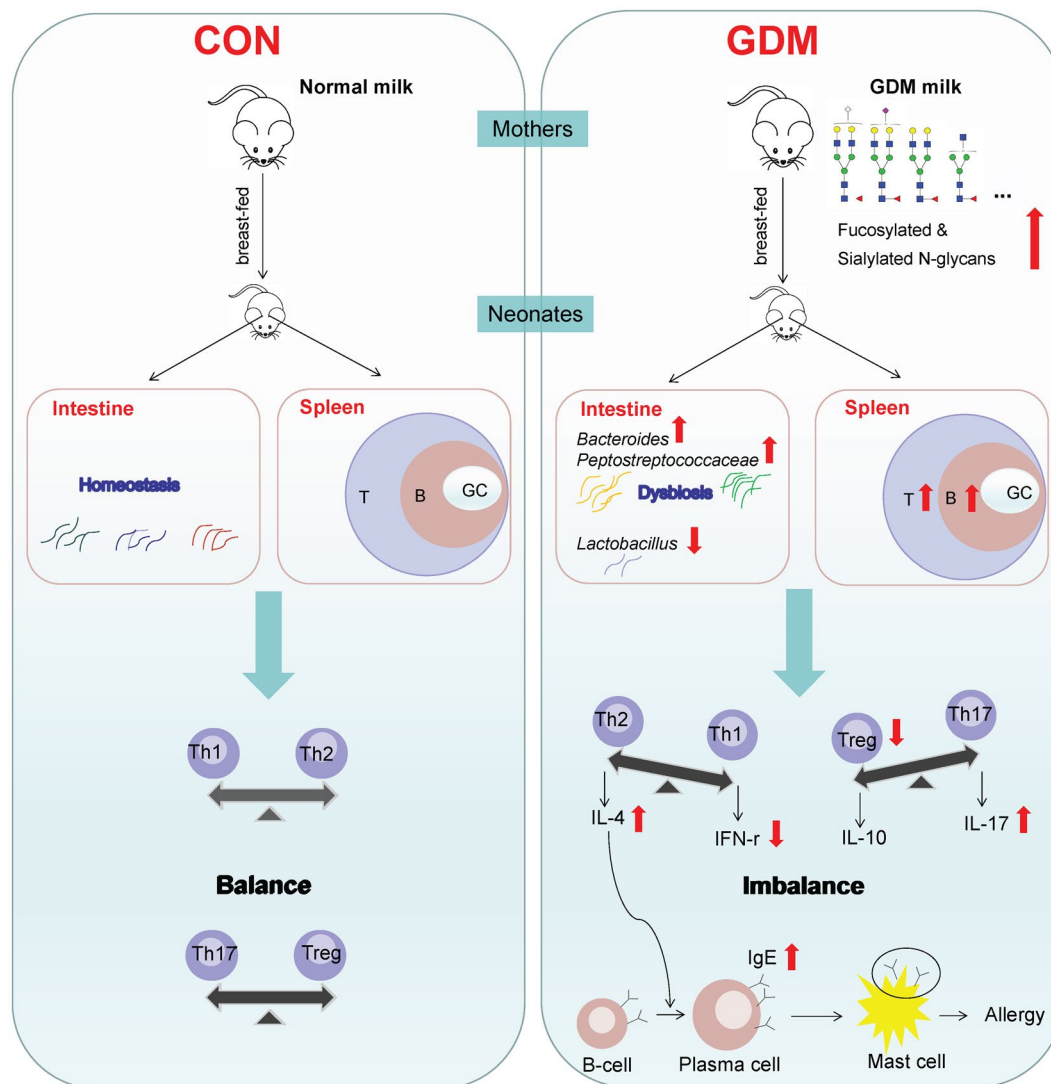


FIGURE 7 Highlights of this study. The GDM maternal mice were found with higher fucosylated and sialylated N-glycans levels in their milk when compared with control mice, these maternal mice-fed neonates harbored more abundant *Bacteroides* and *Peptostreptococcaceae* and reduced abundance of *Lactobacillus* spp. in their gut; these offspring also exhibited a higher proportion of B and T cells in spleen, and an imbalance between Th1/Th2 and Treg/Th17 cells, which resulted in hyperproduction of sIgE in circulation

are present in larger amounts in GDM mouse milk, which explains the overall increases in fucosylation and sialylation of GDM milk N-glycan. This result implies that more detailed studies of the milk glycans of GDM mothers should be conducted, to achieve a better understanding of the impact of milk N-glycans on the gut microbiome of their children (Figure 7).

We next characterized the major differences in the gut microbiome that occurred alongside the differences in milk N-glycans, as well as the differences in the development of the immune system of the offspring. Although due to the species difference between mouse and human, we cannot simply compare the gut microbiota of mice to that of the human infants, however, similar alteration patterns in the

infants of women with GDM and the neonates fed by GDM maternal mice were found. For example, previous studies have shown that newborns of mothers with GDM have gut microbiomes containing decreased abundance of bacterial genera such as *Lactobacillus* and *Bifidobacterium* spp.^{43,44} In the present study, *Lactobacillus* spp. were also found to be relatively less abundant in the gut microbiome of offspring fed by GDM mice. Although it is not a major genus in the gut of mice, the abundance of *Bifidobacterium* spp. in neonatal mice fed by the control and GDM mice was also compared, a decreasing pattern was found in GDM-C offspring (Figure S4). As *Bifidobacteria* and *Lactobacillus* are the major microbial genera in gut of the healthy breast-fed infants,^{2,37} they play important beneficial roles in regulating the growth and

the immune development of the newborns, the decreasing of these bacteria may suggest an unfavorable status for infant health. On the contrary, the abundance of *Bacteroides* spp. were found significantly greater in the gut of both GDM $\rightarrow$ offspring and GDM-C $\rightarrow$ offspring. *Bacteroides* has been known as far back as the 1980s to be one of the most abundant genera in the gut, making up approximately 25% of the microbial community.⁴⁵ Some *Bacteroides* spp., such as *Bacteroides fragilis*, are found prevalent in multiple sample types of maternal and neonatal microbiota in human, reflecting their possible significance to GDM response across generations and body sites.¹² The ability of these bacteria to use complex glycans significantly contributes to their great abundance in the gastrointestinal tract.⁴⁶ A recent study has shown that an extensive enzymatic apparatus encoded by multiple co-regulated genetic loci in the *Bacteroides* genome is involved in the metabolism of complex N-glycans.¹⁴ Therefore, the large amounts of fucosylated and sialylated N-glycans may have provided a major carbon source for *Bacteroides* spp., resulting in their predominance in the gut of GDM-fed neonates.

The gut microbiome plays a vital role in the development and regulation of the immune system, and alterations in the microbiome are associated with the development of many chronic diseases in infants, such as allergic sensitization, eczema, asthma, and necrotizing enterocolitis.^{47,48} It has been shown that poor diversity of the intestinal microbiome may account for the increase in allergic disease in modern society,^{49,50} and our finding that GDM-fed neonates had low alpha diversity is therefore consistent with a similar unhealthy state. Notably, the population of *Bacteroides* spp., mainly *B. acidifaciens*, was much larger, and that of *Lactobacillus* spp. was smaller, in the gut of offspring fed by GDM maternal mice. Previous observations have shown that *Bacteroides* spp., such as *B. fragilis*-like gut commensals can cause pro-inflammatory responses upon gaining access to the systemic compartment and contribute to type 1 diabetes in at-risk subjects,⁵¹ therefore, changes in the abundance of this bacterial genus may contribute to the changes in the immune response of GDM offspring. In addition, similar alteration patterns of gut microbiota were also found in children with allergic disease,⁵²⁻⁵⁴ we were therefore interested in whether this disruption in the microbiome of GDM-fed offspring would affect their immune response. Consistent with our hypothesis, we found that in both GDM $\rightarrow$ offspring and GDM-C $\rightarrow$ offspring, the proportions of B cells and T cells were significantly increased, whereas there was a significant reduction in the Treg population, after challenge with OVA. IL-17 concentration was found to be significantly higher in offspring fed by GDM mothers. Under normal circumstances, the Th helper lymphocyte subsets are in equilibrium. Th17 cells secrete IL-17A (IL-17), which has a pro-inflammatory role and has been implicated in many

inflammatory conditions in humans and mice, whereas Treg cells play a vital role in maintaining immune tolerance and preventing autoimmune disease,⁵⁵⁻⁵⁷ which involves the suppression of Th17-type immune responses.^{58,59} Therefore, the maintenance of immune tolerance by Tregs is key to the prevention of allergic and autoimmune diseases, and our results suggest that the GDM-fed offspring have an immune imbalance that could predispose toward such diseases. We also measured the serum concentrations of IL-17 and IL-10 in the offspring and found that the ratio of IL-10/IL-17 was significantly lower in GDM $\rightarrow$ offspring and GDM-C $\rightarrow$ offspring, further demonstrating the disruption of the balance between Treg and Th17 cells.

However, it is unclear whether there is a relationship between the predominant bacterial genus, *Bacteroides*, and the altered immune response in GDM-fed neonates. A previous study has demonstrated that *B. acidifaciens* is one of the main bacterial species responsible for increasing the proportion of IgA⁺ B cells and activating B cells in cecal patches.⁶⁰ Therefore, we isolated PPs from the mice and incubated them with various concentrations of *B. acidifaciens* supernatant and lysates, aiming to determine the direct effects on lymphocyte responses. The results showed that the numbers of activated B cells and T cells in the PPs of mice were much higher following stimulation with *B. acidifaciens* supernatant. In addition, we measured the concentrations of key cytokines in the culture media and found that IFN- γ concentrations were much lower and IL-4 concentrations were much higher after PP cells were incubated with *B. acidifaciens* supernatant. This suggests an imbalance between Th1 and Th2 cells. It has been reported that an imbalance in Th1 and Th2 cells can induce and aggravate allergic asthma.^{18,19} Th1 and Th2 cells play distinct roles in the development of immunity and disease. Th1 cells stimulate cellular immunity, involving the production of IFN- γ , which is an antagonist of IL-4. Th2 cells stimulate humoral immunity and secrete IL-4, which promotes the proliferation of B cells and induces the production of antibodies, especially IgE, which is a key mediator of allergic reactions.^{61,62} We have also shown that the serum concentrations of OVA sIgE are higher in GDM $\rightarrow$ offspring and GDM-C $\rightarrow$ offspring. Imbalances in Th1/Th2 and Th17/Treg play pathogenic roles in the development of autoimmune diseases.⁶³⁻⁶⁵ Our study is therefore consistent with GDM mother-fed offspring having an imbalance in their immune system that may inhibit the development of immune tolerance, and this is closely associated with overgrowth of gut *B. acidifaciens*, which is likely secondary to significant increases in fucosylated and sialylated milk N-glycans in the GDM mothers.

In conclusion, we report that GDM affects the glycosylation pattern of milk N-glycans in mice. This involves increases in the concentrations of fucosylated and sialylated N-glycans in GDM milk, which might contribute to an immune imbalance in the offspring by increasing the

growth of *Bacteroides* spp. in their guts. Although this was a mouse study, our data show that variations in N-glycan glycosylation in the milk of GDM mothers have profound effects on the intestinal microbiome and immune responses of the offspring. Our findings may provide a theoretical basis for future studies in human populations, especially those aimed at developing specific nutritional care for the children of mothers with GDM.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

J. Zhou carried out the experiments and wrote the draft of this manuscript; Y. Wang, Q. Fan, and Y. Liu analyzed data; M. Li, Y. Bai, M. Liu, and J. Li edited the manuscript; M. Li designed the study; W. Li, W. Dong, and M. Li guided the research. All authors read and approved the final version of this manuscript.

REFERENCES

- Backhed F, Roswall J, Peng Y, et al. Dynamics and stabilization of the human gut microbiome during the first year of life. *Cell Host Microbe*. 2015;17:690-703.
- Madan JC, Hoen AG, Lundgren SN, et al. Association of cesarean delivery and formula supplementation with the intestinal microbiome of 6-week-old infants. *JAMA Pediatr*. 2016;170:212-219.
- Giongo A, Gano KA, Crabb DB, et al. Toward defining the autoimmune microbiome for type 1 diabetes. *ISME J*. 2011;5:82-91.
- Yu ZT, Chen C, Kling DE, et al. The principal fucosylated oligosaccharides of human milk exhibit prebiotic properties on cultured infant microbiota. *Glycobiology*. 2013;23:169-177.
- Koenig JE, Spor A, Scalfone N, et al. Succession of microbial consortia in the developing infant gut microbiome. *Proc Natl Acad Sci USA*. 2011;108(Suppl 1):4578-4585.
- Hadar E, Hod M. Gestational diabetes and pregnancy outcome: do we need an update on diagnostic criteria? *Nutr Metab Cardiovasc Dis*. 2009;19:75-76.
- Zhu WW, Yang HX, Wei YM, et al. Evaluation of the value of fasting plasma glucose in the first prenatal visit to diagnose gestational diabetes mellitus in China. *Diabetes Care*. 2013;36:586-590.
- Damm P, Mathiesen ER. Diabetes: therapy for gestational diabetes mellitus—time for a change? *Nature reviews. Endocrinology*. 2015;11:327-328.
- Kim SY, Sharma AJ, Callaghan WM. Gestational diabetes and childhood obesity: what is the link? *Curr Opin Obstet Gynecol*. 2012;24:376-381.
- Shah BR, Retnakaran R, Booth GL. Increased risk of cardiovascular disease in young women following gestational diabetes mellitus. *Diabetes Care*. 2008;31:1668-1669.
- Kumar R, Ouyang F, Story RE, et al. Gestational diabetes, atopic dermatitis, and allergen sensitization in early childhood. *J Allergy Clin Immunol*. 2009;124:1031-1038.e4.
- Wang J, Zheng J, Shi W, et al. Dysbiosis of maternal and neonatal microbiota associated with gestational diabetes mellitus. *Gut*. 2018;67:1614-1625.
- Ferretti P, Pasolli E, Tett A, et al. Mother-to-infant microbial transmission from different body sites shapes the developing infant gut microbiome. *Cell Host Microbe*. 2018;24:133-145.e5.
- Briliute J, Urbanowicz PA, Luis AS, et al. Complex N-glycan breakdown by gut *Bacteroides* involves an extensive enzymatic apparatus encoded by multiple co-regulated genetic loci. *Nat Microbiol*. 2019;4(9):1571-1581.
- Ravcheev DA, Thiele I. Comparative genomic analysis of the human gut microbiome reveals a broad distribution of metabolic pathways for the degradation of host-synthesized mucin glycans and utilization of mucin-derived monosaccharides. *Front Genet*. 2017;8:111.
- Smilowitz JT, Totten SM, Huang J, et al. Human milk secretory immunoglobulin A and lactoferrin N-glycans are altered in women with gestational diabetes mellitus. *J Nutri*. 2013;143:1906-1912.
- Johnson CC, Ownby DR. Allergies and Asthma: do atopic disorders result from inadequate immune homeostasis arising from infant gut dysbiosis? *Exp Rev Clin Immunol*. 2016;12:379-388.
- Wang J, Wu J, Kong L, et al. BuShenYiQi Formula strengthens Th1 response and suppresses Th2-Th17 responses in RSV-induced asthma exacerbated mice. *J Ethnopharmacol*. 2014;154:131-147.
- Huang C, Zhang Z, Wang L, Liu J, Gong X, Zhang C. ML-7 attenuates airway inflammation and remodeling via inhibiting the secretion of Th2 cytokines in mice model of asthma. *Mol Med Rep*. 2018;17:6293-6300.
- Meng SS, Gao R, Yan BD, et al. Maternal allergic disease history affects childhood allergy development through impairment of neonatal regulatory T-cells. *Respir Res*. 2016;17:114.
- Noval Rivas M, Burton OT, Wise P, et al. Regulatory T cell reprogramming toward a Th2-cell-like lineage impairs oral tolerance and promotes food allergy. *Immunity*. 2015;42:512-523.
- Li C, Sheng A, Jia X, et al. Th17/Treg dysregulation in allergic asthmatic children is associated with elevated notch expression. *J Asthma*. 2018;55:1-7.
- Tao B, Ruan G, Wang D, Li Y, Wang Z, Yin G. Imbalance of peripheral Th17 and regulatory T cells in children with allergic rhinitis and bronchial asthma. *Iranian journal of allergy, asthma, and immunology*. 2015;14:273-279.
- Sifnaos E, Mastorakos G, Psarra K, et al. Gestational diabetes and T-cell (Th1/Th2/Th17/Treg) immune profile. *In Vivo (Athens, Greece)*. 2019;33:31-40.
- Tanoue T, Atarashi K, Honda K. Development and maintenance of intestinal regulatory T cells. *Nat Rev Immunol*. 2016;16:295-309.
- Kwon MS, Lim SK, Jang JY, et al. Lactobacillus sakei WIKIM30 ameliorates atopic dermatitis-like skin lesions by inducing regulatory T cells and altering gut microbiota structure in mice. *Front Immunol*. 2018;9:1905.
- Kim WG, Kang GD, Kim HI, Han MJ, Kim DH. Bifidobacterium longum IM55 and Lactobacillus plantarum IM76 alleviate allergic

- rinitis in mice by restoring Th2/Treg imbalance and gut microbiota disturbance. *Benef Microbes*. 2019;10:55-67.
28. Xu J, Sun H, Huang G, et al. A fixation method for the optimisation of western blotting. *Sci Rep*. 2019;9:6649.
 29. Li J, Zhao F, Wang Y, et al. Gut microbiota dysbiosis contributes to the development of hypertension. *Microbiome*. 2017;5:14.
 30. Testi R, Pulcinelli F, Frati L, Gazzaniga PP, Santoni A. CD69 is expressed on platelets and mediates platelet activation and aggregation. *J Exp Med*. 1990;172:701-707.
 31. Charbonneau MR, O'Donnell D, Blanton LV, et al. Sialylated milk oligosaccharides promote microbiota-dependent growth in models of infant undernutrition. *Cell*. 2016;164:859-871.
 32. Piper KM, Berry CA, Cregan MD. The bioactive nature of human breastmilk. *Breastfeed Rev*. 2007;15:5-10.
 33. Zivkovic AM, German JB, Lebrilla CB, Mills DA. Human milk glycomiome and its impact on the infant gastrointestinal microbiota. *Proc Natl Acad Sci USA*. 2011;108(Suppl 1):4653-4658.
 34. Apweiler R, Hermjakob H, Sharon N. On the frequency of protein glycosylation, as deduced from analysis of the SWISS-PROT database. *Biochem Biophys Acta*. 1999;1473:4-8.
 35. Garrido D, Nwosu C, Ruiz-Moyano S, et al. Endo-beta-N-acetylglucosaminidases from infant gut-associated bifidobacteria release complex N-glycans from human milk glycoproteins. *Mol Cell Proteomics*. 2012;11:775-785.
 36. Allhorn M, Olin AI, Nimmerjahn F, Collin M. Human IgG/Fc gamma R interactions are modulated by streptococcal IgG glycan hydrolysis. *PLoS ONE*. 2008;3:e1413.
 37. Li M, Bai Y, Zhou J, et al. Core fucosylation of maternal milk N-glycan evokes B cell activation by selectively promoting the l-fucose metabolism of gut *Bifidobacterium* spp. and *Lactobacillus* spp. *MBio*. 2019;10(2):e00128-219.
 38. Boerschmann H, Pfluger M, Henneberger L, Ziegler AG, Hummel S. Prevalence and predictors of overweight and insulin resistance in offspring of mothers with gestational diabetes mellitus. *Diabetes Care*. 2010;33:1845-1849.
 39. Vaarasmaki M, Pouta A, Elliot P, et al. Adolescent manifestations of metabolic syndrome among children born to women with gestational diabetes in a general-population birth cohort. *Am J Epidemiol*. 2009;169:1209-1215.
 40. Bellamy L, Casas JP, Hingorani AD, Williams D. Type 2 diabetes mellitus after gestational diabetes: a systematic review and meta-analysis. *Lancet*. 2009;373:1773-1779.
 41. Zhang Y, Yu X, Yuan Y, et al. Positive association of the human frizzled 3 (FZD3) gene haplotype with schizophrenia in Chinese Han population. *Am J Med Genet B Neuropsychiatr Genet*. 2004;129B(1):16-19.
 42. Thurl S, Munzert M, Boehm G, Matthews C, Stahl B. Systematic review of the concentrations of oligosaccharides in human milk. *Nutri Rev*. 2017;75(11):920-933.
 43. Ye G, Zhang L, Wang M, et al. The gut microbiota in women suffering from gestational diabetes mellitus with the failure of glycemic control by lifestyle modification. *J Diabet Res*. 2019;2019:6081248.
 44. Su M, Nie Y, Shao R, et al. Diversified gut microbiota in newborns of mothers with gestational diabetes mellitus. *PLoS ONE*. 2018;13:e0205695.
 45. Salyers AA. Bacteroides of the human lower intestinal tract. *Annu Rev Microbiol*. 1984;38:293-313.
 46. Ji X, Hou C, Zhang X, et al. Microbiome-metabolomic analysis of the impact of *Zizyphus jujuba* cv. Muzao polysaccharides consumption on colorectal cancer mice fecal microbiota and metabolites. *Int J Biol Macromol*. 2019;131:1067-1076.
 47. Azad MB, Konya T, Maughan H, et al. Infant gut microbiota and the hygiene hypothesis of allergic disease: impact of household pets and siblings on microbiota composition and diversity. *Allergy Asthma Clin Immunol*. 2013;9:15.
 48. Zimmermann P, Messina N, Mohn WW, Finlay BB, Curtis N. Association between the intestinal microbiota and allergic sensitization, eczema, and asthma: a systematic review. *J Allergy Clin Immunol*. 2019;143:467-485.
 49. Warner BB, Deych E, Zhou Y, et al. Gut bacteria dysbiosis and necrotising enterocolitis in very low birthweight infants: a prospective case-control study. *Lancet*. 2016;387:1928-1936.
 50. Wang M, Karlsson C, Olsson C, et al. Reduced diversity in the early fecal microbiota of infants with atopic eczema. *J Allergy Clin Immunol*. 2008;121:129-134.
 51. Sofi MH, Johnson BM, Gudi RR, Jolly A, Gaudreau MC, Vasu C. Polysaccharide A-dependent opposing effects of mucosal and systemic exposures to human gut commensal *Bacteroides fragilis* in Type 1 diabetes. *Diabetes*. 2019; 68(10):1975-1989.
 52. Adlerberth I, Carlsson B, de Man P, et al. Intestinal colonization with enterobacteriaceae in Pakistani and Swedish hospital-delivered infants. *Acta Paediatr Scand*. 1991;80:602-610.
 53. Johansson MA, Sjogren YM, Persson JO, Nilsson C, Sverremark-Ekstrom E. Early colonization with a group of *Lactobacilli* decreases the risk for allergy at five years of age despite allergic heredity. *PLoS ONE*. 2011;6:e23031.
 54. Nakayama J, Kobayashi T, Tanaka S, et al. Aberrant structures of fecal bacterial community in allergic infants profiled by 16S rRNA gene pyrosequencing. *FEMS Immunol Med Microbiol*. 2011;63:397-406.
 55. Kolls JK, Linden A. Interleukin-17 family members and inflammation. *Immunity*. 2004;21:467-476.
 56. Gonzalez-Lombana C, Gimblet C, Bacellar O, et al. IL-17 mediates immunopathology in the absence of IL-10 following *Leishmania* major infection. *PLoS Pathog*. 2013;9:e1003243.
 57. Cho JS, Pietras EM, Garcia NC, et al. IL-17 is essential for host defense against cutaneous *Staphylococcus aureus* infection in mice. *J Clin Invest*. 2010;120:1762-1773.
 58. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Pillars article: immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol*. 1995;156:3808-3821.
 59. Chen Y, Kuchroo VK, Inobe J, Hafler DA, Weiner HL. Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis. *Science*. 1994;265:1237-1240.
 60. Yanagibashi T, Hosono A, Oyama A, et al. IgA production in the large intestine is modulated by a different mechanism than in the small intestine: *Bacteroides acidifaciens* promotes IgA production in the large intestine by inducing germinal center formation and increasing the number of IgA+ B cells. *Immunobiology*. 2013;218:645-651.
 61. Wu LC, Zarrin AA. The production and regulation of IgE by the immune system. *Nat Rev Immunol*. 2014;14:247-259.
 62. Kuhn R, Rajewsky K, Muller W. Generation and analysis of interleukin-4 deficient mice. *Science*. 1991;254:707-710.
 63. Talaat RM, Mohamed SF, Bassyouni IH, Raouf AA. Th1/Th2/Th17/Treg cytokine imbalance in systemic lupus erythematosus (SLE) patients: correlation with disease activity. *Cytokine*. 2015;72:146-153.

64. Eisenstein EM, Williams CB. The T(reg)/Th17 cell balance: a new paradigm for autoimmunity. *Pediatr Res*. 2009;65:26r-31r.
65. Wu S, Zhou Y, Liu S, et al. Regulatory effect of nicotine on the differentiation of Th1, Th2 and Th17 lymphocyte subsets in patients with rheumatoid arthritis. *Eur J Pharmacol*. 2018;831:38-45.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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