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Nutritional Immunology

Infant Microbiota Communities and Human Milk Oligosaccharide Supplementation Independently and Synergistically Shape Metabolite Production and Immune Responses in Healthy Mice

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A B S T R A C T

Background: Multiple studies have demonstrated associations between the early-life gut microbiome and incidence of inflammatory and autoimmune disease in childhood. Although microbial colonization is necessary for proper immune education, it is not well understood at a mechanistic level how specific communities of bacteria promote immune maturation or drive immune dysfunction in infancy.
Objectives: In this study, we aimed to assess whether infant microbial communities with different overall structures differentially influence immune and gastrointestinal development in healthy mice.
Methods: Germ-free mice were inoculated with fecal slurries from *Bifidobacterium longum* subspecies *infantis* positive (BIP) or *B. longum* subspecies *infantis* negative (BIN) breastfed infants; half of the mice in each group were also supplemented with a pool of human milk oligosaccharides (HMOs) for 14 d. Cecal microbiome composition and metabolite production, systemic and mucosal immune outcomes, and intestinal morphology were assessed at the end of the study.
Results: The results showed that inoculation with a BIP microbiome results in a remarkably distinct microbial community characterized by higher relative abundances of cecal *Clostridium senu stricto*, *Ruminococcus gnavus*, *Cellulosilyticum* sp., and *Erysipelatoclostridium* sp. The BIP microbiome produced 2-fold higher concentrations of cecal butyrate, promoted branched short-chain fatty acid (SCFA) production, and further modulated serotonin, kynurenine, and indole metabolism relative to BIN mice. Further, the BIP microbiome increased the proportions of innate and adaptive immune cells in spleen, while HMO supplementation increased proliferation of mesenteric lymph node cells to phorbol myristate acetate and lipopolysaccharide and increased serum IgA and IgG concentrations.
Conclusions: Different microbiome compositions and HMO supplementation can modulate SCFA and tryptophan metabolism and innate and adaptive immunity in young, healthy mice, with potentially important implications for early childhood health.

Keywords: human milk oligosaccharides, HMO, immunity, gastrointestinal tract, neonatal, microbiome

Introduction

It is now well understood that the gastrointestinal (GI) microbiome plays a fundamental role in immune, GI, and metabolic development. Symbiosis between the host and the microbiome in early life is critical in shaping both short- and long-term health trajectories [1]. To date, numerous studies have demonstrated associations between an aberrant gut microbiome composition in infancy and diseases including obesity [2], food allergy [3], atopic dermatitis [4], asthma [5], and diabetes [6].

Abbreviations: BIN, *Bifidobacterium longum* subspecies *infantis* negative; BIP, *Bifidobacterium longum* subspecies *infantis* positive; GF, germ-free; GI, gastrointestinal; HMO, human milk oligosaccharide; MLN, mesenteric lymph node; PMA, phorbol myristate acetate; SCFA, short-chain fatty acid.

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In addition to other life stages, microbiota development in infancy is principally shaped by diet [7–9]. Breastfed infants tend to have a less diverse gut microbiome and harbor higher abundances of *Bifidobacterium* species due to their ability to metabolize human milk oligosaccharides (HMOs). HMOs are complex glycans, which make up the third most abundant component in human milk but are nondigestible by the human host [9,10]. In addition to exerting prebiotic functions for *Bifidobacterium* and some *Bacteroides* species [10], HMOs can shape the microbiome and immune system by limiting pathogen adhesion to epithelial cells [11,12], promoting intestinal cell maturation [13,14], and reinforcing the gut barrier via their effect on short-chain fatty acid (SCFA) production [15,16], among other mechanisms [17]. Taxonomic composition of the microbiome also changes with the introduction of solid foods and weaning from human milk. As substrate availability for resident microbiota shifts, taxa within the Bacillota phylum increase and *Bifidobacterium* species tend to decrease [9,18]. Although diet has been shown in some cases to be the most significant factor shaping microbiome structure in infancy [7], composition is also influenced by mode of delivery, antibiotic usage [19], furry pet exposure [20], and family lifestyle and industrialization [21–24].

Maturation of the gut microbiome and the immune system parallel one another during early life [1]. Microbial colonization is necessary for immune education, both for induction of tolerance as well as coordination of an optimal immune response to pathogen invasion [1,25,26]. We have learned much of this through studies in germ-free (GF) animals that demonstrate lower numbers of $CD4^+$ and $CD8^+$ T cells [27], a deficient intestinal mucus barrier [28], impaired IgA secretion [29], and abnormal gut-associated lymphoid tissue development as well as altered intestinal morphology and nutrient absorption [30]. Although it is understood that microbes are absolutely necessary for proper immune development, there remains a lack of understanding at a mechanistic level how specific communities of bacteria promote immune maturation or drive immune dysfunction in infancy.

Recent studies have proposed that the infant gut microbiome has changed in recent decades due to industrialization and subsequent decrease in microbial exposure [22,31–34]. Such shifts in the early microbial profile have further been implicated, at least partially, in global rises in rates of autoimmune and inflammatory diseases. To investigate such hypotheses, scientists have monocolonized or supplemented animals with high concentrations of commensal microbes thought to be disappearing in industrialized societies, such as *Bifidobacterium longum* subspecies *infantis (B. infantis)*, and measured response to an immunologic or morphologic challenge [35–40]. Although such studies have produced critical findings, they have not answered the question of how unperturbed microbial communities with different overall structures influence immune development in infancy in absence of disease or insult. To investigate this question, we leveraged a GF mouse model inoculated with different infant microbial communities and assessed downstream GI tract development, microbial structure, metabolite production, and immune development.

Methods

Fecal slurry preparation

Fecal samples were obtained from 4 breastfed infants from Rochester, New York. One of the infants was 6 wk old and 3 were 6 mo old. Samples were collected in diapers, transferred to a sterile tube, and immediately stored in home freezers before

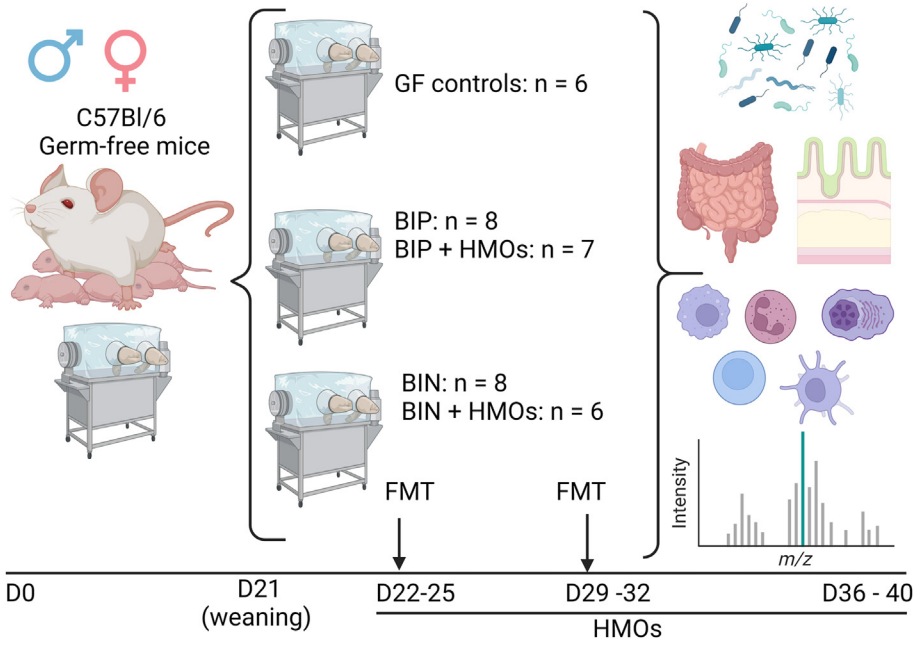

FIGURE 1. Study design: 21-d-old GF mice were gavaged with fecal samples from breastfed infants that were either positive (BIP) or negative (BIN) for *B. infantis*. Fecal slurries were gavaged on experimental days 1 and 8 corresponding to animal ages of 21 and 29 d. Half of the mice received a daily gavage of a pool of HMOs for 14 d, creating 4 groups: *1*) BIP, *2*) BIP + HMO, *3*) BIN, and *4*) BIN + HMO. Body weight was monitored throughout the duration of the study. At days 36–40, mice were killed and samples collected for microbiome, metabolomic, immunologic, and morphological analyses. BIN, *B. infantis* negative; BIP, *B. infantis* positive; GF, germ free; HMO, human milk oligosaccharide.

being transferred to −80°C at the laboratory, as described previously [41]. The samples were assayed for *B. infantis* via qPCR as described previously [22] and were then designated as *B. infantis* positive (BIP) or *B. infantis* negative (BIN) samples using primers previously described [42]. A fecal microbiota slurry was generated from each infant sample and comprised of 10% feces (wt/wt) in phosphate-buffered saline (PBS) with 0.1% resazurin and 0.05% L-cysteine.

Mouse studies

Three to 3.5-wk-old male and female mice were housed in germ-free (GF) isolators upon weaning at 23°C in 12-h light/dark conditions with ad libitum access to sterile food (ENVIGO 2020SX) and water. A pool of HMOs was isolated and purified in LB's Laboratory at University of California–San Diego [43]. Half of the mice were gavaged daily with this HMO pool for 14 d. HMOs (15 mg) were co-administered in a total volume of 50 μL of PBS. Mice, including GF controls, not receiving HMOs were also administered 50 μL of 1× PBS.

Mice were gavaged with fecal slurries generated from infant samples, as described earlier, on experimental days 1 and 8 corresponding to animal ages of 22-25 and 29-32 days. Mice were gavaged with 1 slurry generated from 1 infant, rather than a pooled sample, in an attempt to maintain dynamics of the individual donor microbial communities in the mice. GF animals were gavaged with the vehicle alone, 0.1% resazurin and 0.05% L-cysteine. Fecal samples were obtained from mice on day 4; DNA was extracted and assayed for *B. infantis* by qPCR to confirm presence or absence, resulting in the following groups: *1*) BIP, 2) BIP + HMO, *3*) BIN, and *4*) BIN + HMO. Separate GF isolators were used for GF mice, those receiving BIP slurries with or without HMOs and those receiving BIN slurries with or without HMOs. Body weight was monitored throughout the duration of the study. At days 36 to 40, mice were euthanized for sample collection and processed for different assays described further. Rodent studies were conducted in accordance with Institutional animal care and committee (IACUC) guidelines at University of Arkansas for Medical Sciences.

DNA extraction

DNA was extracted from 100 mg mouse cecal contents or feces with metal beads (2.38 mm) and 1 mL Inhibitex buffer in a 2-mL screw-cap tube. Samples were vortexed for 1 min and placed in Precellys homogenizer and operated at 5.3 m/s for 2

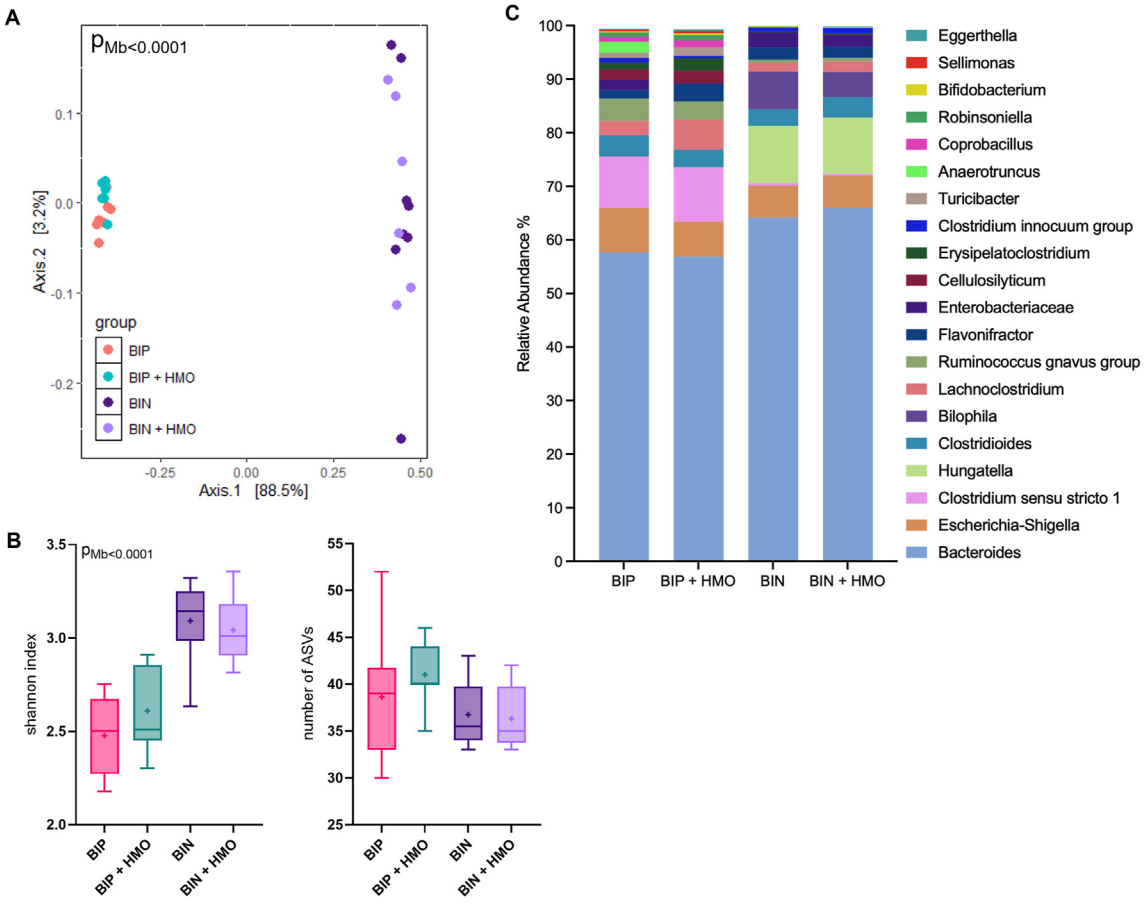

FIGURE 2. Cecal microbiome composition measured by 16S rRNA gene sequencing: (A) Beta diversity: effects of Mb and HMO on overall microbial structure analyzed using PERMANOVA performed on Bray–Curtis dissimilarity distances. (B) Alpha diversity as assessed by Shannon index and number of ASVs. Data were analyzed using a 2-way ANOVA including interaction. Mb, main effect of presence/absence of *B. infantis* in donor microbiome; HMO, main effect of HMO supplementation; Mb × HMO, interaction effect of microbiome and HMO supplementation. (C) Mean relative abundance of bacterial genera present in each study group at ≥0.1%. Animals used were as follows: BIP (3 males, 5 females), BIP + HMO (1 male, 6 females), BIN (5 males, 3 female), and BIN + HMO (3 males, 3 females). ANOVA, analysis of variance; ASV, amplicon sequencing variant; BIN, *B. infantis* negative; BIP, *B. infantis* positive; HMO, human milk oligosaccharide; PERMANOVA, permutational multivariate analysis of variance.

rounds of 30 s each. Samples were incubated at 70°C for 10 min, and QIAamp Fast DNA Stool Mini Kit manufacturer's instructions (Qiagen No. 51604; Valencia) were followed for DNA extraction.

B. infantis and 16S qPCR

We assessed *B. infantis* presence and abundance using *B. infantis*–specific primer set targeting Blon_0915 region in a probe-based qPCR analysis [42]. To derive relative abundance of *B. infantis*, universal primers that anneal to conserved regions of the 16S rRNA was also used to quantify total 16S copies via qPCR as previously described [22]. Universal 16S qPCR was additionally used to compare total bacterial load at the end of the study between GF controls and gavaged animals.

Microbiome analyses

DNA extracted from cecal contents were submitted to the University of Rochester Genomics Research Center for sequencing. The V4 region of bacterial 16S rRNA genes were amplified through the use of primers V4f (5′-GTGYCAGCMGCCGCGGTAA-3′) and V4r (5′-GGACTACNVGGGTWTCTAAT-3′) [44], followed by amplicon pooling, bead-based normalization, and sequencing on the Illumina MiSeq platform with MiSeq reagent version 3 (2 × 250 nt paired-end reads; Illumina). Because of data quality issues in reverse reads, only forward reads were processed, assigned taxonomy, and diversity metrics were calculated using QIIME 2 version 2022.2 [45]. Further details are provided in Supplemental Methods.

Metabolomics

Cecal contents were analyzed for the concentrations of a comprehensive panel of 33 tryptophan pathway metabolites and 8 fatty acids at the Metabolic Invention Center at the University of Victoria, British Columbia, Canada, as outlined in Supplemental Methods.

Cell proliferation

Proliferation assay was carried out as described previously [46]. We plated cells from mesenteric lymph node (MLN) (5×10^4) and spleen ($1–3 \times 10^5$) in duplicate wells of a 96-well plate with complete media, stimulated with nonspecific antigens, concanavalin A, phorbol myristate acetate (PMA) + ionomycin or LPS, and incubated at 37°C and 5% carbon dioxide for 72 h to determine the cell-mediated immune response. At the end of the incubation period, 20 µL AlamarBlue (DAL11000; Thermo Fisher Scientific) was added to each well and fluorescence was quantified with an Omega Polar Star Absorbance Reader.

Flow cytometry

Spleen and MLN were collected in Roswell Park Memorial Institute (RPMI) with 20% fetal bovine serum on ice. Single-cell suspensions were obtained as previously described [47]. Cells collected from spleen (5×10^5) and MLN ($1–2.5 \times 10^5$) were counted and stained for dendritic cells ($B220^-NK1.1^-CD11c^+MHCII^+$), monocytes/macrophages ($B220^-NK1.1^-Ly6G^-CD11b^+$), neutrophils ($B220^-NK1.1^-CD11b^+Ly6G^+$), T cells (i.e., CD3, CD4, CD8), B cells (B220), and plasma cells ($B220^-CD138^+$) as described previously [46]. The UAMS Flow Cytometry Core Facility conducted data acquisition using BD LSRFortessa. Gating for the cell types was carried out as published previously [46].

Ig and cytokine ELISA

Serum IgA, IgG, and IgM were measured using ELISA kits from Invitrogen (Catalog #EMIGA), Cayman Chemical (Catalog

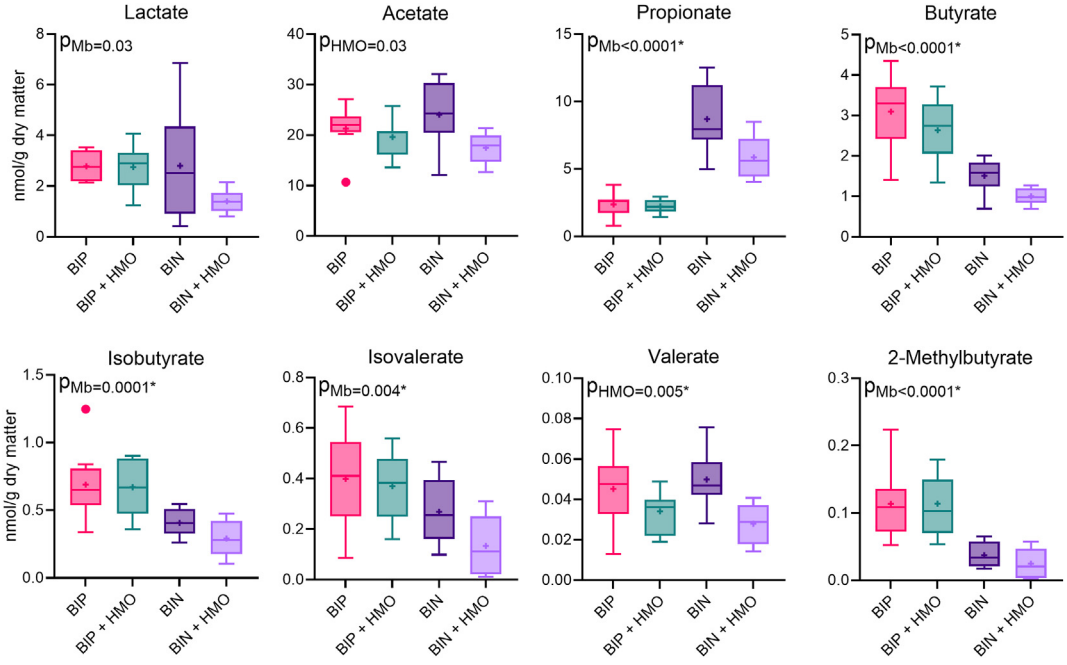

FIGURE 3. Lactate and short-chain fatty acid concentrations in cecal contents. Data were analyzed using a 2-way ANOVA including interaction. Mb, main effect of presence/absence of *B. infantis* in donor microbiome; HMO, main effect of HMO supplementation; Mb × HMO, interaction effect of microbiome and HMO. *Significance of main or interaction effects after Bonferroni correction. Animals used were as follows: BIP (3 males, 5 females), BIP + HMO (1 male, 6 female), BIN (5 male, 3 female), and BIN + HMO (3 males, 3 females). BIN, *B. infantis* negative; BIP, *B. infantis* positive; HMO, human milk oligosaccharide.

#501240), and Invitrogen (Catalog #88-50470), respectively, as per the manufacturer's instructions. Inflammatory and anti-inflammatory cytokines were quantified using U-PLEX Custom Biomarker Multiplex Assay kit from Meso Scale Discovery and as per the manufacturer's instructions, details of which can be found in Supplemental Methods.

GI tract analysis

Length of the entire small intestine (duodenum, jejunum, ileum) and large intestine was measured while cecum weight was noted. Cecal and large intestinal contents were collected and frozen in liquid nitrogen. Small intestine distal region (1 cm), cecum, and proximal large intestine (1 cm) were fixed in formalin for histology. Tissues fixed in formalin were cut, embedded in paraffin blocks, and stained with hematoxylin and eosin. Histomorphometric analyses for small intestine villus height, crypt depth, cecal and large intestine gland length were performed by a board-certified pathologist (TL) with Aperio Imaging software as published previously [46]. Pen tool was used to measure villi and the crypt depths from crypts that were immediately adjacent or as near as possible to measured villi. Crypts and villi that were intact and were entirely within the plan of section were quantified.

Statistical analyses

Data, aside from β-diversity and differential abundance analysis of the microbiome, were analyzed in GraphPad Prism Version 9.3.1 using a 2-way analysis of variance (ANOVA) with Mb, referring to presence or absence of *B. infantis* in donor microbiome, and HMO supplementation (HMO) as main effects and their interaction (Mb × HMO) included in the model. If an interaction was deemed significant, multiple comparison test for pairwise group comparisons was adjusted by Tukey method. Bonferroni correction was used to adjust for multiple comparisons on main and interaction effects when analyzing cecal metabolite data. Figures visualizing metabolite data will display any P value of <0.05, but those P values that remained significant after Bonferroni correction are denoted with an asterisk. For all other analyses, data were considered significant at $P < 0.05$. For data presented as box plots, the center line represents the median, the center point represents the mean, and the box extends from the 25th to the 75th percentile. The top whisker represents the 75th percentile plus 1.5 × IQR or the largest value, and the bottom whisker represents the 25th percentile minus 1.5 × IQR or the smallest value. Points outside of the whiskers represent outliers. All outliers were included in statistical analysis.

Microbiome β-diversity and differential abundance analyses were performed in R. The effect of Mb, HMO, and Mb × HMO

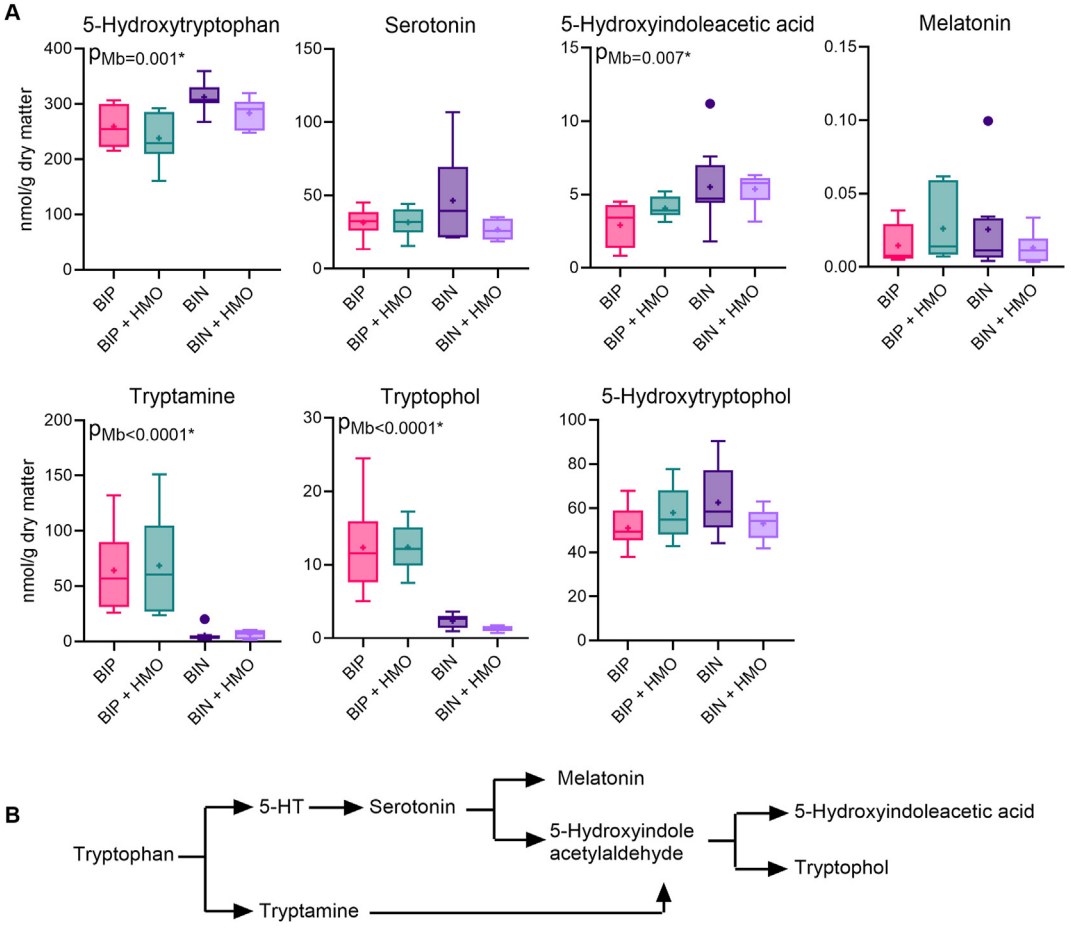

FIGURE 4. Cecal serotonin pathway metabolites. (A) Serotonin metabolite concentrations in cecal contents. (B) Serotonin metabolic pathway. Data were analyzed using a 2-way ANOVA including interaction. Mb, main effect of presence/absence of *B. infantis* in donor microbiome; HMO, main effect of HMO supplementation; Mb × HMO, interaction effect of microbiome and HMO supplementation. *Significance of main or interaction effects after Bonferroni correction. Animals used were as follows: BIP (3 males, 5 females), BIP + HMO (1 male, 6 female), BIN (5 male, 3 female), and BIN + HMO (3 males, 3 females). BIN, *B. infantis* negative; BIP, *B. infantis* positive; HMO, human milk oligosaccharide.

interaction on overall microbial structure were evaluated using permutational multivariate ANOVA performed on Bray–Curtis Dissimilarity calculated in QIIME 2. The effects of Mb and HMO on taxonomic abundance of the cecal bacterial genera was performed using linear models after log transformation in MaAsLin2 [48]. *P* values presented are false discovery rate corrected. *P* values for individual effect terms in ANOVA and permutational multivariate ANOVA are expressed as P_{Mb} and P_{HMO} for Mb and HMO main effects and $P_{Mb \times HMO}$ for Mb × HMO interaction, respectively. Analysis was restricted to taxa present at a mean relative abundance of ≥0.01% across all gavaged animals. The data sets generated during and/or analyzed for this study are available from the corresponding author upon reasonable request.

Results

Colonization with donor fecal microbiota results in 2 structurally different microbial communities

To assess effects of different infant fecal microbiome compositions on metabolite production and immune markers, we inoculated GF mice with stool from breastfed infants that were either naturally colonized or not colonized with *B. infantis*, as determined by qPCR, which is an example of a keystone commensal disappearing in industrialized communities [21,32,34]. This yielded 2 groups based on donor infant microbiomes: BIP or BIN (Supplemental Figure 1A). Mice were each gavaged with fecal material from 1 of the 4 donors (2 BIP and 2 BIN in total); taxonomic composition of individual donor samples differs in other taxa beyond *B. infantis* and is available in Supplemental Figure 1A–C. Half of the mice in each group were also gavaged with a pool of HMOs for 14 d. This resulted in 4 study groups: BIP, BIP + HMO, BIN, and BIN + HMO (Figure 1).

To examine overall microbiome composition among the groups, 16S rRNA sequencing was performed on cecal contents. Overall microbial structure was significantly different between BIP and BIN groups at the end of the study (Figure 2A). α-Diversity analyses demonstrated that BIP animals had a less diverse microbiota when accounting for both richness and evenness, but the total number of bacterial species detected in the cecal microbiota did not differ among study groups (Figure 2B). HMO supplementation had no effect on α- or β-diversity measures of the microbiota. The cecal microbiota among groups was largely

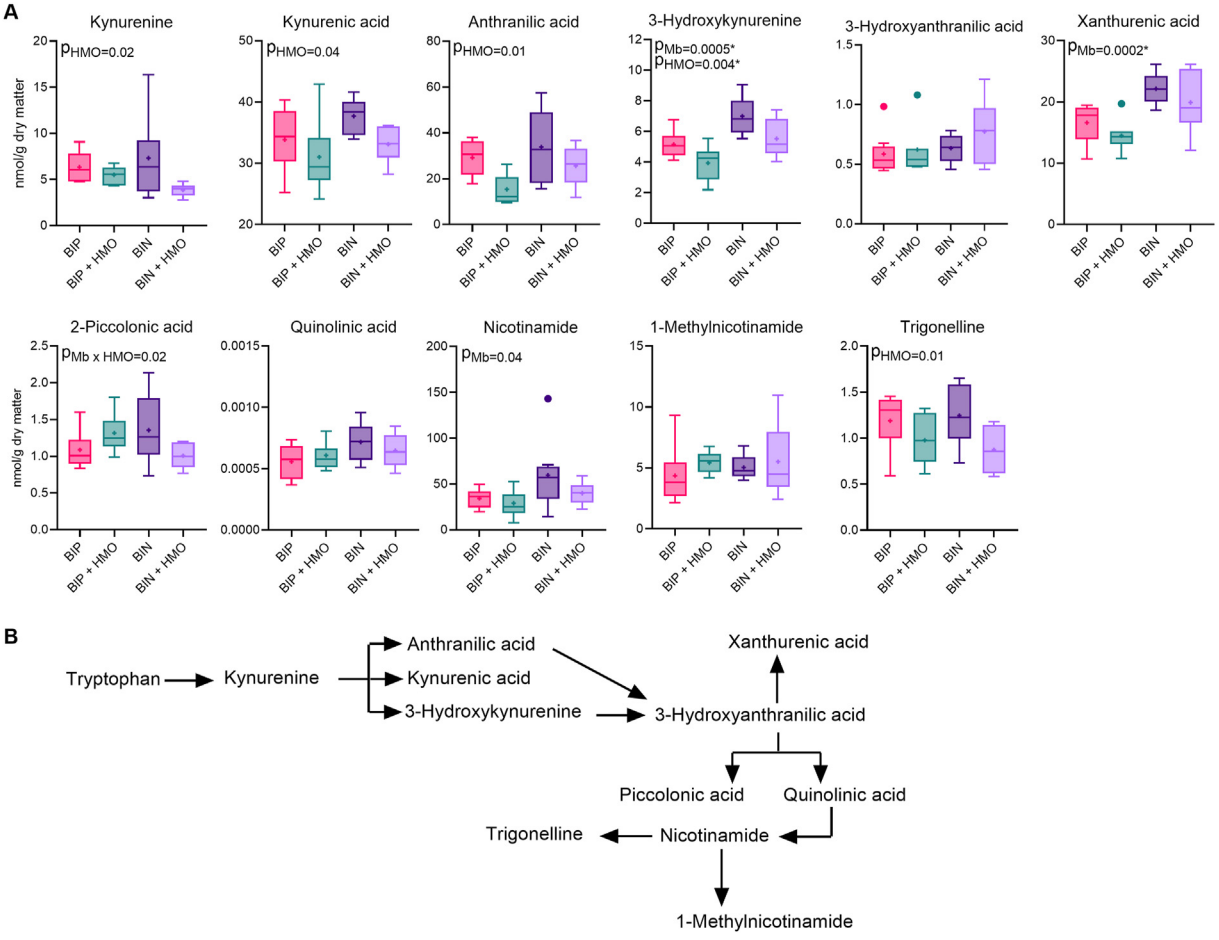

FIGURE 5. Cecal kynurenine pathway metabolites. (A) Kynurenine metabolite concentrations in cecal contents. (B) Kynurenine metabolic pathway. Data were analyzed using a 2-way ANOVA including interaction. Tukey multiple comparison test was used when interaction effect was significant. Mb, main effect of presence/absence of *B. infantis* in donor microbiome; HMO, main effect of HMO supplementation; Mb × HMO, interaction effect of microbiome and HMO supplementation. *Significance of main or interaction effects after Bonferroni correction. Animals used were as follows: BIP (3 males, 5 females), BIP + HMO (1 male, 6 female), BIN (5 male, 3 female), and BIN + HMO (3 males, 3 females). BIN, *B. infantis* negative; BIP, *B. infantis* positive; HMO, human milk oligosaccharide.

dominated by *Bacteroides* spp., which accounted for >50% of the microbial community across groups on average (Figure 2C). Several bacterial genera were differentially abundant between BIP and BIN groups, irrespective of HMO supplementation (Supplemental Figure 2). Although *Bacteroides* spp. dominated the microbiota of all mice, BIN animals had a higher abundance relative to BIP animals irrespective of HMO supplementation (mean ± SD: BIP: 57.3% ± 3.67%; BIN: 65.0% ± 3.91%). Among several other low abundance taxa, BIP animals harbored higher concentrations of *Clostridium senu stricto*, *Ruminococcus gnavus*, and *Erysipelatoclostridium*, *Coprobacillus*, and *Bifidobacterium* spp. *Enterobacteriaceae* spp. were also, on average, >2-fold more abundant in BIN compared with BIP animals. However, an even more notable difference was present within the BIP group where BIP + HMO mice harbored a 10-fold lower abundance of *Enterobacteriaceae* spp. compared with BIP mice not receiving HMOs. Further, *Hungatella* and *Bilophila* spp. were more abundant in BIN animals and almost completely absent in BIP animals. HMO supplementation had an independent effect on just 3 taxa—*Romboutsia*, *Eggerthella*, and *Enterobacteriaceae* spp. Interestingly, total *Bifidobacterium* spp. accounted for <1% of the microbiome in all animals.

Presence or absence of *B. infantis* within the fecal microbiome of the mice was assessed on day 4 after initial oral gavage via qPCR (Supplemental Figure 3A). No *B. infantis* was detected in BIN or BIN + HMO groups. Although *B. infantis* was detectable in the BIP groups via qPCR throughout the study, it was present at a very low concentration (<0.01% mean relative abundance) (Supplemental Figure 3A, B). Thus, any downstream differences between BIP and BIN groups were unlikely to be a direct effect of *B. infantis* but rather due to different cecal microbial communities (Figure 2) resulting from gavage with different infant microbial communities previously shaped by presence or absence of *B. infantis* (Supplemental Figure 1A–C). Therefore, we proceeded with the opportunity to assess how different human infant-like microbiome compositions differentially affect the gut metabolome and immune and GI tract development in healthy mice. All analyses, unless otherwise noted, assessed the main effects of the Mb, referring to presence/absence of *B. infantis* in donor microbiome, and HMO supplementation (HMO) as well as the interaction of Mb × HMO effects.

BIP microbiome drives cecal butyrate and branched-chain fatty acid production

Donor microbiome had opposite effects on both cecal propionate and butyrate, with BIP animals having 2-fold higher cecal concentrations of butyrate (mean ± SD—BIP: 2.88 ± 0.86 nmol/g; BIN: 1.29 ± 0.43 nmol/g) but lower concentrations of propionate, independent of HMO supplementation (Figure 3). BIP

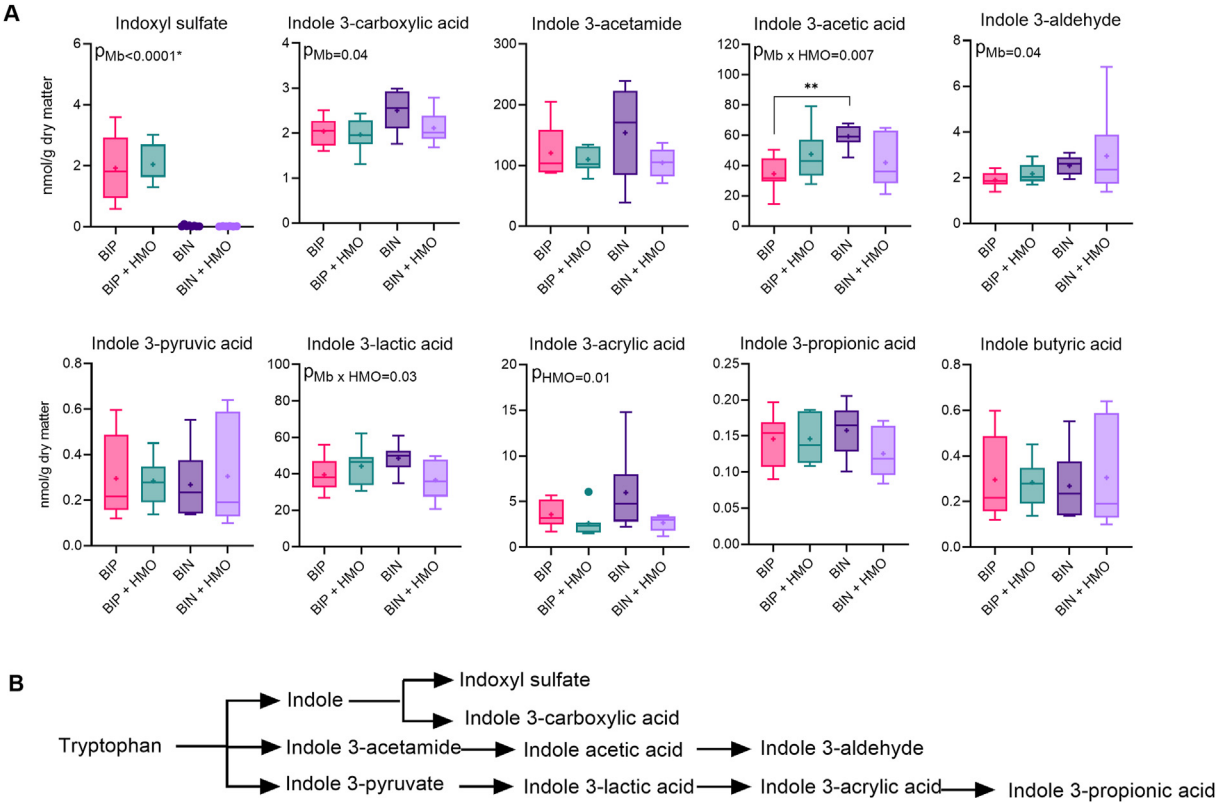

FIGURE 6. Cecal indole concentrations. (A) Indole concentrations in cecal contents. (B) Indole metabolic pathway. Data were analyzed using a 2-way ANOVA including interaction. Tukey multiple comparison test was used when interaction effect was significant. Statistical significance of pairwise group comparisons was noted at *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. Mb, main effect of presence/absence of *B. infantis* in donor microbiome; HMO, main effect of HMO supplementation; Mb × HMO, interaction effect of microbiome and HMO supplementation. *Significance of main or interaction effects after Bonferroni correction. Animals used were as follows: BIP (3 males, 5 females), BIP + HMO (1 male, 6 females), BIN (5 male, 3 female), and BIN + HMO (3 males, 3 females). BIN, *B. infantis* negative; BIP, *B. infantis* positive; HMO, human milk oligosaccharide.

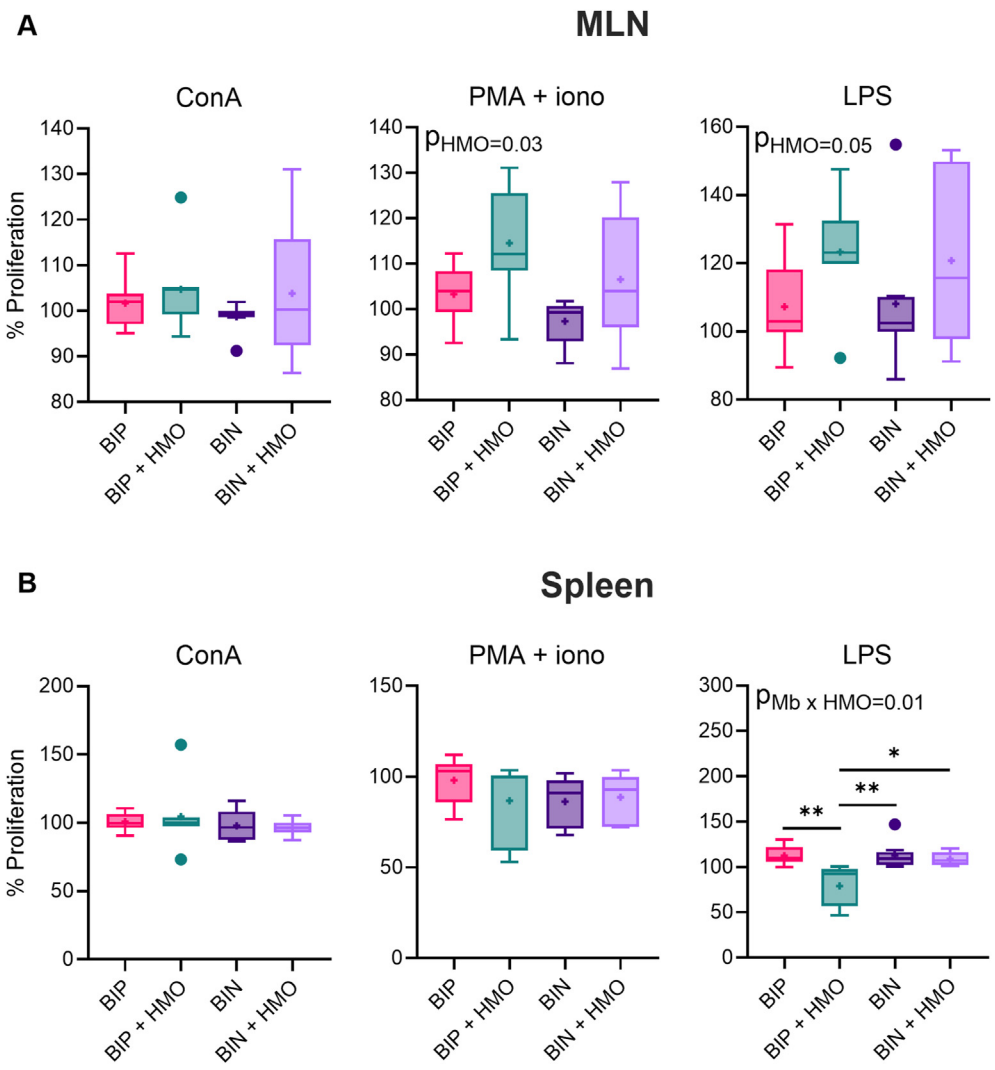

FIGURE 7. Cell proliferation in MLN and spleen. (A) Proliferation in MLN in response to ConA, PMA + ionomycin, and LPS. (B) Splenocyte response to ConA, PMA + ionomycin, and LPS. Data were analyzed using a 2-way ANOVA including interaction. Tukey multiple comparison test was used when interaction effect was significant. Statistical significance of pairwise group comparisons was noted at $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. Mb, main effect of presence/absence of *B. infantis* in donor microbiome; HMO, main effect of HMO supplementation; Mb × HMO, interaction effect of microbiome and HMO supplementation. Animals used were as follows: BIP (3 males, 5 females), BIP + HMO (1 male, 6 female), BIN (5 males, 3 females), and BIN + HMO (3 males, 3 females). BIN, *B. infantis* negative; BIP, *B. infantis* positive; ConA, concanavalin A; HMO, human milk oligosaccharide; LPS, lipopolysaccharide; MLN, mesenteric lymph node; PMA, phorbol myristate acetate.

animals also had higher cecal concentrations of branched SCFA, isobutyrate, isovalerate, and 2-methylbutyrate, while mice supplemented with HMOs had lower cecal concentration of valerate. Initial analyses identified a Mb effect on cecal acetate and lactate; however, these effects no longer remained significant after correction for multiple comparisons.

Cecal tryptophan metabolism is markedly shifted by inoculation with different microbial communities

Some bacterial species, including *Bifidobacterium* spp., possess the genetic machinery to metabolize aromatic amino acids into byproducts that can have potent immunologic effects during early life [49]. Thus, we measured concentrations of cecal aromatic amino acids and derivatives as well as metabolites in 3 pathways of downstream tryptophan metabolism—serotonin, kynurenine, and indole pathways. Concentration of cecal tolyl sulfate, an aromatic amino acid degradation product, was significantly higher in BIP groups with relatively little to no production measured in BIN mice (Supplemental Figure 4). After controlling for multiple comparisons, there were no other significant Mb or HMO effects on concentrations of cecal phenylalanine, tyrosine, and tryptophan or lysine derivative, pipecolic acid (Supplemental Figure 4).

In terms of serotonin pathway metabolites (Figure 4A), we found that BIN animals, independent of HMO supplementation, had higher cecal concentrations of 5-hydroxytryptophan (mean ± SD—BIP: 249.4 ± 41.6 nmol/g; BIN: 300.1 ± 30.1 nmol/g) and 5-hydroxyindoleacetic acid (mean ± SD—BIP: 3.44 ± 1.27 nmol/g; BIN: 5.46 ± 2.16 nmol/g) but lower concentrations of

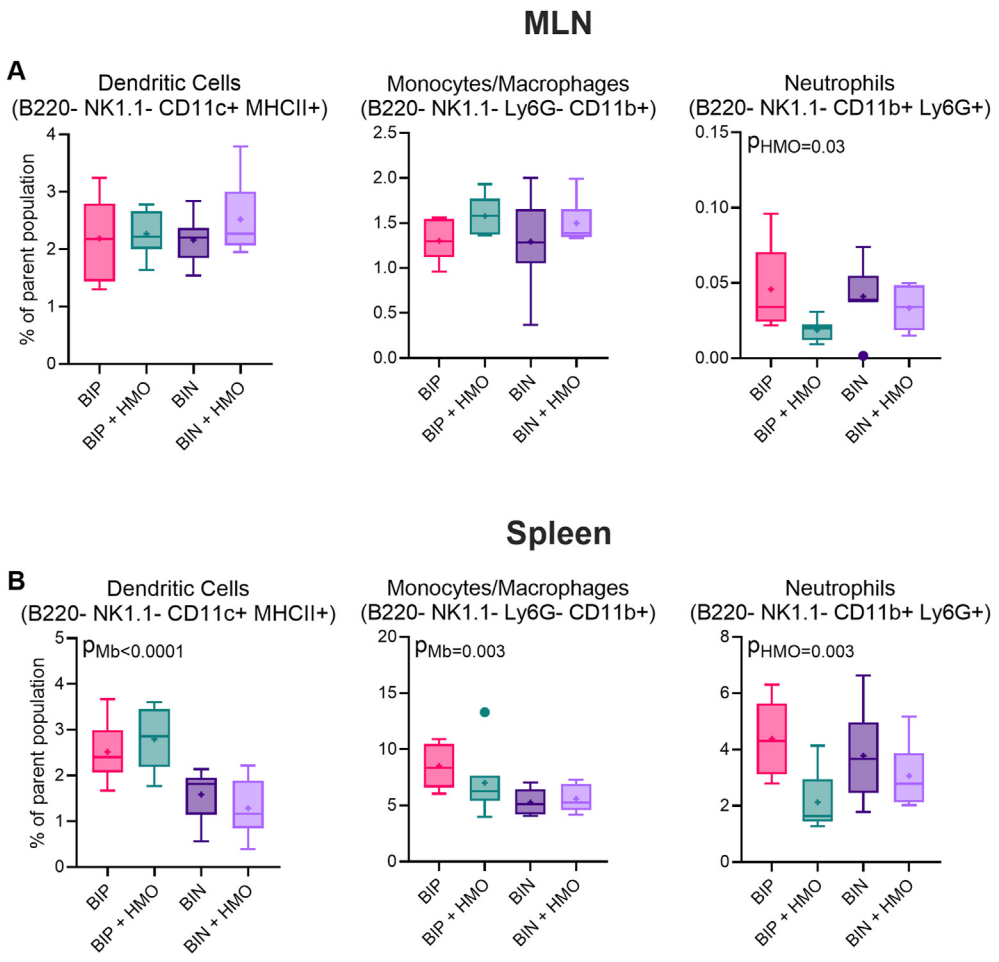

FIGURE 8. Innate immune cell composition. Dendritic cells ($B220^{-}NK1.1^{-}CD11c^{+}MHCII^{+}$), monocytes/macrophages ($B220^{-}NK1.1^{-}Ly6G^{-}CD11b^{+}$) and neutrophils ($B220^{-}NK1.1^{-}CD11b^{+}Ly6G^{+}$) population from (A) MLN and (B) spleen. Data were analyzed using a 2-way ANOVA including interaction. Tukey multiple comparison test was used when interaction effect was significant. Mb, main effect of presence/absence of *B. infantis* in donor microbiome; HMO, main effect of HMO supplementation. Animals used were as follows: BIP (3 males, 5 females), BIP + HMO (1 male, 6 female), BIN (5 males, 3 females), and BIN + HMO (3 males, 3 females). MLN, mesenteric lymph nodes; BIN, *B. infantis* negative; BIP, *B. infantis* positive; HMO, human milk oligosaccharide; MLN, mesenteric lymph node.

cecal tryptamine and tryptophol relative to BIP groups (Figure 4B). Further analysis of kynurenine pathway metabolites (Figure 5A) demonstrated that BIN mice had higher cecal concentrations of xanthurenic acid (Figure 5B). There were also independent effects of both Mb and HMO on 3-hydroxykynurenine; the BIP + HMO group had the lowest mean concentration and BIN animals had the highest. We lastly measured concentrations of 10 cecal indoles (Figure 6A, B). The most significant finding was a higher concentration of indoxyl sulfate in BIP animals.

BIP microbiome and HMOs downregulate LPS-induced splenic cell proliferation and increase splenic B and plasma cells

To assess the impact of the BIP compared with BIN microbiome and HMO supplementation on immune development, we first stimulated single-cell suspensions derived from MLNs and spleen with concanavalin A, PMA + ionomycin, and LPS and measured subsequent proliferation. In MLN, HMO supplementation led to higher proliferation after PMA + ionomycin and LPS stimulation (Figure 7A). In the spleen, there was an Mb × HMO interaction effect with cells derived from the spleen of BIP + HMO mice, showing less proliferation after LPS stimulation relative to the 3 other groups of mice (Figure 7B).

Next, we measured composition of innate and adaptive immune cells in both MLN and spleen via flow cytometry. There was a Mb effect on neutrophils in both MLN and spleen, which was driven primarily by a lower percentage of neutrophils in the BIP + HMO group (Figure 8A, B). In the spleen, BIP mice had a higher percentage of both dendritic cells as well as monocytes/macrophages relative to BIN animals, independent of HMO supplementation (Figure 8B). In MLN, we identified no Mb or HMO effects on $CD8^{+}$ T, B, or plasma cell populations; however, percentages of $CD4^{+}$ T cells were lower in BIP than those in BIN mice (Figure 9A). More prominent effects of microbiome and diet were identified in splenic cell populations. BIP groups had higher percentages of splenic $CD8^{+}$ T cells and B cells (Figure 9C,

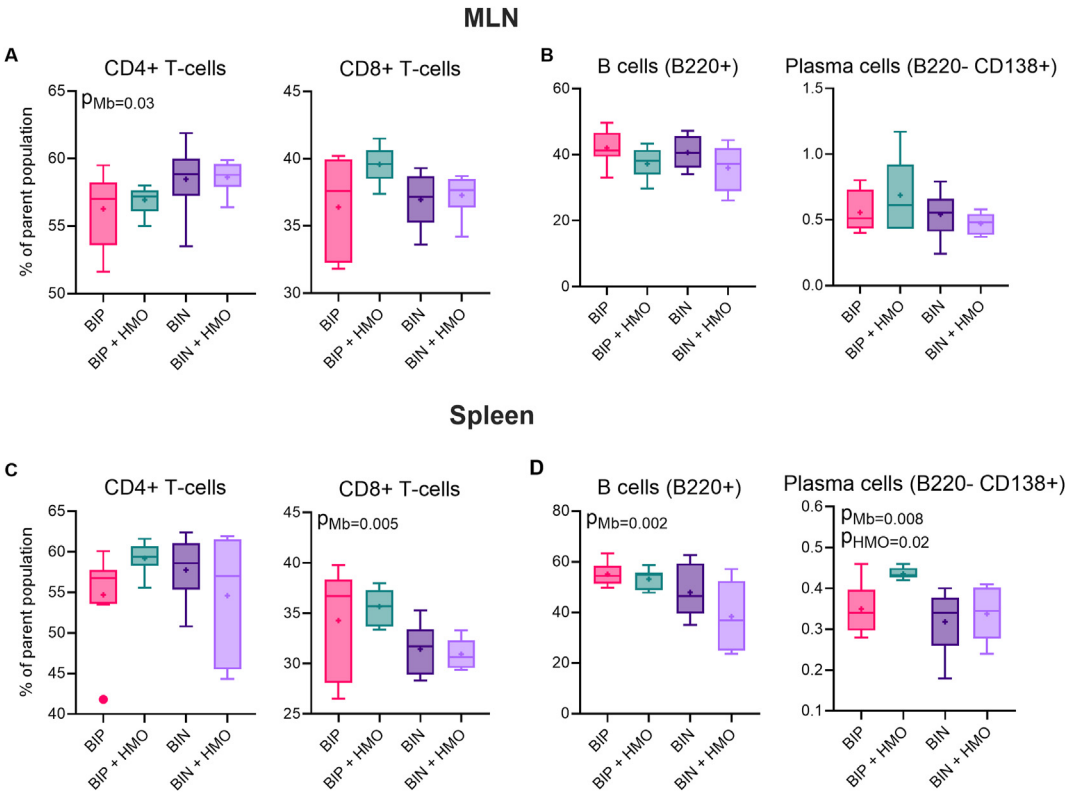

FIGURE 9. Adaptive immune cell composition. T cells ($CD3^+CD4^+$ and $CD3^+CD8^+$), B cells ($B220^+$), and plasma cells ($B220^-CD138^+$) population from (A) MLN and (B) spleen. Data were analyzed using a 2-way ANOVA including interaction. Tukey multiple comparison test was used when interaction effect was significant. Mb, main effect of presence/absence of *B. infantis* in donor microbiome; HMO, main effect of HMO supplementation. Animals used were as follows: BIP (3 males, 5 females), BIP + HMO (1 male, 6 females), BIN (5 males, 3 females), and BIN + HMO (3 males, 3 females). MLN, mesenteric lymph nodes; BIN, *B. infantis* negative; BIP, *B. infantis* positive; HMO, human milk oligosaccharide; MLN, mesenteric lymph node.

D). There were additionally independent Mb and HMO effects on percentages of splenic plasma cells driven by the BIP + HMO mice, which had the highest percentages among the groups.

Finally, we assessed cytokine and Ig concentrations in serum as additional markers of immune system development in these mice. IL-17A/F, IL-4, and IL-12p70 were below the detection level, and no differences among groups were identified for serum IL-10 or interferon-γ (Figure 10A). However, BIN + HMO mice had significantly greater concentrations of serum TNF-α relative to BIN mice receiving no HMOs (Figure 10A). Interestingly, HMO supplementation increased both total IgA and IgG concentrations in serum (Figure 10B).

Gut morphology, but not body weight, is influenced by microbiome composition and HMO supplementation

Body weights of the animals were monitored throughout the course of the study, and we detected no Mb or HMO effects on percent body weight increase on day 14 of the study (Supplemental Figure 5). Finally, we were interested in how a BIP compared with BIN microbiome and HMO supplementation influence development of the GI tract (Figure 11). Small and large intestine lengths were measured at the end of the study. Independent of HMO supplementation, there were modest Mb effects on small intestine (SI) length, which appeared to be driven by BIP mice who showed the lowest mean SI length. Conversely, BIP mice demonstrated greater large intestine length relative to BIN mice, independent of HMO supplementation (Figure 11A). Using histomorphometric analysis, we identified independent Mb and HMO effects on villi height (Figure 11B), likely driven by the BIP + HMO group, which had the lowest mean villus height. Further, BIP mice without HMO supplementation had a significantly greater crypt depth than all other study groups (Figure 11B). Cecum and large intestine gland depth were not different among all study groups (Figure 11C).

Discussion

Host–microbe interactions in early life play a critical role in influencing long-term immune and metabolic function. We extend previous studies on the immune effects of the early life microbiome, showing that the gut microbiota differentially influences metabolite production and provide novel findings of its effect on proportions of mucosal and systemic innate and adaptive immune cell populations in healthy mice. Notably, inoculation with a BIP microbiome combined with HMO supplementation reduced LPS-induced cell proliferation and increased proportion of plasma cells in the spleen. HMO supplementation had little effect on microbiome composition but independently decreased neutrophil populations and enhanced

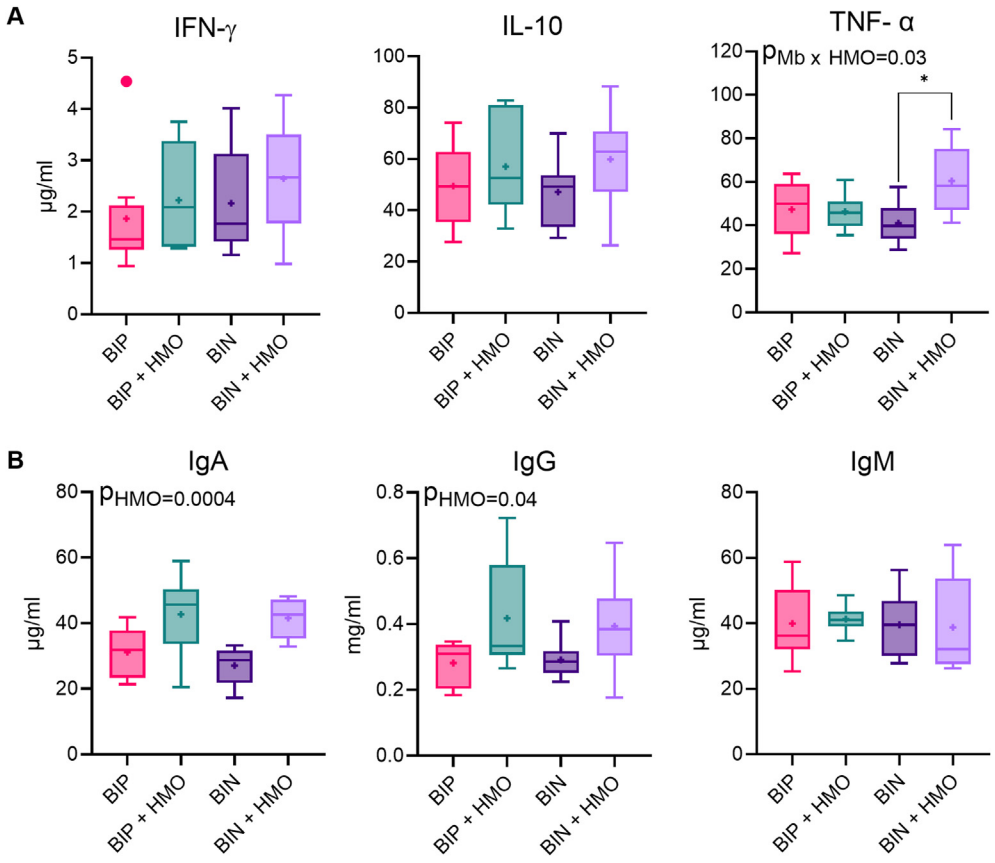

FIGURE 10. Serum cytokines and immunoglobulins. Serum (A) cytokines and (B) immunoglobulins. Data were analyzed using a 2-way ANOVA including interaction. Tukey multiple comparison test was used when interaction effect was significant. Statistical significance of pairwise group comparisons was noted at $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. Mb, main effect of presence/absence of *B. infantis* in donor microbiome; HMO, main effect of HMO supplementation; Mb × HMO, interaction effect of microbiome and HMO supplementation. Animals used were as follows: BIP (3 males, 5 females), BIP + HMO (1 male, 6 females), BIN (5 males, 3 females), and BIN + HMO (3 males, 3 females). BIN, *B. infantis* negative; BIP, *B. infantis* positive; HMO, human milk oligosaccharide.

Ig production, suggesting alternative mechanisms of action irrespective of microbiota effects. A summary of independent microbiome and diet effects are outlined in Table 1.

Upon assessment of the cecal microbiota at the end of the study, we identified an overall different microbial structure in BIP relative to BIN mice independent of HMO supplementation, which likely resulted from significant differences in the infant donor microbiome composition. BIP mice harbored higher relative abundances of cecal *Clostridium senu stricto*, *Ruminococcus gnavus*, *Cellulosilyticum* spp., and *Erysipelatoclostridium* spp., among others, while BIN animals had higher concentrations of *Hungatella*, *Bilophila*, and *Bacteroides* species. Despite high concentrations in donor fecal samples, total *Bifidobacterium* spp. abundance was surprisingly low in cecal contents of all animals, and *B. infantis* concentrations were similarly low in BIP groups. Thus, any downstream effects found to be different between BIP and BIN groups were unlikely to be a direct effect of colonization with *B. infantis* but rather a result of different infant microbial communities shaped by presence or absence of *B. infantis in* the donor infants. Low colonization of *Bifidobacterium* spp. could, in part, be due to inefficient engraftment of members of this genus upon gavage or competition with other bacteria capable of metabolizing the relatively large amounts of fiber present in mouse feed pellets, perhaps resembling gut microbiome dynamics of an infant whose diet has mostly transitioned to solid foods with some continued breastfeeding. Similarly, differences in the host immune system or GI environment of mice and humans [50], such as mucin and pH, may have also influenced concentrations of *Bifidobacterium* spp. A recent study gavaged GF mice with a fecal slurry from a human infant followed by gavage of *B. infantis* (1×10^6 to 1×10^8 CFU every 10–14 h) plus HMO for 3 days and then HMO alone [51]. Even on a polysaccharide-deficient diet, the median abundance of *B. infantis* did not reach 25%, suggesting that *B. infantis* dynamics are likely different in mice and human infants in the presence of other gut microbes. Dominant bacteria appearing in the mice in our study such as *Bacteroides* spp., *Clostridium senu stricto*, *Ruminococcus gnavus*, *Escherichia* spp., and *Lachnoclostridium* sp. are common in older infants and young children [7,52,53], ages that often overlap with weaning from human milk and diversification of diet. Although HMO supplementation had little effect on microbial composition, there were notable Mb and HMO effects on cecal abundance of *Enterobacteriaceae*, which was the lowest among BIP + HMO mice. Abundance of *Enterobacteriaceae* species is higher in infants with necrotizing enterocolitis [54] as well as IgE- and non–IgE-mediated food allergy [55,56].

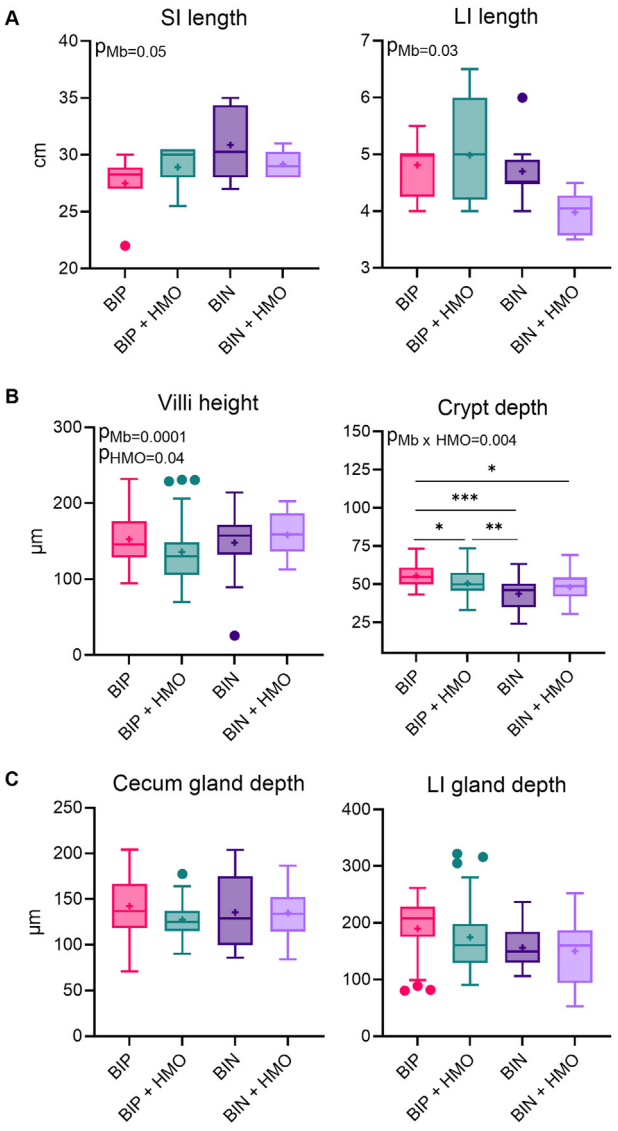

FIGURE 11. Intestine weights and morphologic characteristics. (A) Small and large intestine lengths; (B) villi height and crypt depth; (C) cecum and large intestine gland depth. Data were analyzed using a 2-way ANOVA with Tukey multiple comparison test. Mb, main effect of presence/absence of *B. infantis* in donor microbiome; HMO, main effect of HMO supplementation; Mb × HMO, interaction effect of microbiome and HMO supplementation. Statistical significance of pairwise group comparisons was noted at $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. Animals used were as follows: BIP (3 males, 5 females), BIP + HMO (1 male, 6 females), BIN (5 males, 3 females), and BIN + HMO (3 males, 3 females). BIN, *B. infantis* negative; BIP, *B. infantis* positive; HMO, human milk oligosaccharide.

Therefore, a microbiome resembling that of BIP animals—containing high concentrations of *Clostridium senu stricto* and *Ruminococcus gnavus* and low concentrations of *Bilophila* and *Hungatella* spp.—in the presence of HMOs may also increase colonization resistance for pathogens responsible for hyperactive immune responses.

Diet, microbial metabolism, and host health are inextricably linked [57–60]. In this study, we identified several metabolites to be differentially abundant between BIP and BIN mice. BIP animals produced 2-fold higher concentrations of butyrate and significantly higher concentrations of multiple branched SCFA, while the BIN microbiome produced higher concentrations of propionate, all irrespective of HMO supplementation. A strong body of evidence supports a role for butyrate in supporting intestinal barrier function and induction of regulatory T cells [16, 61]. Branched SCFA are generally products of amino acid fermentation by the microbiota [62] and have been associated with metabolic disease in adults. However, data from younger cohorts demonstrate that fecal branched SCFA concentrations increase over time in early childhood, and higher concentrations around preschool age have been identified in farm children at low risk for allergic disease [63] and associated with protection against allergic sensitization [64]. Further, BIP mice demonstrated increased production of tryptophan metabolites tryptophol, tryptamine, and indoxyl sulfate. Tryptophol has been shown to increase gut barrier integrity in a mouse model of dextran sulfate sodium (DSS) colitis [65]. Tryptamine, likely increased due to higher abundance of *Ruminococcus gnavus* in BIP animals [66], and indoxyl sulfate are known ligands of the aryl hydrocarbon receptor [67], which is expressed in innate and adaptive immune cells and can regulate dendritic cell differentiation, antigen presentation, and T cell immunity. Indoxyl sulfate has specifically been shown to inhibit T_H2 differentiation in vitro [68] and induce trained immunity in monocytes via epigenetic mechanisms [69]. However, little research overall is available on relevance of indoxyl sulfate on gut function and immune development in early life. On the contrary, BIN animals harbored higher cecal concentrations of 2 kynurenine metabolites—3-hydroxykynurenine and xanthurenic acid. The kynurenine pathway has a demonstrated role in T cell physiology, specifically the reduction of T_H1 [70] and promotion of T regulatory cell differentiation [71,72]. Xanthurenic acid, in particular, has been inversely correlated with measures of intestinal inflammation in mice and inflammatory bowel disease in humans [73] and may represent a host mechanism to ameliorate inflammatory responses to resident microbiota or other stimuli.

These collective differences in cecal microbial composition and metabolite production led to marked differences in the immune system. Notably, we identified a significantly greater percentage of splenic dendritic cells in BIP animals, perhaps due to aryl hydrocarbon receptor activation by tryptamine and indoxyl sulfate as previously discussed. These findings highlight a mechanism by which a BIP-like microbiome may enhance antigen presentation and promote peripheral tolerance, which is important for prevention of autoimmune and other inflammatory diseases. BIP animals also had expanded populations of splenic $CD8^+$ T and B cells, the latter of which could be due to increased production of butyrate, a known modulator of B cell metabolism [74,75]. The Mb effect on plasma cell abundance was primarily driven by the BIP + HMO group, which had the highest splenic plasma cell population. These findings may underpin previous work showing that both the early life microbiome composition [76,77] and breastfeeding [78] influence efficacy of vaccine response. Our findings further showed an attenuated proliferation response in splenocytes of BIP + HMO mice after LPS stimulation, which has been shown to protect against later asthma development in mice [79]. *Bifidobacterium* strains have in fact been previously shown to inhibit LPS-induced inflammation in intestinal epithelial cells [80]. LPS immunogenicity also varies across microbial species, thus differential

TABLE 1
Summary of independent microbiome and diet effects on metabolite, immune, and morphologic findings.

Outcomes	Microbiome (Mb) effect		HMO effect	
	BIP	BIN	HMO^+	HMO^-
SCFA	↑Butyrate ↑Isobutyrate ↑Isovalerate	↑Propionate	—	↑Valerate
Tryptophan metabolites	↑Tryptamine ↑Tryptophol ↑Indoxyl sulfate	↑5-Hydroxytryptophan ↑5-Hydroxyindoleacetic acid ↑3-Hydroxykynurenine ↑Xanthurenic acid	↑3-Hydroxykynurenine	—
MLN cell proliferation	—	—	↑ with PMA + iono and LPS stimulation	—
Innate immunity	↑Dendritic cells (spleen) ↑Monocytes/macrophages (spleen)	—	—	↑Neutrophils (MLN and spleen)
Adaptive immunity	↑$CD8^+$ T cells (spleen) ↑B cells (spleen) ↑Plasma cells (spleen)	↑$CD4^+$ T cells in MLN	↑Plasma cells (spleen)	—
Immunoglobulins	—	—	↑IgA and IgG (serum)	—
Organ lengths	↑LI length	↑SI length ↑Villi height	↑Villi height	—

Abbreviations: BIN, *B. infantis* negative; BIP, *B. infantis* positive; HMO, human milk oligosaccharide; iono, ionomycin; LI, large intestine; LPS, lipopolysaccharide; MLN, mesenteric lymph node; PMA, phorbol myristate acetate; SCFA, short-chain fatty acid; SI, small intestine.
Interaction effects are not listed in the table.

exposure to other resident microbiota between groups could have further influenced the host response to LPS [31].

Several studies have demonstrated the impact of HMOs in immune development in early life [17]. In this study, we showed that HMO supplementation independently decreased neutrophil populations in both MLN and spleen. Acidic HMOs have in fact have been shown to reduce platelet–neutrophil complex formation and neutrophil activation ex vivo [81], effects that have the potential to suppress intestinal inflammation in infants. HMO supplementation increased cell proliferation in MLN after both PMA + ionomycin and LPS stimulation as well as total serum IgA and IgG concentrations. We have recently shown higher T cell proliferation and enhanced vaccine response in human milk fed compared with formula fed piglets [47] and a direct effect of HMOs on higher vaccine response in a GF mouse model [46]. These findings collectively demonstrate that microbiome composition and HMO supplementation synergistically influence innate immune activation and plasma cell populations but independently shape neutrophil, T and B cell populations and Ig production in healthy mice.

Finally, gut morphologic analyses showed an increased villi height and decreased small intestine crypt depth in BIN compared with BIP mice, suggesting that BIN animals had increased digestive and absorptive capacity in the small intestine, supporting previous findings that the microbiome influences nutrient absorption and energy regulation [82,83]. We further hypothesize that the increased crypt depth and colon length in BIP animals may have been due to increased production of butyrate, which is known to promote intestinal proliferation in animal models [84–86].

We acknowledge that this study does come with limitations. First, we gavaged mice with individual fecal slurries from different infants rather than using 1 pooled fecal slurry for all mice in each respective group in an attempt to maintain initial microbial community dynamics that supported *B. infantis* colonization in human infants. Although cecal microbiome composition was comparable among mice within each group at the end of the study, the time at which metabolite and immune outcomes were also measured, initial differences in microbiome composition of BIN infant donors could have impacted immunologic programming immediately after gavage. Further, we used only 2 fecal slurries each for BIP and BIN groups. Given the lack of an established core infant gut microbiome in the literature, the donor compositions in this study may not be representative of BIP and BIN infants around the world. The lack of *B. infantis* colonization in these mice, likely due to nutritional substrates and other host factors, limited our investigation of direct effects of this commensal microbe on early development. Recapitulation of *B. infantis* abundance in a mouse to a level seen in human infants remains a methodologic hurdle. There are also differences in functional capacity of species and strains within the same bacterial genus, thus, there is likely more variability within the microbiota among animal groups that cannot be detected with 16S rRNA sequencing. Furthermore, we were only able to use forward reads in this study due to poor sequencing quality of the reverse reads; the use of overlapping reads could have enhanced diversity assessment and taxonomic assignment in this study. Finally, given the relatively small sample size, we did not include sex as an effect in our models, but this would be important in future studies.

In summary, our findings extend current knowledge about the infant microbiome and immune development, demonstrating that different microbiome compositions, with and without HMO supplementation, robustly modulate bacterial metabolite production and drive different innate and adaptive immune trajectories in healthy mice, with potentially important and translatable implications for early childhood health.

Author contributions

The authors' responsibilities were as follows – KMJ, AES, LY: conceived the overall study and supervised the research; PT, ECD, MG, FR: performed experiments; RF: processed and stained the gut tissues for histomorphometry analyses; CS: provided support with germ-free mouse facility; TL: conducted histomorphometry analyses; LB: provided HMOs; AES, KMJ: provided the infant fecal samples; ECD: completed the data analysis and provided the figures; ECD, AES, KMJ, LY: contributed to literature search and data analyses and drafted the manuscript with additional input from all authors; and all authors: read and approved the final manuscript.

Conflict of interest

The authors report no conflicts of interest.

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Data availability

Data described in the manuscript will be made available upon request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tjnut.2024.07.031.

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