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ORIGINAL ARTICLE

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Gut bacteria at 6 months of age are associated with immune cell status in 1-year-old children

Morten Nilsen[1] | **Unni Cecilie Nygaard**[2] | **Petter Brodin**[3,4] | **Karin Cecilie Lødrup Carlsen**[5,6] | **Cecilie Fredheim**[1] | **Guttorm Haugen**[6,7] | **Gunilla Hedlin**[3,8] | **Christine Monceyron Jonassen**[1,9] | **Unni Lise Albertsdottir Jonsmoen**[1] | **Tadepally Lakshmikanth**[3] | **Björn Nordlund**[3,8] | **Axel Olin**[10] | **Eva Maria Rehbinder**[11] | **Håvard O. Skjerven**[5,6] | **Lars Snipen**[1] | **Anne Cathrine Staff**[6,7] | **Cilla Söderhäll**[3,8] | **Riyas Vettukattil**[5,6] | **Knut Rudi**[1]

[1]Faculty of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, Ås, Norway

[2]Section for Immunology, Department of Method Development and Analytics, Norwegian Institute of Public Health, Oslo, Norway

[3]Department of Women's and Children's Health, Karolinska Institutet, Stockholm, Sweden

[4]Pediatric Rheumatology, Karolinska University Hospital, Solna, Sweden

[5]Division of Paediatric and Adolescent Medicine, Oslo University Hospital, Oslo, Norway

[6]Faculty of Medicine, Institute of Clinical Medicine, University of Oslo, Oslo, Norway

[7]Division of Obstetrics and Gynaecology, Oslo University Hospital, Oslo, Norway

[8]Astrid Lindgren Children's Hospital, Karolinska University Hospital, Stockholm, Sweden

[9]Genetic Unit, Centre for Laboratory Medicine, Østfold Hospital Trust, Kalnes, Norway

[10]Human Evolutionary Genetics, Institut Pasteur, Paris, France

[11]Department of Dermatology and Vaenorology, Oslo University Hospital, Oslo, Norway

Correspondence
Morten Nilsen, Faculty of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, Ås, Norway.
Email: morten.nilsen@nmbu.no

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Abstract

Age-related gut bacterial changes during infancy have been widely studied, but it remains still unknown how these changes are associated with immune cell composition. This study's aim was to explore if the temporal development of gut bacteria during infancy prospectively affects immune cell composition. Faecal bacteria and short-chain fatty acids were analysed from 67 PreventADALL study participants at four timepoints (birth to 12 months) using reduced metagenome sequencing and gas chromatography. Immune cell frequencies were assessed using mass cytometry in whole blood samples at 12 months. The infants clustered into four groups based on immune cell composition: clusters 1 and 2 showed a high relative abundance of naïve cells, cluster 3 exhibited increased abundance of classical- and non-classical monocytes and clusters 3 and 4 had elevated neutrophil levels. At all age groups, we did observe significant associations between the gut microbiota and immune cell clusters; however, these were generally from low

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abundant species. Only at 6 months of age we observed significant associations between abundant (>8%) species and immune cell clusters. *Bifidobacterium adolescentis* and *Porphyromonadaceae* are associated with cluster 1, while *Bacteroides fragilis* and *Bifidobacterium longum* are associated with clusters 3 and 4 respectively. These species have been linked to T-cell polarization and maturation. No significant correlations were found between short-chain fatty acids and immune cell composition. Our findings suggest that abundant gut bacteria at 6 months may influence immune cell frequencies at 12 months, highlighting the potential role of gut microbiota in shaping later immune cell composition.

KEYWORDS

blood, gut microbiota, human, short-chain fatty acids, monocytes/macrophages, neutrophils, T cells

1 | INTRODUCTION

Immune cell development and bacterial colonization are vital in maintaining homeostasis in human intestines. Humans are constantly exposed to microbial antigens and diverse dietary fibres, which influence gut and immune development.[1,2] A disruption of the gut microbiota colonization may lead to a loss of key bacteria, resulting in gut microbiota dysbiosis, and thus affect immune development. For instance, loss of *Bifidobacterium* has been observed to be associated with decreased autoimmune activity,[3] and reduction of other early-life bacteria such as *Faecalibacterium*, *Lachnospira*, *Veillonella* and *Rothia* and the resulting gut microbial dysbiosis has been linked to a higher risk of developing asthma and allergic disease.[4]

A healthy neonatal immune system needs to develop a balance between the various immune cells, both innate and adaptive and between functional subpopulations such as T helper, T cytotoxic and regulatory T cells (Tregs) subpopulations.[5] Among these, a balance of Th1 and Th2 cells is important for pathogen clearance.[5] However, the early neonatal immune system is skewed towards T helper 2 (Th2) cells.[5,6] Observations that early colonizing bacteria, such as *Bifidobacterium infantis*, can help skew the Th polarization towards Th1, suggest a link between gut microbiota and immune modulatory effects, including influence on Th1/Th2 balance.[1]

The first year of life involves a major temporal development of the gut microbiota. Several factors induce these changes, including delivery mode,[7,8] breastfeeding[7,9,10] and introduction to solid foods.[11] One major change in the early-life gut microbiota is the increased abundance of *Bifidobacterium*, mainly due to breastmilk, and *Bifidobacterium's* ability to utilize and degrade human milk oligosaccharides (HMOs).[7] The *Bifidobacterium* remains the most abundant gut bacterium until weaning, whereas its abundance decreases synchronously with an increased abundance of *Firmicutes* and *Bacteroides*, which become the more abundant genera.[11]

The short-chain fatty acid (SCFA) composition in the gut is altered as a result of bacterial changes in the first year of life,[12] which may affect immune development.[13] Butyrate, one of the major SCFAs produced by the gut microbiota, is the colonocytes' main energy source.[14] Both propionate and butyrate have been shown to induce Treg generation by polarizing CD4+ T cells to Foxp3+ Tregs, which may be due to their ability to inhibit histone deacetylase (HDACs).[13,15] In addition, SCFAs may impair Th2 polarization[16] and promote polarization of Th1 and Th17 cells.[13,17] The production of SCFAs by gut bacteria is not confined to a bacterial order, but in general, *Clostridiales* are effective butyrate producers, *Bacteroides* mainly produces propionate, and most enteric bacteria can produce acetate.[18]

It is not known how these age-related changes in bacterial composition and SCFA levels in the gut are associated with the development of immune cell composition and function during infancy. Therefore, the aim of this study was to explore if the first-year temporal development of the infant gut bacteria and SCFAs are associated with immune cell composition in blood in children at 1 year of age.

2 | MATERIALS AND METHODS

We longitudinally analysed faecal SCFAs and gut microbiota composition of 67 infant cohort participants to associate them with immune cell profiles at 12 months of age. The biological samples were derived from the Preventing Atopic Dermatitis and ALLergy in children study (PreventADALL).[19,20]

2.1 | Clinical samples

The present data and biological samples are from the general population-based PreventADALL study,[19] which included approximately 2400 mother-child pairs from Norway and Sweden. The primary aim of PreventADALL was to test strategies for primary prevention of allergic disease development, and secondarily to identify early life factors involved in the development of non-communicable diseases. The present sub-sample of 67 infant cohort participants was collected from Oslo, Norway, and was included due to the first availability of complete faecal collection sets for all time points, as well as successful blood samples collected at 12 months of age. For more details on infant variables and biological sampling, see Appendix S1.

Informed written consent from all pregnant mothers was received upon inclusion at mid-gestation, and from both parents upon inclusion of the newborn child. The PreventADALL study has been approved by the Regional Ethical Committee (REK) for Medical and Health Research Ethics in South-Eastern Norway (2014/518) as well as in Sweden (2015/4:3) by the Regional Ethics Committee at Karolinska Institutet, Stockholm.

Faecal samples were collected longitudinally at birth (meconium), 3, 6 and 12 months of infant age. Bacterial composition from faecal samples at these ages was determined by reduced metagenome sequencing (RMS). Faecal SCFA composition was determined by gas chromatography. Blood samples were collected from the infants at 12 months of age, and immune cell frequencies were determined by mass cytometry (CyTOF). An outline of the analytical strategy is presented in Figure 1.

2.2 | Reduced metagenome sequencing and inferred metabolic potential

We included the first available participants with faecal samples available from all timepoints (at birth, 3, 6 and 12 months) as well as available immune cell profiling data. After receiving the faecal samples, they were directly put in −20°C freezers, and within a week stored at −80°C until further analysis (Appendix S1). We performed fragment generation, processing and sequencing of the RMS library as previously described.[21] In short, the DNA was cut by MseI and EcorI, and adapters for amplification were ligated to the fragments. The fragments were amplified and quantified, and thereafter sequenced on the Illumina HiSeq 3000 by the Norwegian Sequencing Center (NSC) in Oslo, Norway. The fragments were annotated taxonomically using the Kraken2 taxonomic classification system with the HumGut database HumGut_05.[22] All samples with higher fragment count than the highest negative control were included. The number of sequenced fragments from each species was corrected and normalized as previously described.[21] The taxonomic composition at species level was matched to the VMH database as previously described,[21] but in this study, we inferred metabolites instead of carbon sources to predict bacterial fermentation products.

2.3 | SCFA analysis by gas chromatography

Faecal samples were processed and analysed as described previously.[12] In short, faecal aliquots were mixed with an internal standard, centrifuged and thereafter filtered with a 0.2 μM filter before being applied to the gas chromatograph.

2.4 | Whole blood immune cell populations determined by mass cytometry

Within 20–30 min after collection of EDTA blood, 150-μL infant blood was mixed with 'Stabilizer' component of whole blood processing kit; Cells were fixed, and RBCs lysed using Wash #1 and #2 buffers (Whole blood processing kit; Cytodelics AB, Sweden) as per the manufacturer's recommendations. Post-fix/lysis of cells, ~1×10^6 cells/sample were plated onto a 96-well round bottom plate using standard cryoprotective solution (10% DMSO and 90% FBS) and cryopreserved at −80°C. Whole blood analysis was performed at the Karolinska Institutet, Sweden, as mentioned below.

On the day of barcoding and staining of cells, cells were thawed at 37°C using RPMI medium supplemented with 10% foetal bovine serum (FBS), 1% penicillin-streptomycin and benzonase (Sigma-Aldrich, Sweden). Briefly, cells were barcoded using an automated liquid handling robotic system (Agilent Technologies, Santa Clara, CA, USA)[23] using the Cell-ID 20-plex Barcoding kit (Standard Biotools) as per the manufacturer's recommendations.

Following cell pooling batch-wise, cells were washed and then FcR blocked using blocking buffer (Cytodelics,

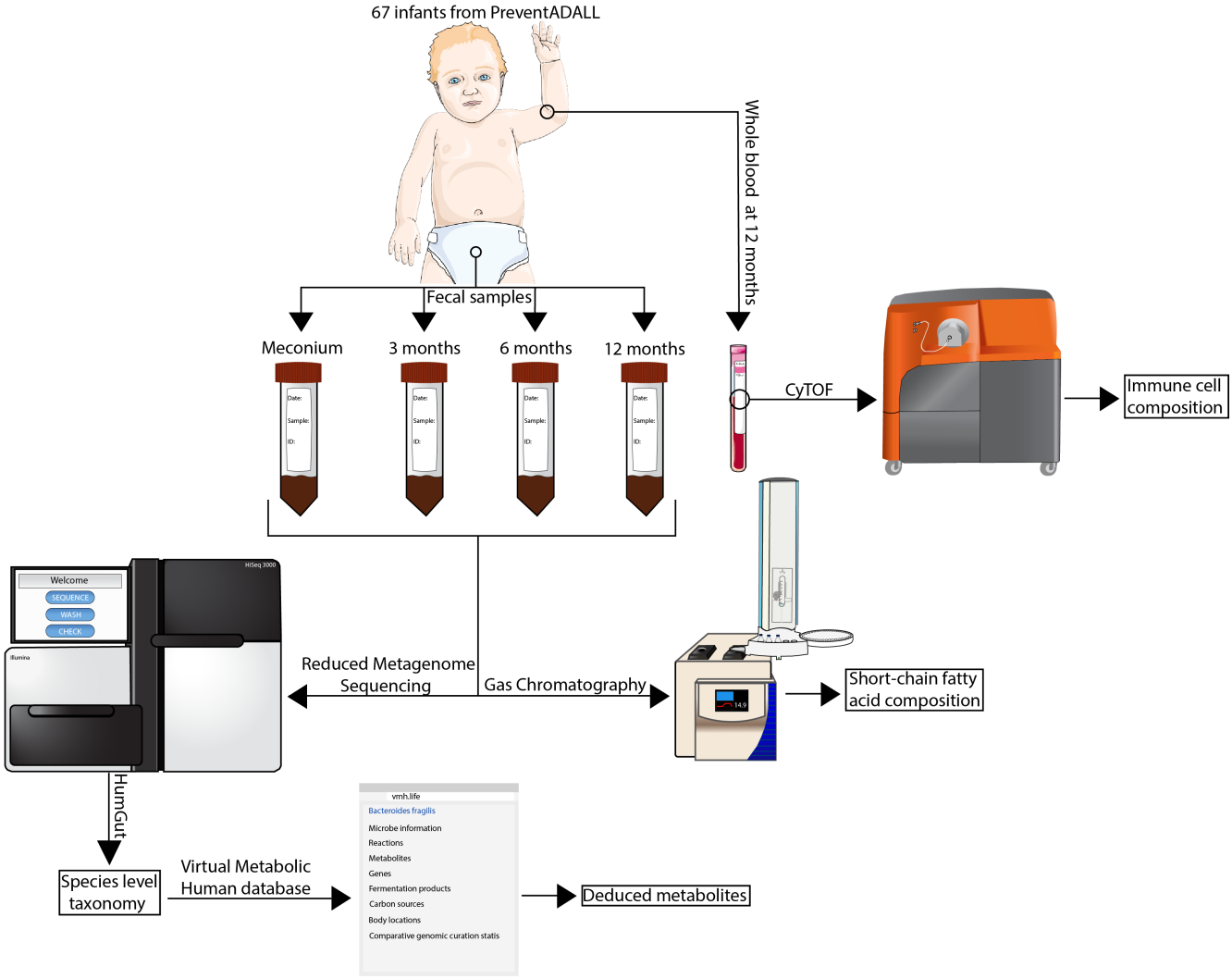


FIGURE 1 The analytical strategy of the current study. Faecal samples from infants were sampled at 4 timepoints during the first year of life (at birth, 3, 6 and 12 months of age) and blood was sampled from infants at 12 months of age. Detailed immune cell frequencies in whole blood were determined by mass cytometry, while the faecal samples were used to determine gut bacterial composition and taxa by reduced metagenome sequencing (RMS) and short-chain fatty acids (SCFA) composition using gas chromatography. We deduced potential metabolites by mapping taxonomy from RMS to the Virtual Metabolic Human database (VMH, www.vmh.life). Figure items of the infant and blood tube are edited from Servier Medical Art (SMART, smart.servier.com).

AB, Sweden) for 10 min at room temperature. Cells were incubated for another 30 min at 4°C after the addition of a cocktail of metal-conjugated antibodies targeting the surface antigens. Cells were washed twice with cell staining buffer (CSB) and then fixed overnight using 2% formaldehyde made in PBS (VWR, Sweden). For acquisition by CyTOF (within 2 days after staining), cells were stained with DNA intercalator (0.125 μM Iridium-191/193 or MaxPar® Intercalator-Ir, Standard Biotools) in 2% formaldehyde made in PBS for 20 min at room temperature. After two washes with CSB, PBS and milli-Q water, cells were filtered through a 35-μm nylon mesh and diluted to 750,000 cells/mL. Cells were acquired at a rate of 300-500 cells/s using a super sampler (Victorian Airship, USA) connected to a CyTOF2 (Standard Biotools) mass cytometer, CyTOF software version 6.0.626 with noise reduction, a lower convolution threshold of 200, event length limits of 10–150 pushes and a sigma value of 3 and flow rate of 0.045 mL/min. Based on single cell expression of the 38 surface markers, automated assignment of cells into the 28 cell subpopulations was performed according to Chen et al.[24]

The antibody panel used is shown in Table S2. Purified antibodies for mass cytometry were obtained in carrier/protein-free buffer and then coupled to lanthanide metals using the MaxPar antibody conjugation kit (Standard Biotools) as per the manufacturer's recommendations. Following the protein concentration determination by measurement of absorbance at 280 nm on a nanodrop, the metal-labelled antibodies were diluted in Candour

PBS Antibody Stabilization solution (Candor Bioscience, Germany) for long-term storage at 4°C.

2.5 | Data analysis

2.5.1 | K-means clustering

To identify groups of participants with similar immune cell profiles at 12 months, we performed a non-hierarchical clustering. We identified the number of clusters based on using gap statistics, the silhouette method, and with a particular focus on the elbow method of the frequencies of the 28 immune cell subsets. The aim of the elbow method is to get the lowest value of k, with a low sum of squared error (SSE). This was found by plotting the SSE with an increasing k and observe where the decrease of SSE starts plateauing.[25] The gap statistic was proposed for clustering algorithms, such as k-means clustering, where it compares the within-cluster variation to a null reference distribution of the data.[26] The best k based on this method is the lowest k that maximizes the gap. The last method, silhouette, computes how well a data point object lies within the cluster. A high average silhouette of the data points within the cluster is the optimal number of k.[27] Based on this, we performed a k-means clustering of the individuals based on immune cell subpopulation compositions with $k=4$, hereafter referred to as "clusters" (Figure S2). These methods were performed in the RStudio environment v. 4.0.3 using the fvbiz_nbclust and kmeans functions from the factoextra-package v. 1.0.7.[28]

2.5.2 | Multi-level pattern analysis

We analysed the four clusters of participants described above for differences in immune cell frequencies, relative abundances of bacterial species and measured and deduced metabolite levels. In order to shed some light on how the bacterial composition is associated with the clusters, we employed an analysis from ecology. This method is used to identify indicator taxa, i.e., taxa that are typically associated with different environments. We used methods implemented in the R package indicspecies v. 1.7.9[29] to search for bacterial taxa associated with the clusters. We used the multipatt function on all the infants for all age categories, with the "r.g" function that calculates a group-equalized point biserial correlation. The infants were used as 'sites' while k-means clustering groups were used as site groups. Cluster 1 was defined by the immune cell composition of 10 infants, cluster 2 by 21 infants, cluster 3 by 16 infants and cluster 4 by 20 infants. Details regarding the number of infants with sequences of sufficient quality within each cluster for each age category are found in Appendix S1.

3 | RESULTS

3.1 | Taxonomic and SCFA composition

Bifidobacterium was the most abundant genus at 3 and 6 months of age, representing 55% at 3 months of age and declining to 15% at 12 months (Figure 2A). One of the abundant genera in newborns was *Bacteroides*, representing 24% on average just after delivery. However, the relative abundance of *Bacteroides* declined to 7% at 3 months of age, while gradually increasing to 24% again at 12 months. From 6 months to 12 months, there was a shift from a *Bifidobacteria*-dominated gut, to a gut comprised mostly of *Bacteroides, Faecalibacterium*, *Clostridium*, *Sutterella*, *Prevotella* and *Blautia*, in addition to *Bifidobacteria*.

In general, acetate had the highest relative abundance of the SCFAs for all age categories and decreased with increasing age. The average relative abundance of faecal acetate was 84% in newborns, 92% at 3 months, 81% at 6 months and 66.5% at 12 months (Figure 2B). Propionate decreased from 13.8% newborns to 3.7% at 3 months, and thereafter increased in relative abundance to 9.5% at 6 months and to 11.2% at 12 months. Butyrate increased in relative abundance among all age categories, representing 0.8% in newborns, 2.3% at 3 months, 7.2% at 6 months and 18.9% at 12 months.

3.2 | Immune cell abundancy profiles at 12 months of age

As expected, the immune cell type with the highest relative abundance at 12 months of age was neutrophils, constituting approximately 31% of all immune cells in the blood (Figure S1). Following neutrophils, the naïve CD4T and the naïve B cells were present at 21% and 13% respectively. The naïve and memory Tregs had a relative abundance of 1.6% and 0.5% respectively (Figure 3C). Neutrophils were negatively correlated with several immune cells in infants at 12 months of age, while naïve CD4T and naïve Tregs were positively correlated with each other, as shown in Figure 3A ($p<0.05$, Spearman correlation, p-value adjusted with the Holm's method).

We observed a separation of infants into four clusters based on immune cell composition at 12 months of age (Figure S2). Of the 28 immune cell subsets determined, 13 were identified as indicators for one or several of these four clusters (multi-level pattern analysis, $p<0.05$), as

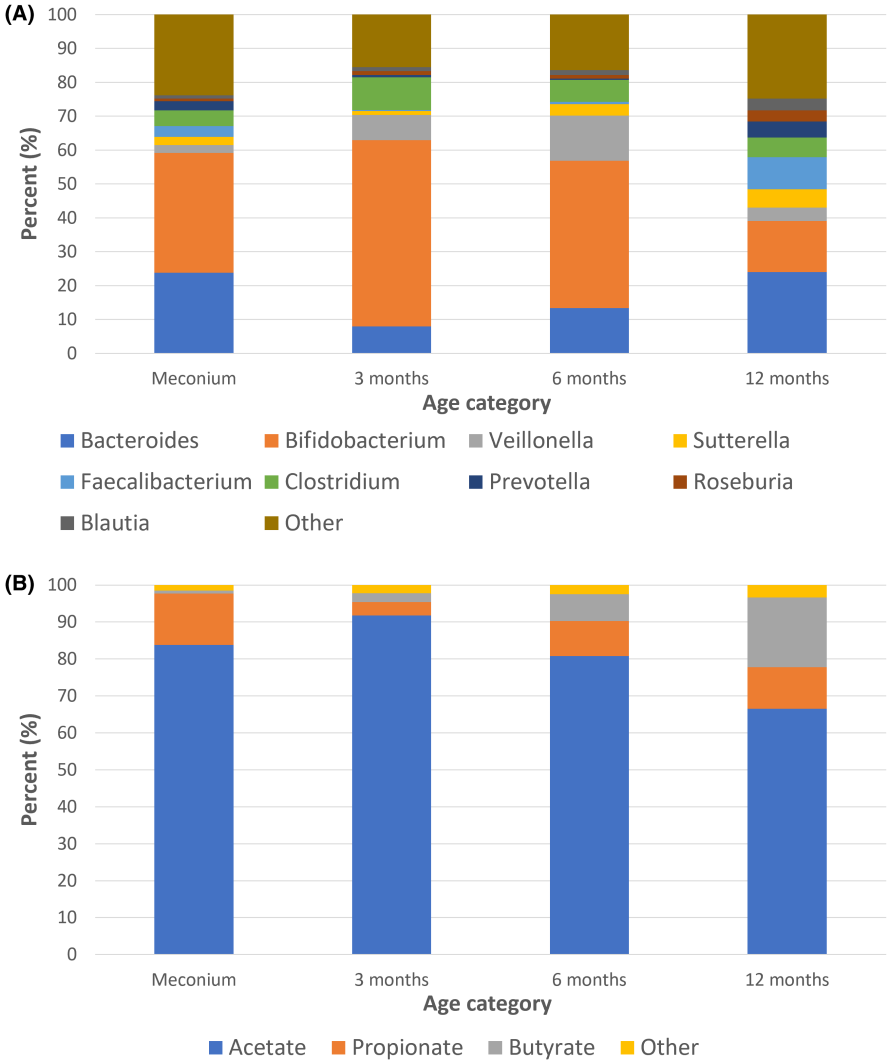


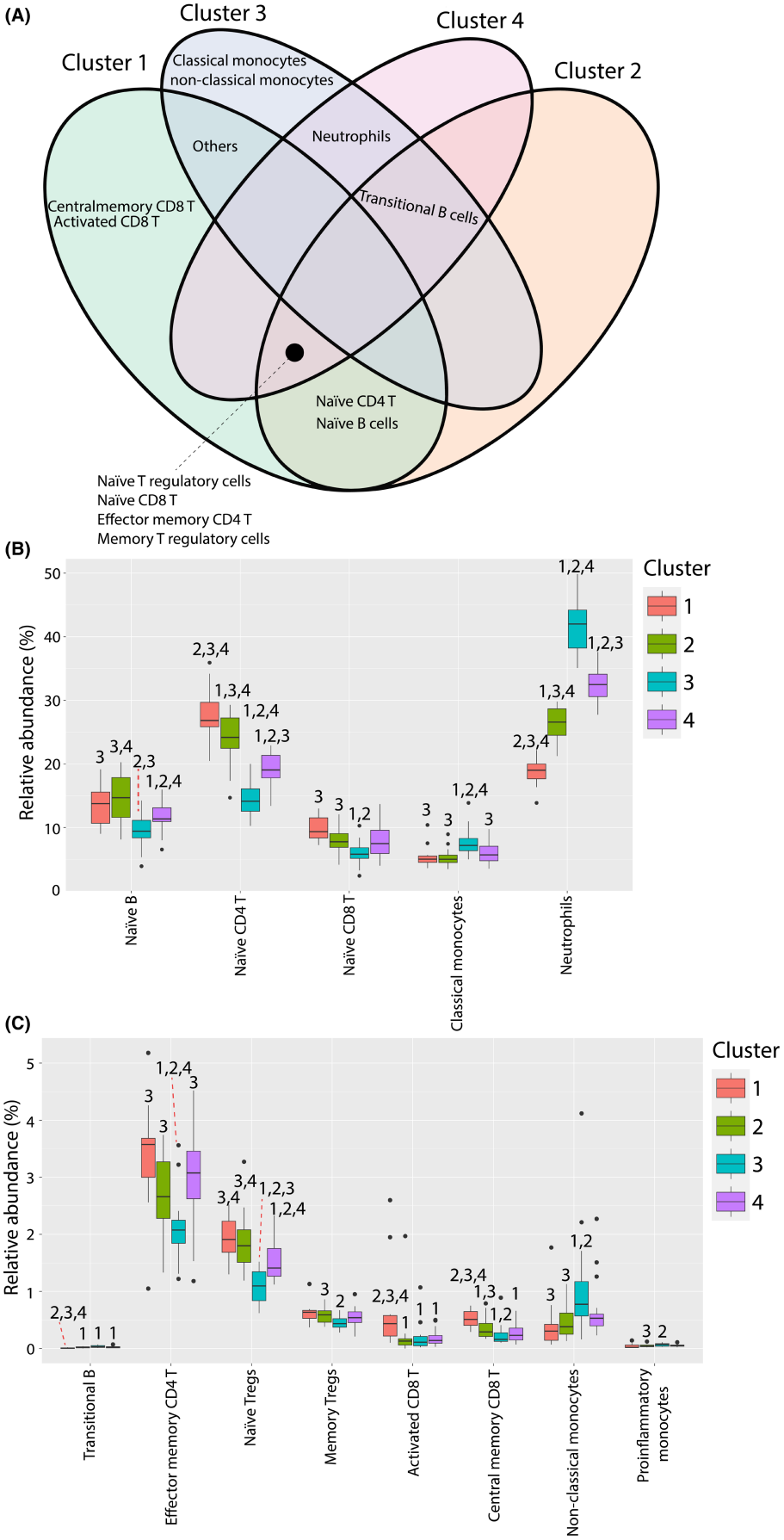
FIGURE 2 Faecal bacterial composition at genus level and SCFA ratios for the different age categories of investigated infants. The bar chart represents the average percentages of the different genera (A) and SCFA (B) for the age categories. The 'Other' group for taxonomy includes low abundant genera (<1% within all age categories), while for SCFAs it includes isobutyric, isovaleric and valeric acids.

shown in Figure 3A. In general, clusters 1 and 2 had a significantly higher frequency of naïve cells (naïve B-cells, naïve CD4T and naïve T-regulatory cells, Wilcoxon rank-sum test, $p < 0.05$, Figure 3B,C) compared to clusters 3 and 4. Additionally, clusters 1 and 2 had a significantly higher frequency of naïve CD8T and central memory CD8T, compared to cluster 3 (Wilcoxon rank-sum test, $p < 0.05$). Clusters 3 and 4 were observed to have a significantly higher frequency of neutrophils (Wilcoxon rank-sum test, $p < 0.05$), while cluster 3 had a significantly higher abundance of classical- and non-classical monocytes (Wilcoxon rank-sum test, $p < 0.05$) compared to the other three clusters.

3.3 | Correlations between immune cells at 12 months of age and bacterial composition at different age categories

To explore bacterial predictors, we analysed the bacterial exposure in infants sampled at birth, 3, 6 and 12 months and their associations with clusters defined by immune cell populations at 12 months of age. We observed 39 faecal taxa from newborns that were associated with one or more of the clusters, 32 taxa at 3 months, 104 at 6 months and 25 taxa at 12 months (Table S1). Seven taxa were associated with the clusters and overlapped between the age categories. *Bacteroides* sp. *CAG:98* and *Clostridiales*

FIGURE 3 Immune cell indicators and significant differences. The figure illustrates a Venn diagram (A) of the indicator immune cells (multi-level pattern analysis, $p < 0.05$) for the four different clusters derived by k-means clustering. Additionally, the relative abundance of immune cells with significant differences between the clusters are illustrated in (B) and (C) ($p < 0.05$, Kruskal-Wallis & $p < 0.05$ pairwise Wilcoxon test with *p*-adjusted for using Benjamini-Hochberg method). (B) illustrates immune cells with an average relative abundance >5% within a given cluster, while (C) represents immune cells with a relative abundance <5% for all the clusters. The relative abundance (*y*-axis) of the immune cells (*x*-axis) for each cluster (coloured) is shown. Significant differences are noted against the clusters, ex. 1 represents that the relative abundance of the immune cell for a given cluster is significantly different from cluster 1.



bacterium VE202.26 were indicator species for cluster 1 in newborns (meconium) and at 6 months of age, and *an uncultured succinatimonas* in cluster 3. *Clostridiales bacterium UBA1390*, *Muribaculaceae bacterium isolate 001 NCI* and *Ruminococcaceae bacterium UBA6392* overlapped between 3 and 6 months of age in cluster 1. *Bifidobacterium adolescentis* overlapped between 6 and 12 months of age to cluster 1.

Of all the indicator bacteria, only four bacteria were abundant (>8% average relative abundance) indicator species. These four were all abundant indicator species at 6 months of age. These were annotated as *Bifidobacterium adolescentis*, and an uncultured *Porphyromonadaceae* bacterium that was associated with cluster 1, with a mean relative abundance of 9.4% and 10.1% respectively. *Bacteroides fragilis* and *Bifidobacterium longum* are associated with clusters 3 and 4 with mean abundances of 8.3% and 25.5% respectively (Figure 4A,C). While all these four were deemed as indicator species, none were significantly different between the clusters (Wilcoxon rank-sum test, $p > 0.05$), but all followed a trend of having a higher mean relative abundance within their clusters (Figure 4C). There were also some significant differences in low abundant indicator species between the groups (>5%, Figure 4D).

Moreover, to assess if there were any direct correlations between the indicator species and immune cells, a Spearman correlation analysis was performed. From cluster 1, *B. adolescentis* were significantly negatively correlated to plasmablasts, while CD151 negative γδT were negatively correlated to *Porphyromonadaceae*, but were positively correlated to CD 56 dim NK cells and activated CD8T (Figure 4B). While not a significant correlation (Spearman, with *p*-adjusted for using Benjamini-Hochberg >0.05), *B. adolescentis* did show a negative correlation to naïve CD4T and naïve T regulatory cells, while *Porphyromonadaceae* were negatively correlated to naïve B and naïve CD4 T cells, and both trending towards a negative correlation to neutrophils.

For *B. fragilis*, we observed a significant positive correlation to central memory CD4T and MAIT cells, and a significant negative correlation to CD161 negative γδT cells, CD4+CD8+ double positive cells, naïve B-cells and IgD-positive memory B cells (Spearman, with *p*-adjusted for using Benjamini-Hochberg <0.05). While for *B. longum*, we observed negative correlations to naïve CD4T and Naïve T regulatory cells, and positive correlations to transitional B cells, non-classical monocytes and plasmacytoid dendritic cells (Spearman, with *p*-adjusted for using Benjamini-Hochberg, $p < 0.05$). Generally, the direct correlations between bacteria at 6 months and individual immune cell frequencies at 12 months were weak.

Of the predicted bacterial fermentation products retrieved from the VMH database, we observed that the bacterial composition associated with cluster 1 at 6 months had the potential to produce a higher abundance of methane (CH_4) and valeric acid (M03134) compared to the bacterial composition in infants with bacteria associated with the other clusters (multi-level pattern analysis, $p < 0.05$). However, we did not observe any faecal SCFAs at any given age category that were directly associated with the clusters at 12 months ($p > 0.05$, Kruskal-Wallis rank sum test).

3.4 | Immune cell enumeration and atopic dermatitis outcome by 12 months of age

To explore potential links between atopic dermatitis (AD) and immune cells, we analysed if there were any differences in immune cell composition at 12 months between infants who had AD at any given timepoint by 12 months of age. Between infants with documented AD ($n = 9$) and no AD ($n = 58$), we did not observe any statistical differences in the immune cell composition (Wilcoxon test, FDR corrected with Benjamini-Hochberg, $p > 0.05$) or immune cell clusters associated with AD at 12 months (Chi-square test, $p < 0.05$, Appendix S1). Moreover, delivery mode, early food intervention, or breastmilk feeding at 6 months could not explain the separation of the infants to the four immune cell groups (Chi-square test, $p > 0.05$, Appendix S1).

4 | DISCUSSION

We identified four distinct immune cell composition-based clusters in infants at 12 months of age and found associations with their gut bacterial compositions at different age categories (birth, 3, 6 and 12 months of age). Only at 6 months, we could find associations for abundant species (8% and higher), including associations between *Bifidobacterium adolescentis*, an uncultured *Porphyromonadaceae*, *Bifidobacterium longum* and *Bacteroides fragilis* with immune cell profiles at 12 months of age, and some direct correlations between bacteria and immune cell subsets.

The observation that *Bifidobacterium adolescentis* and the uncultured *Porphyromonadaceae* were associated with a cluster characterized by a higher relative abundance of naïve immune cells (naïve CD4T, naïve CD8T, naïve B cells and naïve Tregs) and trended towards a positive correlation (although, a non-significant one) is as far as we know novel. *Bifidobacterium adolescentis* is of particular interest, as this bacterium is commonly observed as a member of the adult gut microbiota, while its presence in infants has earlier been linked to allergic

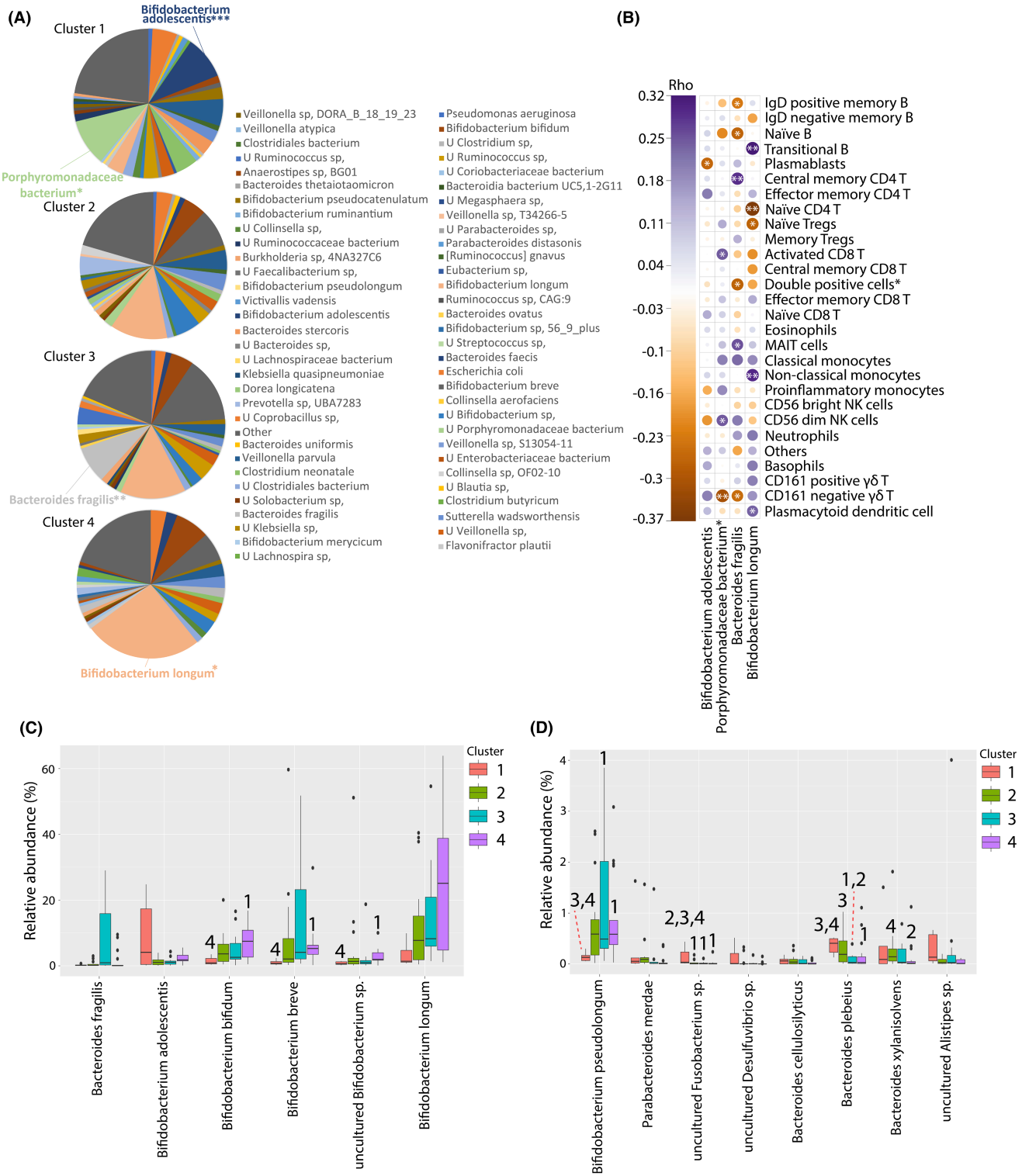


FIGURE 4 Indicator bacteria, significant bacteria and their correlations. The figure illustrates the (A) relative abundance (<0.05%) of bacteria at 6 months of age, within each cluster. Abundant indicator bacteria (>8%) that are associated with the immune cell subpopulation clusters are marked with an asterisk (*$p < 0.05$, **$p < 0.01$, ***$p < 0.005$), which was determined by multi-level pattern analysis. The correlation (Spearman's rho) between the indicator species and specific immune cells is illustrated in (B) (Spearman's rank correlation coefficient). Significant bacteria between each cluster ($p < 0.1$ Kruskal-Wallis and $p < 0.05$ pairwise Wilcoxon-rank test, p-value is adjusted for using Benjamini-Hochberg) are illustrated in (C) and (D) for bacteria >0.2% in relative abundance. Bacteria with a relative abundance >5% are represented in (C) and <5% in (D). Significant differences are noted against the clusters, ex. 1 represents that the relative abundance of the bacteria within a given cluster is significantly different from cluster 1. 'U' in front of the bacterial taxonomy represents 'uncultured'. In (B), *Double positive cells represent CD4+CD8+ cells.

disease development.[30] A higher abundance of naïve immune cells may indicate a less mature immune system. Interestingly, in animal models, both *B. adolescentis* and *Porphyromonadaceae* have been associated with increased induction of Th17 cells.[31–35] The Th17 cells represent a unique subset of the CD4+ T-cells, characterized by their IL-17 production[36] and may play a role in allergic and autoimmune diseases.[37] We speculate that the presence of *B. adolescentis* and *Porphyromonadaceae* observed at 6 months of age may contribute to a delayed/decreased maturation of immune cells (as indicated by our results) and skewing of Th-cell polarization to Th17 cells, instead of what is conceived as a healthy Th1/Th2/Th17 balance. The small blood volume drawn and our antibody panel, however, did not allow identification of these Th subsets.

Our novel finding that the high relative abundance of *Bifidobacterium longum* associated with transitional B cells, and negatively correlated to naïve CD4T, and naïve Tregs, may indicate that infants with a higher relative abundance of *B. longum* have a more matured immune cell composition compared to infants in the other immune cell profile groups. Indications that *B. longum*, subspecies *infantis* may affect T-cell development are supported by previous reports of *B. longum* subspecies *infantis* silencing Th2 cells and skewing Th-cell polarization towards Th1 in infants.[1] Such development pattern during the first year of life is beneficial due to the Th2 skewing at birth.[5]

Bacteroides fragilis at 6 months of age had a non-significant positive correlation to classical monocytes and neutrophils, and a significantly positive correlation to central memory CD4T and MAIT cells. These associations may suggest that *B. fragilis* may stimulate recruitment of the innate immune cells. *B. fragilis* can promote CD4+T cell-dependent immune responses as the bacterium also helps with induction of IL-10 secretion from CD4+T cells.[38] *B. fragilis* produces zwitterionic polysaccharides (ZPS) which include polysaccharide A (PSA) that can contribute to establishing a healthy Th1/Th2 balance in early life by decreasing IL-4 secretion from Th2 T cells.[39] Moreover, as previous studies have reported that the production of vitamins from *B. fragilis* may activate MAIT cells,[40,41] this may explain their positive association with MAIT cells observed in our study. However, the specific interactions between *B. fragilis* and immune cell subpopulations remain unclear. While *B. fragilis* is mostly a commensal bacterium in the human gut, their lipopolysaccharide (LPS) generation and *B. fragilis* toxin (fragilysin) production may cause bacteraemia.[42]

The strengths of the present study are the multitude of variables connected to the infants, the combination of metagenomics, selected metabolomics and identification of high-dimensional immune profiling on a single cell level subpopulation from the infants. These contribute to a more comprehensive understanding of how microbial factors may influence the infant's immune system. However, it is important to acknowledge the limitations of this study. Firstly, the small population size restricts the generalizability of the findings to a larger population. The limited sample size may affect the statistical power of the study and may not capture the full spectrum of potential outcomes. Moreover, the study's focus on immune cell subsets at a single time point does exclude an important part, which is the longitudinal development of immune cell composition over time. Future studies with a larger sample size, and longitudinal designs would provide a more comprehensive understanding of the link between the temporal gut microbiota development and immune system development early in life. Moreover, investigating the potential of the identified microbial species using metagenomic and metatranscriptomic approaches could shed light on specific mechanisms by which they influence immune cell development and function. Integration of multi-omics data, including metabolomics, transcriptomics, single-cell proteomics and cytokines, would provide a more holistic view of the gut microbiome-immune system interplay. Overall, while this study offers valuable insights into the interplay between gut bacteria and the immune system of infants, its limitations emphasize the need for further research to validate and expand upon our findings.

In conclusion, we found associations between *B. adolescentis*, an uncultured *Porphyromonadaceae*, *B. longum* and *B. fragilis* at 6 months of infant age and immune cell profiles at 12 months of age. Although the associations detected in our work were weak, they support the notion that the early gut microbiota composition may affect immune cell development during the first year of life.

AUTHOR CONTRIBUTIONS

Conceptualization of the project was done by MN, UCN and KR; Methodology: MN, KR, UN, PB, AO, TL and LS; Laboratory work: UJ, MN, CF, PB, AO and TL; Software for analysis: PB, AO, TL, LS, MN and KR; Validation: KC, LS, KR and UN; investigation: CF, UJ, MN, UCN and KR; data curation: RV, PB, AO, TL and UCN; writing-original draft preparation: MN and UN; writing-review and editing: All. Visualization: MN, LS and KR; Supervision: KC, LS and KR; project administration: KCLC, GH, CJ, BN, HS, ER and KR. All authors have read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The raw sequencing data and relative abundances of immune cells are available upon request in TSD UiO (University of Oslo).

ORCID

Morten Nilsen https://orcid.org/0000-0002-9498-1262
Unni Cecilie Nygaard https://orcid.org/0000-0002-2955-9970

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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