



Differences in Systemic IgA Reactivity and Circulating Th Subsets in Healthy Volunteers With Specific Microbiota Enterotypes

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Changes in the intestinal microbiota have been associated with the development of immune-mediated diseases in humans. Additionally, the introduction of defined bacterial species into the mouse intestinal microbiota has been shown to impact on the adaptive immune response. However, how much impact the intestinal microbiota composition actually has on regulating adaptive immunity remains poorly understood. Therefore, we studied aspects of the adaptive immunity in healthy adults possessing distinct intestinal microbiota profiles. The intestinal microbiota composition was determined via Illumina sequencing of bacterial 16S rRNA genes extracted from the feces of 35 individuals. Blood B-cell and T-cell subsets from the same individuals were studied using flow cytometry. Finally, the binding of fecal and plasma Immunoglobulin A (IgA) to intestinal bacteria (associated with health and disease) *Bacteroides fragilis*, *Prevotella copri*, *Bifidobacterium longum*, *Clostridium difficile*, and *Escherichia coli* was analyzed using ELISA. Unsupervised clustering of microbiota composition revealed the presence of three clusters within the cohort. Cluster 1 and 2 were similar to previously-described enterotypes with a predominance of *Bacteroides* in Cluster 1 and *Prevotella* in Cluster 2. The bacterial diversity (Shannon index) and bacterial richness of Cluster 3 was significantly higher than observed in Clusters 1 and 2, with the *Ruminococaceae* tending to predominate. Within circulating B- and T-cell subsets, only Th subsets were significantly different between groups of distinct intestinal microbiota. Individuals of Cluster 3 have significantly fewer Th17 and Th22 circulating cells, while Th17.1 cell numbers were increased in individuals of Cluster 1. IgA reactivity to intestinal bacteria was higher in plasma than feces, and individuals of Cluster 1 had significant higher plasma IgA reactivity against *B. longum* than individuals of Cluster 2. In conclusion, we identified three distinct fecal microbiota clusters, of which two clusters resembled previously-described “enterotypes”. Global T-cell and B-cell immunity seemed unaffected, however, circulating Th subsets and plasma IgA reactivity were significantly different between Clusters. Hence, the impact of intestinal bacteria composition on human B cells, T cells and IgA reactivity appears limited in genetically-diverse and environmentally-exposed humans, but can skew antibody reactivity and Th cell subsets.

Keywords: intestinal microbiota, enterotypes, IgA+ B cells, $\gamma\delta$ T cells, IgA reactivity, helper-T cells, 16S sequencing

INTRODUCTION

All mucosal surfaces of the human body are colonized by microorganisms — termed the human “microbiota” (1). The number and composition of the microbiota (microbiota profile) varies greatly dependent on the microbial location on/in the human host, with variation also being observed at identical locations within different hosts (2). The total number of bacteria on/in the human host is estimated at the same order of magnitude as the total number of eukaryotic cells in the human body ($\sim 3.10^{13}$) (3), with the largest microbial load being found in the intestinal tract, where more than 1,000 bacterial species may be found (4). The vast majority of intestinal bacteria appear to be commensals, providing the host with otherwise inaccessible nutrients, whilst in addition, preventing colonization and translocation of pathogens within the host (1). The challenge therefore, is for the host to maintain immunological tolerance to its resident microbiota without activating inflammatory processes (5).

In the gut, immunological tolerance is thought to be maintained via for example, the prevention of bacterial translocation through the protective intestinal epithelial layer via the production of mucus, antibacterial peptides and secretory immunoglobulin A (IgA) (6). IgA is an immunoglobulin that is “specialized in mucosal protection” (7) and the most abundant antibody produced and secreted in the human body, mainly in the intestine (8). The human *IGH* locus contains two IgA subclasses, with IgA2 being more resistant to the action of secreted (neutralizing) bacterial proteases, as it has a shorter “hinge region” than IgA1 (9). IgA secreting plasma cells can be generated upon terminal differentiation of activated B cells, within organized intestinal lymphoid structures with cognate T-cell help (T-dependent; TD). Alternatively, B cells can mature into IgA secreting plasma cells following T-cell independent (TI) activation in the lamina propria (10, 11). Both pathways also generate IgA memory B cells with TD-derived B cells expressing CD27 and the TI derived B-cells being CD27-IgA+ (12). Once secreted, transcytosis of IgA across the gut epithelium transports it to the lumen of the intestine, where it is able to bind to bacteria (6). The majority of antibodies produced in the intestine are antigen-specific (13), with those derived from TI responses showing a high degree of polyreactivity (14). As is evident from studies in mice raised in sterile conditions (germ free; GF), the intestinal microbiota can shape both immunological tolerance and systemic immunity, resulting in lower IgA levels, fewer CD4+ and CD8+ T cells and fewer organized lymphoid structures (Peyer’s patches) in the intestines, as well as fewer germinal centers in the spleens of GF mice (15).

High-throughput sequencing of the 16S rRNA gene of bacteria has greatly facilitated research into the inter-individual and inter-location diversity of the human microbiota (16). Inter-individual differences in the microbiota appear to be influenced by host genotype and environmental factors such as diet or antibiotic use (17–20), with alterations in the intestinal microbiota having been linked to various immunological diseases such as atopic disorders, inflammatory bowel disease (IBD), arthritis, type 1 diabetes and multiple sclerosis (MS) (21–25). Nevertheless, the

existence of a “core” human microbiota has been reported, with individuals being clustered based on the composition/profile of their microbiota. With respect to the intestinal microbiota, three clusters (“Enterotypes”), have been observed, dependent on the relative predominance of the bacterial genera *Bacteroides*, *Prevotella*, and *Ruminococcus* (26).

Although the great majority of published microbiota studies have only described various correlations between specific microbiota profiles and disease, experimental studies using mouse models have demonstrated that changes in the intestinal microbiota can actually affect adaptive immune responses. Specifically, the introduction of specific pathogen free (SPF) bacteria into germfree (GF) mice has been shown to result in lower concentrations of interleukin 4 (IL-4), IL-5 and eosinophil numbers in an OVA-induced allergy model (27). In addition, administration of probiotic bacteria in these mice reduced serum IgE levels and Th2 related cytokines, whilst increasing FoxP3 expression in CD4+ T cells in the mesenteric lymph node (28). Other researchers showed that colonization with segmented filamentous bacteria (SFB) induced the differentiation of T helper 17 (Th17) cells and regulatory T cells (Treg) (29, 30).

However, these observations were made in inbred animal models under highly controlled conditions. Therefore, it remains unclear how well these findings translate to actual microbiota-immune interactions in humans (31, 32). To gain more insight into the relationship between the composition of the intestinal microbiota and associated human immune responses, we studied the local and systemic adaptive immune response of healthy adult volunteers harboring distinct intestinal microbiota profiles.

METHODS

Human Subjects

Blood and feces samples of 35 adult individuals were collected after written informed consent was obtained (Table 1). All individuals completed a questionnaire to gather information about Body Mass Index (BMI), diet, habit, intake of antibiotics, and lifestyle. Individuals with gastrointestinal, auto-immune and intake of antibiotics in the last 6 months were excluded from the study. Fecal samples were either frozen immediately at -80°C or stored in the fridge overnight before transporting it to the laboratory. This study was performed in accordance with the Declaration of Helsinki and the guidelines of the Medical Ethics Committees of the Erasmus MC.

Intestinal Microbiota Sequencing and Analysis

Bacterial genomic DNA was isolated from approximately 100 mg feces using the NorDiag-Arrow DNA extraction kit (Autogen, MA, United States). Next, the V3-V4 region of the 16S rRNA gene was amplified by PCR (Qiagen, Hilden, Germany) using the following forward: 5'-ACTCCTACGGGAGGCAGCAG-3' and reverse: 5'-ACTACHVGGGTWTCTAAT-3' primers. Subsequently, the amplicon (460 bp) was normalized using the SequalPrep Normalization kit (Thermo Fisher Scientific, Waltham, MA) and sequenced using the Illumina MiSeq platform (2X 300PE, v3) (Illumina, San Diego, CA).

TABLE 1 | Demographics of participants per cluster.

Cluster (n)	Cluster 1 (n = 7)	Cluster 2 (n = 8)	Cluster 3 (n = 20)
Age in yrs (median; range)	21 (18–35)**	26 (20–46)*	30 (20–38)
Male Gender (n; %)	6 (86.2%)	6 (75.2%)	8 (40.0%)
Allergic (n; %)	5 (71.4%)	3 (37.6%)	10 (50.0%)
Pets in household (n; %)	3 (42.9%)	2 (25.0%)	7 (35.7%)
Smoking (n; %)	2 (28.6%)	0	1 (5.0%)
Vegetarian (n; %)	1 (14.3%)	0	1 (5.0%)
BMI (median; IQR)	21.04 (19.9–23.5)	22.68 (19.4–23.1)	23.03 (22–24.8)
WBC (median; IQR)	7.1 (4.9–7.5)	7.2 (5.6–10.4)	7.3 (5.7–8.6)
Specific IgE (median; IQR)	4.23 (0.1–23.6)	0.61 (0.17–22.9)	4.60 (0.02–13.5)
CONSUME REGULARLY			
Coffee (n; %)	4 (57.1%)	7 (87.7%)	13 (65.4%)
Probiotics (n; %)	0	0	1 (5.0%)
Alcohol (n; %)	5 (71.4%)	4 (50.0%)	15 (75.2%)

BMI, body mass index in kg/m²; WBC, white blood cell count in million cells/ml; Specific IgE reactivity against most common airborne allergens, >0.35 is considered positive; IQR, Interquartile range; yrs, years; consume regularly is defined as at least once a week. **Cluster 3 vs. Cluster 1 and *Cluster 3 vs. Cluster 2; significant difference calculated with Mann-Whitney U-test. *P < 0.05; **P < 0.01.

16S profiles were generated using an in-house developed pipeline. Briefly, singleton reads were discarded. Subsequently, to normalize for differences in number of reads per sample, subsampling to 19,000 reads per sample was performed. Similar 16S rRNA gene amplicon sequences with a minimum cluster identity of 97% were clustered in operational taxonomic units (OTUs) using UPARSE (USEARCH, v8) (33). OTUs were then aligned to the SILVA 16S rRNA reference gene database using RDP classifier v2.2 with a confidence threshold of 0.90 (34, 35). In a quality control step OTUs with an abundance of <0.005% were deleted. This Targeted Locus Study project (BioProject PRJNA515953) has been deposited at DDBJ/EMBL/GenBank under the accession KCRL00000000. The version described in this paper is the first version, KCRL01000000. Data analysis was performed using R studio v3.4.1, Vegan v2.4-5 and Phyloseq v1.22.3 package. Samples were clustered by unsupervised hierarchical clustering using Bray-Curtis distance calculations. The robustness of Clusters were assessed by silhouette validation (36).

Blood Sampling and Flow Cytometric Immunophenotyping

Peripheral blood (9 ml) was obtained from all 35 donors. Samples were centrifuged for 10 min at 820×g to obtain plasma, which was stored at –80°C for later use.

Absolute counts of blood CD3⁺ T cells, CD16⁺/56⁺ NK cells, and CD19⁺ B-cells were obtained with a diagnostic lyse-no-wash protocol from 50 µl blood (BD Biosciences, San Jose, CA). Following red blood cell lysis of 1–2 ml blood, detailed immunophenotyping of B and T cells was performed by 11-color

flow cytometry using fluorochrome-conjugated antibodies listed in **Table S1**.

The following subsets were defined within total B cells (CD19⁺): naive B cells (CD27–CD38^{dim}IgD⁺), Ig class switched memory B cells (CD27–CD38^{dim}IgD–IgM- and CD27+CD38^{dim}IgD–IgM-) and plasma blasts (CD27+CD38^{hi}). Furthermore, total IgA⁺ class switched memory B cells, as well as IgA1⁺ and IgA2⁺ memory B cells were determined (**Figure S3**). Within total T cells (CD3⁺), CD4⁺, CD8⁺, and γδT cell lineages were defined. Within the CD4⁺ T-cell lineage, Treg cells (CD25+CD127–), follicular T helper (Tfh) cells (CD45RA–CXCR5⁺), Th1 cells (CD45RA–CCR6–CXCR3+CCR4–), Th2 (CD45RA–CCR6–CXCR3–CCR4⁺), Th17.1 (CD45RA–CCR6+CXCR3+CCR4–), Th17 (CD45RA–CCR6+CXCR3–CCR4+CCR10–) and Th22 cells (CD45RA–CCR6+CXCR3–CCR4+CCR10⁺) were identified (**Figure S3**). Data were acquired on an LSRFortessa (BD Biosciences) with standardized instrument settings (37) and analyzed using FACS DIVA software v8 (BD Biosciences).

IgA Isolation From Feces and Plasma Samples

Approximately five grams of feces were collected from all study subjects and frozen at –80°C before use. Feces samples were thawed and homogenized in 25 ml extraction buffer consisting of Phosphate Buffered Saline (PBS; pH 7.4, 0.5% Tween-20, and 0.5% NaAc) and the mixtures were centrifuged for 20 min at 4°C and 1500×g. Two milliliters of supernatant was taken and 20 µl protease inhibitor cocktail (Sigma-Aldrich, Saint Louis, MO) added, with the mixture being centrifuged for 10 min at 10,000×g. The resulting homogenized feces mixtures were stored at –20°C.

IgA isolation from homogenized feces and plasma samples was performed using Mobicol Affinity Chromatography spin columns (35 µm pores; Mobitec, Göttingen, Germany) with high affinity for both the IgA1 and IgA2 subclasses. Following filling of the columns with 100 µl CaptureSelect IgA affinity resin (Thermo Fisher Scientific), either 500 µl homogenized feces or 200 µl plasma was added to the column and incubated for 1 h. After three wash steps using PBS pH 7.4, IgA was eluted with 0.1M glycine pH 3.

Quantification of IgA Concentrations and IgA Reactivity to Bacterial Antigens

IgA concentrations in plasma and feces samples were determined by ELISA using coating flat-bottom 96-well plates overnight with 50 µl of 2 µg/ml Affinipure goat anti-human IgA α-chain (Jackson ImmunoResearch, West Grove, PA) in carbonate buffer. Subsequently, all wells were washed to remove unbound antibody. Fifty microliters of sample or IgA standard (Sigma-Aldrich) was added to each well and incubated for 2 h at room temperature. Bound antibodies were detected using peroxidase-conjugated goat anti-human IgA (Jackson ImmunoResearch) at a concentration of 0.8 µg/ml in PBS pH 7.4 with 1 mM Tris-EDTA and 0.05% Tween-20 and TMB ELISA substrate (ThermoFisher Scientific) according to manufacturer's instructions. Optical

density (OD) was measured at 450 nm. For each plate, a PBS only negative control were used to obtain the background signal, which was subtracted from each sample measurement prior to calculation of the sample concentration using the standard curve in Soft-max Pro software v6.4 (Molecular Devices, Silicon Valley, CA).

To test IgA reactivity, the following bacterial strains were obtained from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures: (1) *Bifidobacterium longum* (20088) - Gram positive - one of the earliest colonizers of the human intestine; (2) *Prevotella copri* (18205) - Gram negative - *Prevotella* enterotype; (3) *Clostridium difficile* (1296) - Gram positive - spore-forming potential human pathogen, associated with diarrhea, and colitis after antibiotic therapy; (4) *Bacteroides fragilis* (2151) - Gram positive - *Bacteroides* enterotype; and (5) *Escherichia coli* - Gram negative - the best understood of all microbiota microorganisms. Axenic culture of bacteria was performed under recommended conditions on CDC blood agar with Kanamycin and Vancomycin (BD Biosciences) for *P. copri* and on blood agar for all other bacteria. 100 μ l bacterial suspension with OD600 of 0.5 was used to coat flat-bottom 96-well plates at 4°C overnight. 25 ng/ml sample IgA, calculated as mentioned above was used. As positive control IgA isolated from human colostrum (Sigma-Aldrich) showed reactivity to the five bacteria tested and was measured at concentrations of 25, 50, and 100 ng/ml on each plate. Absorbance was measured at 450 nm and background signal was subtracted. The absorbance of each sample was expressed relative to that of the positive control (mean OD sample/ mean OD pos ct) for normalization and to enable comparison of results from various samples and those obtained from different ELISA plates.

Statistics

Statistical analyses were performed using the Mann-Whitney U test, χ^2 test or Kruskal-Wallis, as indicated in the Figure legends. $P < 0.05$ were considered statistically significant.

RESULTS

Study Cohort

Thirty-five adult volunteers were included in this study with a median age of 28 years (range 18–46 years). The study cohort consisted of 20 males and 15 females with an average BMI of 22.9. Two participants were vegetarian. Total T-, B-, and NK-cell numbers of all individuals were within the normal range. Detailed information about the study subjects can be found in **Table S2**.

Three Groups of Individuals With Distinct Intestinal Microbiota Profiles

To study intestinal microbiota profiles, we collected feces and sequenced the 16S rRNA gene of extracted DNA. We obtained an average of 44,708 reads (SD = 11,905; range = 19,699–78,703 reads) per sample and identified in total 1,324 OTUs. Subsampling of 19,000 reads per sample yielded a total of 677 OTUs. The predominant phyla in our cohort feces samples were *Bacteroidetes* and *Firmicutes*, in line with previous studies

(2, 38). Within *Bacteroidetes*, *Bacteroidaceae*, and *Prevotellaceae* were the dominant bacterial families, with the most abundant bacterial families within the *Firmicutes* being the *Lachnospiraceae* and *Ruminococcaceae*. Further classification to the genus level revealed 228 distinct genera of which 57% could be assigned to a genus. The remaining 43% were assigned to a higher taxonomic level such as the family, order or class.

The abundance of these 228 genera was used to perform unsupervised hierarchical clustering with complete linkage based on Bray-Curtis dissimilarity distance (**Figure 1A**), and three distinct clusters were determined by calculating the average silhouette (36) (**Figure S1**). The median BMI, White Blood Cell count (WBC), % of males and allergic individuals did not significantly differ between the three clusters. The median age of Cluster 3 is significantly higher than that of Cluster 1 and 2 with $P < 0.01$ and $P < 0.05$, respectively (**Table 1**). Dietary intake, e.g., vegetarian diet, consumption of coffee, probiotics, or alcohol did not differ between the three clusters, nor did other lifestyle and environmental factors, such as smoking or pets in the household. The clustering was not grossly affected by the inclusion of the two vegetarians in the study cohort, as was shown by unsupervised clustering after exclusion of vegetarian individuals (**Figure S2**).

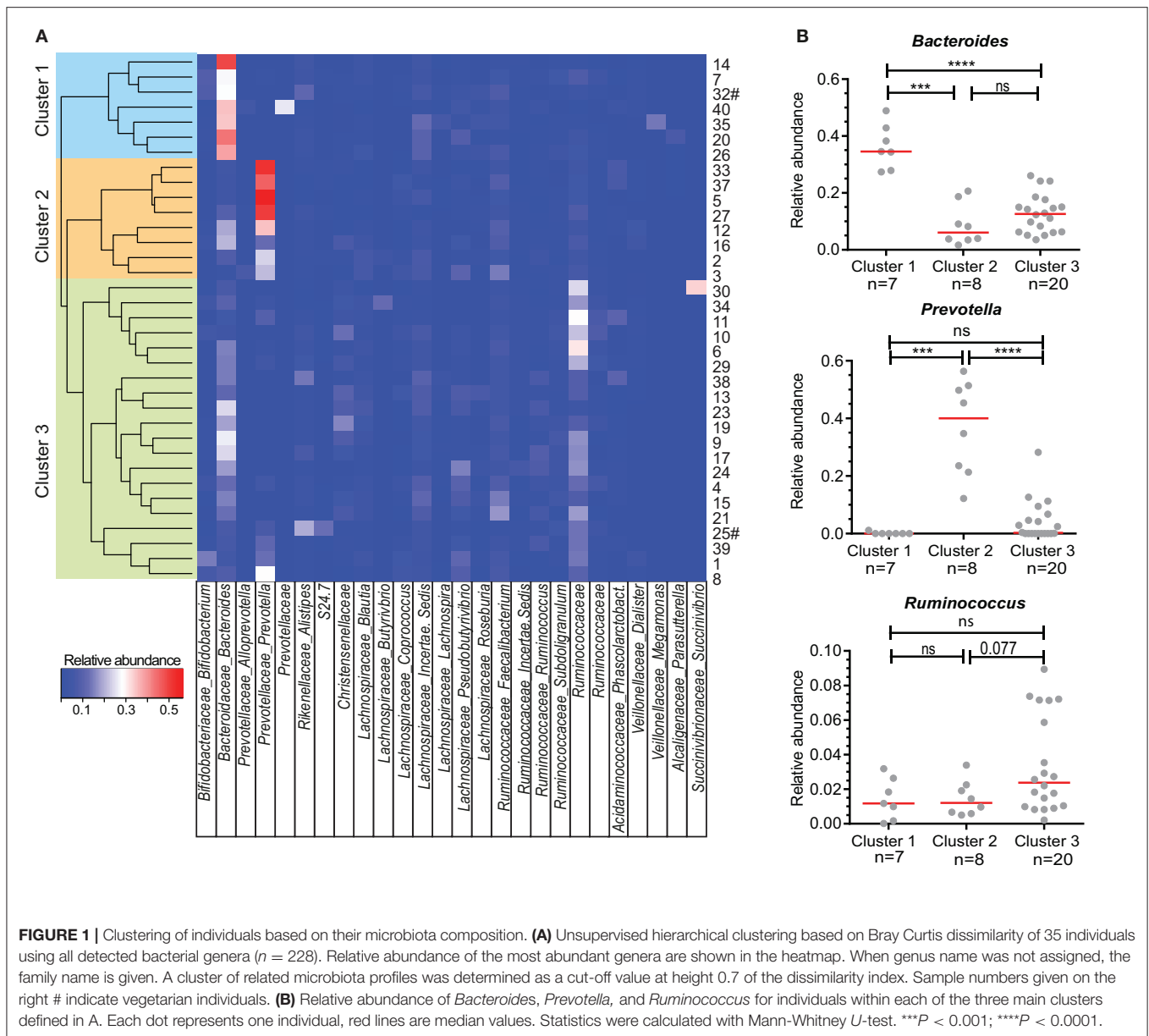
In previous studies, individuals could be clustered into three distinct enterotypes (26), which was based on the most dominant bacteria present in their feces, namely *Bacteroides*, *Prevotella* and *Ruminococcus*. Within our own cohort, individuals in “Cluster 1” had significantly more *Bacteroides* in their feces than individuals in Clusters 2 and 3 (**Figure 1B**). On the other hand, *Prevotella* was significantly more abundant in Cluster 2 than in Clusters 1 and 3. Finally, although *Ruminococcus* was not significantly higher in abundance in Cluster 3 individuals (**Figure 1B**), a trend could be seen with increased numbers of *Ruminococcaceae*.

To further characterize the composition of the microbiota within the three clusters detected, we selected the 20 most abundant genera in our cohort and quantified these per cluster (**Figure 2A**). The second most abundant genus in Cluster 1 was from the *Lachnospiraceae* family with *Bacteroides* also being highly abundant in Cluster 2. Of note, *Prevotella* was undetectable in 4 out of 8 individuals from Cluster 1 and in 5 out of 20 individuals of Cluster 3. The most abundant genus in the microbiota of participants in Cluster 3 could not be assigned at the genus level, but was of the family *Ruminococcaceae* (*Ruminococcaceae* I; **Figure 2A**). *Ruminococcus* is part of the *Ruminococcaceae* family.

The three clusters differed in bacterial diversity and richness (**Figures 2B,C**). Cluster 3 had the most diverse microbiota with 810 unique observed species. This was higher than in Cluster 1 and 2 in which there were 489 and 518 unique species, respectively ($P < 0.0001$ and $P < 0.01$).

Blood T Cells, Memory B Cells and Microbiota Clusters

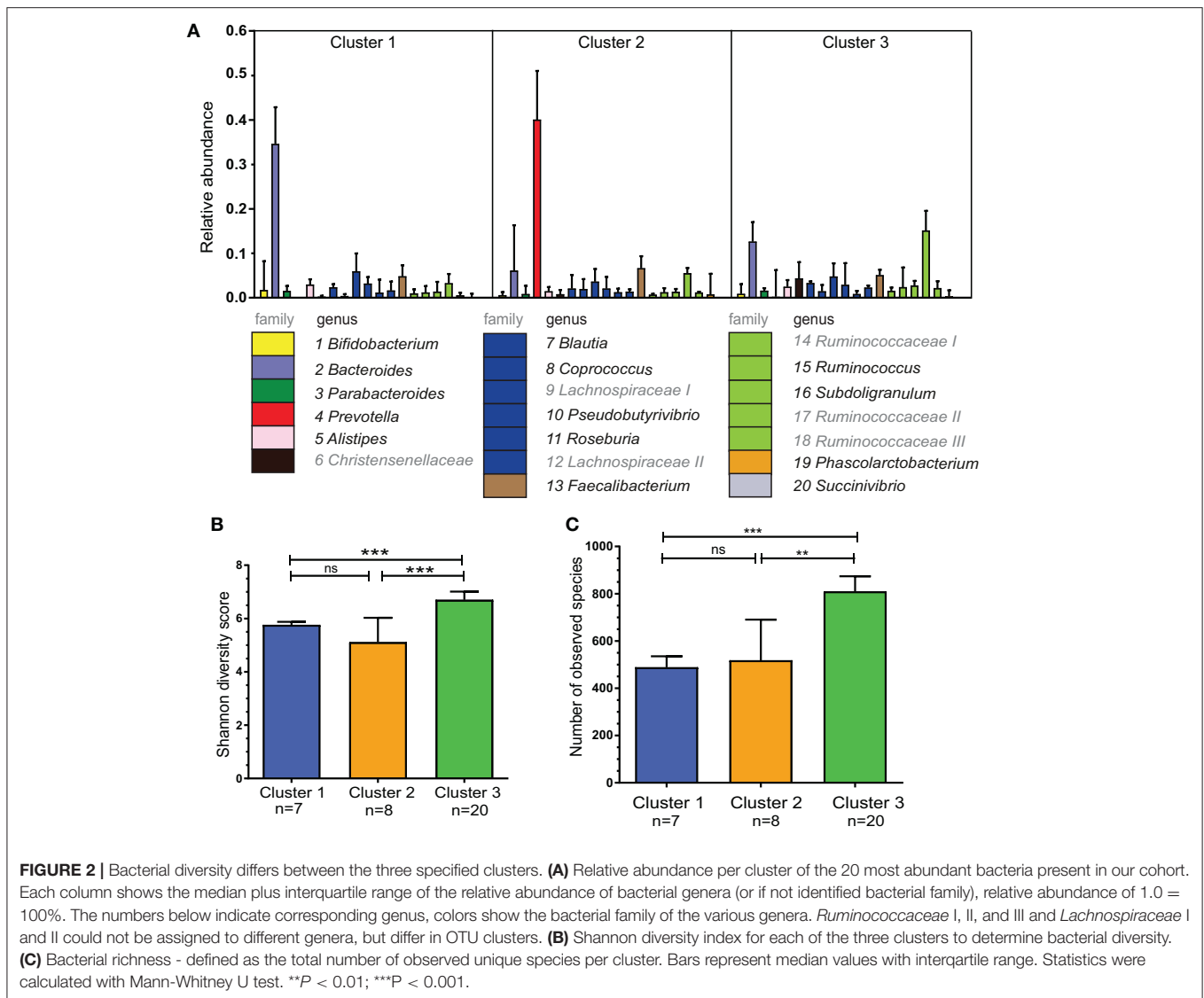
The intestinal microbiota in animal models has been shown to affect IgA+ B cells and CD8+ T cells (39) as well as



CD4+ Th subset numbers (30). Therefore, T- and B-cell subsets were both analyzed in our human cohort. The total counts of CD3+ T cells, as well as CD4+, CD8+ and $\gamma\delta$ T cells, were similar between individuals of Cluster 1, 2, and 3 (Figure 3A). Multiparameter gating strategies were performed to define CD4+ T-helper cell subsets (Th1, Th2, Th17, Th17.1, Th22), and to differentiate follicular helper T cells (Tfh) and regulatory T cells (Treg) (Figure S4). Th17 and Th22 cells were significantly lower in individuals of Cluster 3 than to Clusters 1 and 2 ($P < 0.0001$ and $P < 0.05$, respectively). Th17.1 cells were significantly higher in Cluster 1 than in Clusters 2 and 3 ($P < 0.05$ and $P < 0.01$, respectively). Absolute numbers of Th1, Th2, Tfh, and Treg subsets, as

well as the Th1/Th2 ratios did not differ significantly between Clusters (Figure 3A).

Considering the previously published role of secreted immunoglobulins (Igs) in intestinal immune responses, we determined the cell counts of total, naive and memory B-cells, as well as plasmablasts, in peripheral blood. Total B-cell, naive B-cell and plasmablast counts were similar in individuals with distinct microbiota composition (Figure 3B). Memory B cells were defined as CD27+ class-switched (cs; IgD-IgM-) and CD27-cs memory B cells, and within these subsets, IgA-expressing memory B cells were defined as CD27+IgA+ and CD27-IgA+ B cells. CD27-IgA+ memory B-cell numbers were lower than CD27+IgA+ memory B cells in all 3 Clusters. However,



these counts did not differ significantly between the 3 Clusters (**Figure 3B**). To further distinguish between IgA+ memory B cells originating in the intestine or at other mucosal surfaces, we determined the expression of surface IgA1 and IgA2 on blood B cells (40). The IgA1+/IgA2+ ratios of memory B cells ranged from 2.5 to 3 and were not significantly different between the 3 Clusters (**Figure 3C**).

Thus, detailed blood T- and B-cell analysis showed differences in Th17, Th22, and Th17.1 cells between individuals with distinct intestinal microbiota clusters (enterotypes).

Specific IgA to Intestinal Bacteria in Feces and Plasma

For a more functional analysis of B-cell responses to the intestinal microbiota, we analyzed the binding of IgA isolated from feces and plasma samples to indicator human intestinal

bacterial species, including those possessing Gram-positive and Gram-negative cell walls and associated with health and disease. These strains included the commensal bacteria *Bacteroides fragilis*, *Prevotella copri*, *Bifidobacterium longum* and the potential pathogens *Escherichia coli* and *Clostridium difficile*. Plasma IgA reactivity against all of these bacteria was higher than fecal IgA reactivity, and all differences, except for *B. fragilis*, were statistically significant ($P < 0.05$) (**Figure 4A**). The levels of binding of fecal IgA as assessed by relative absorbance were higher for the commensal species (*Bacteroides fragilis*, *Prevotella copri*, and *Bifidobacterium longum*) than for the pathogen *C. difficile* (**Figure 4B**). These differences were nearly all significant, with the exception of IgA binding levels between *B. longum* and *C. difficile* ($P = 0.066$). There was a trend in higher fecal IgA binding to *E. coli* in individuals of Cluster 2, but this was not significant (C1 vs. C2, $P = 0.054$; C2 vs. C3, $P = 0.058$;

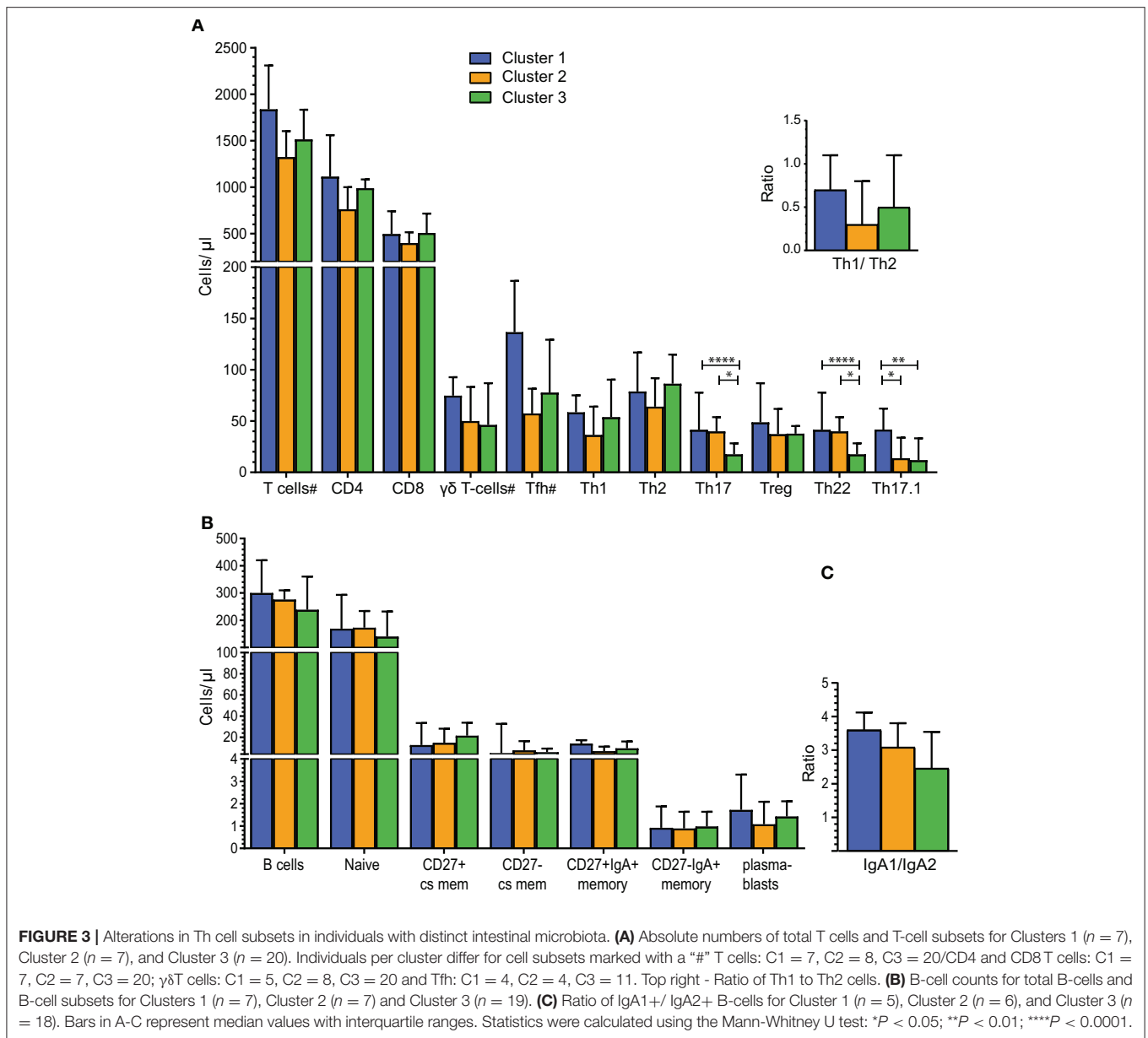


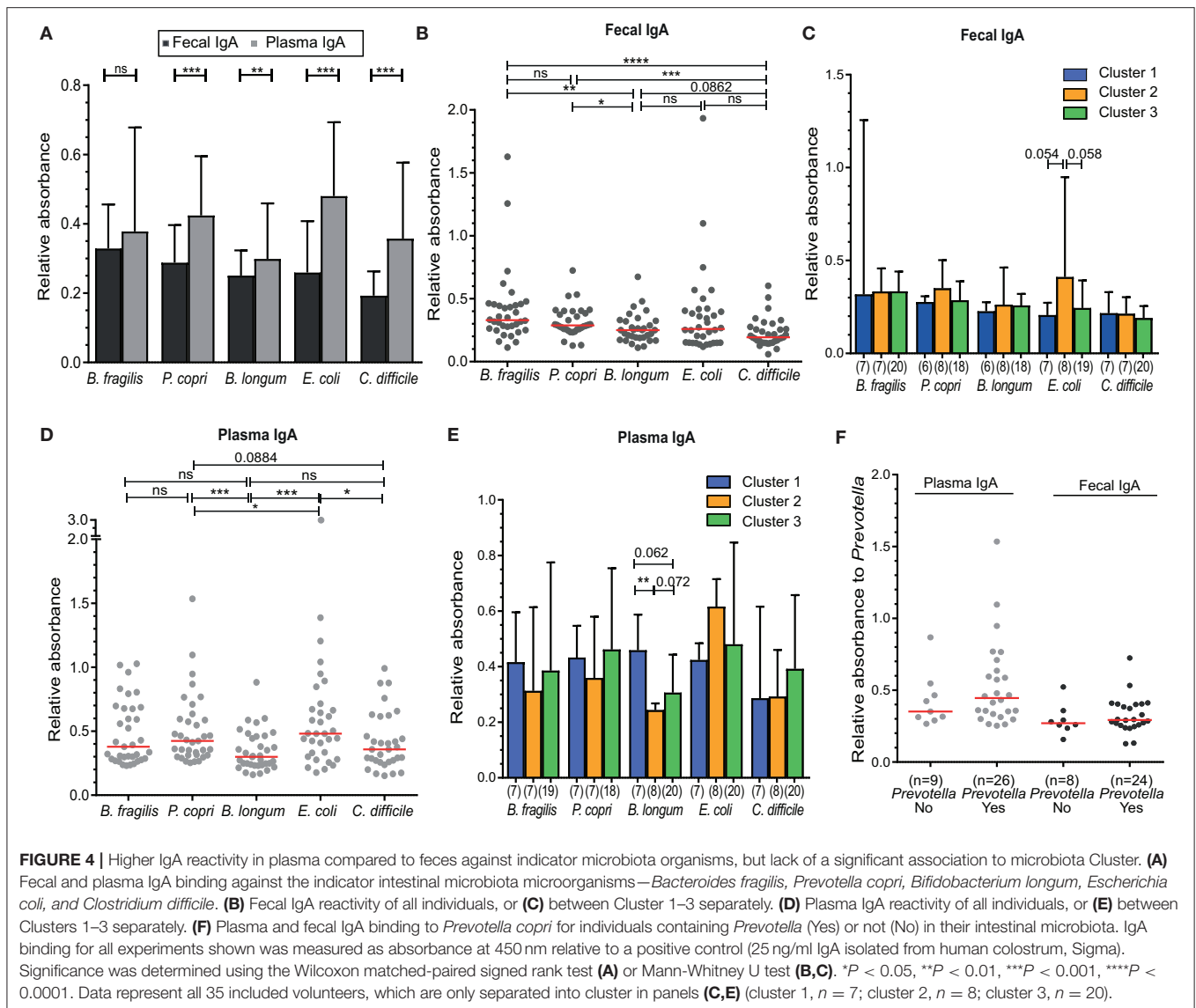
Figure 4C). Further, fecal IgA reactivity levels did not correlate to blood CD27+ and CD27-IgA+ memory B-cell counts (**Figure S5**).

Relative plasma IgA binding was highest for *E. coli*, in contrast to fecal IgA (**Figure 4D**). IgA binding to *B. longum* was lower than binding to the other two commensal bacteria (*B. fragilis* and *P. copri*) in both feces ($P = 0.0016$ and $P = 0.0182$) and plasma ($P = 0.0105$ and $P = 0.0009$). Plasma IgA binding to *B. longum* was lower in individuals of Cluster 2, which was significant when compared to Cluster 1 ($P < 0.01$) and not significant when compared to Cluster 3 ($P = 0.072$) (**Figure 4E**).

The abundance of *Prevotella* species differed greatly between individuals, with levels below our protocol detection limit

in 11 cases ($<0.005\%$) to $\sim 55\%$ of reads (**Figures 1B, 2A**). Therefore, we directly compared *Prevotella*-specific IgA between 11 individuals that showed undetectable levels of *Prevotella* to that of *Prevotella*-carriers. Both fecal and plasma IgA reactivity did not differ between individuals with (yes) or without (no) *Prevotella* as part of their intestinal microbiota (**Figure 4F**).

Taken together, our results demonstrate that IgA reactivity to specific intestinal bacteria is detectable in both the feces and blood plasma of healthy individuals. However, the plasma IgA levels appear to be higher than fecal IgA levels for all 5 fecal indicator microorganisms. In addition, the levels of specific IgA to *B. longum* were significantly lower in individuals of Cluster 2 as compared to individuals of Cluster 1.



DISCUSSION

As a first step in tackling the complexity of examining effects of intestinal microbiota profiles on adaptive immunity, we studied the immune response to 5 indicator bacteria and T- and B-cell subsets in individuals grouped in 3 defined microbiota profile clusters. Using this approach, we provide further evidence that the fecal microbiota composition from healthy individuals may be grouped into three clusters, characterized by a dominance of the *Bacteroides* or *Prevotella* genera or the *Ruminococcus* family as previously reported (26). Whereas, no differences were apparent in the B-cell compartment, Th subsets were different between the clusters with Th17 and Th22 cell numbers being lower in Cluster 3 (*Ruminococcus* enterotype), and Th17.1 cells significantly higher in Cluster 1 (*Bacteroides* enterotype). Intestinal IgA reactivity was similar between individuals with distinct intestinal microbiota, whereas

plasma IgA reactivity to *B. longum* was reduced in individuals of Cluster 2 (*Prevotella* enterotype).

We found three microbiota clusters, characterized by a dominance of the *Bacteroides* or *Prevotella* genera or the *Ruminococcus* family. The classification of specific enterotypes as three clusters as initially defined by Arumugam et al. (26), is still debated. Subsequent studies since have reported two, or even four major clusters (41, 42). The commonly identified enterotypes in most publications are those clusters defined by either a higher abundance of *Bacteroides* or *Prevotella* species, with the abundance of *Prevotella* being linked to diets with a higher fiber intake and *Bacteroides* to protein-rich diets (41). Therefore, *Prevotella* and *Bacteroides* have been suggested as potentially useful biomarkers in studies determining diet and lifestyle choices in humans (43). We did not observe differences between individuals with distinct microbiome in the parameters we collected about food intake. However, we had only a limited

number of questions about food intake and life style in our questionnaire and did not include questions to estimate protein or fiber intake.

In contrast to the initial description of enterotypes, a significantly higher abundance of bacteria belonging to the *Ruminococcus* genus was not observed in our cohort Cluster 3. Although the family *Ruminococcaceae* was shown to be most abundant in this Cluster, we could not be certain that this family identification comprised bacteria from the genus *Ruminococcus* or another genus from the same family. Interestingly, in previous studies the differences between *Bacteroides* and *Ruminococcus* clusters were shown to be less well-defined than originally thought, while the *Prevotella* cluster appears easier to define (44).

Despite the differences observed in microbiota profiles, we found no significant differences in blood B-cell subsets. Previously, infants colonized with *E. coli* and *Bifidobacterium* have been shown to carry more circulating CD20+CD27+ memory B cells in their blood (45). However, in our study, we did not find alterations of the B-cell compartment in blood between individuals with distinct intestinal microbiota. The composition of the intestinal microbiota in children is variable especially in the first years of life and becomes more stable in adults after 3–5 years (46). This might explain why we did not observe the types of differences previously reported in infants when using our adult cohort.

We found reduced Th17.1 cells in individuals of Cluster 2 and 3, as well as lower Th17 and Th22 cells in individuals of Cluster 3. Studies using mouse models have shown that segmented filamentous bacteria induce Th17 cell development (30, 47). In humans, Th17 cells have been described in the pathogenesis of IBD (48), and reduced Th22 cells were found in inflamed colon tissue of IBD patients (49). In contrast, Th22 and Th17 cells were increased in rheumatoid arthritis (RA) (50). Interestingly, in newly diagnosed RA patients *Prevotella* bacteria were increased and *Bacteroides* decreased. More specifically, the species *Prevotella copri* was found in 75% of RA patients, while this was only found in 21% of healthy individuals (24). In healthy controls we did not find alterations in *Prevotella* linked to Th17 and Th22 cells.

The balance between Th17 and Treg cells is influenced by the microbiome and important for the homeostasis in the gut (51). Han and co-workers have shown that Treg cell numbers were positively correlated to the abundance of *Ruminococcaceae* in the intestine of patients with graft vs. host disease. However, a decrease in Th17 cells with increased *Ruminococcaceae* abundance was not statistically significant (51). In our cohort were Th17 cells lower in individuals of Cluster 3 (*Ruminococcaceae* cluster), which confirms the trend observed by Han and co-workers. We did not find a difference in Treg cell numbers between individuals with high (Cluster 3) and low (Cluster 1 and 2) *Ruminococcaceae* abundance. Thus, in our cohort reductions in Th17- and Th22-cell numbers were not linked to increased Treg numbers, although it is possible that intestinal cell numbers may differ at specific local sites in the intestine.

Possible immunomodulatory capacities related to *Ruminococcus* and *P. copri* have not yet been fully described, although polysaccharide A (PSA) from the capsule of *B. fragilis* have been shown to affect systemic Th1/Th2 ratios in mice (52). Additionally, Round et al. showed that PSA can alter T-cell differentiation via the induction of Tregs (53). However, profound differences exist between the microbiota and immune responses between mice and humans, which may limit the translation of mouse microbiota studies into the human situation (54). In our cohort, we found no statistically significant alterations in Treg numbers or Th1/Th2 ratios in individuals with a high abundance of *Bacteroides* in their feces. This may be explained by the fact that we studied a relatively small cohort, which might not have been sufficient to detect more subtle differences in the immune response and microbiota profiles of human populations that are exposed to many different interpersonal and environmental influences. Further, we were not able to identify *Bacteroides* at the species level, and were consequently not able to examine differences in the abundance of *B. fragilis* between individuals.

Not only bacterial colonization, but also persistent infections with herpes viruses, esp. CMV and EBV, have been shown to affect memory T-cell, but not B-cell numbers in peripheral blood (55–57). These effects included the CD4, CD8, and $\gamma\delta$ T cell lineages in CMV positive individuals. As we did not determine viral seropositivity in our study cohort, we cannot exclude a confounding effect of CMV infection. However, in our experience (56), as well as in publications from others, it is evident that >80–90% of adults are seropositive for CMV. Hence, it is unlikely that the difference in CMV serostatus would have affected the changes in T-cell numbers that we observed.

IgA reactivity to 5 indicator bacterial strains was higher in plasma than in fecal matter, although differences between individuals with distinct microbiota composition were only found for plasma IgA reactivity to *B. longum*. A potential explanation for higher plasma IgA reactivity as compared to fecal IgA could be that IgA in the intestine binds commensal bacteria with low affinity and in a polyreactive manner, possibly leading to weaker binding in our ELISA assays. In contrast, systemic immune activation may lead to strong IgA responses in peripheral blood. For example, it has been shown that recombinant IgA from intestinal IgA memory B-cells is not only polyreactive to antigens such as insulin, dsDNA and LPS, but also against different commensal bacteria (14, 58).

A correlation between IgA and the abundance of the intestinal bacteria was not found. Although the relative bacterial 16S rRNA gene quantities calculated are dependent on the number of 16S rRNA gene copies per bacterial species, we did not correct for differences in gene copy numbers between all possible fecal bacteria detected. This was due to the fact that gene copy numbers vary per individual bacterial species and current limitations in Illumina sequencing technology mean that it is not possible to accurately identify bacteria to the species level. Therefore, differences in 16S rRNA gene copy numbers could have theoretically influenced our results and masked a potential correlation between bacterial species abundance and IgA reactivity. Additionally, our ELISA methodology utilized a

single representative species of 5 different bacterial genera for IgA determination, and correlations of IgA activity were obtained using only a single species from each genus under investigation.

Interestingly, we found *Prevotella*-reactive IgA antibodies in plasma and feces of individuals that apparently lacked *Prevotella* in the intestinal microbiome. There is a possibility that the abundance of this genus was below the limit of detection in our microbiome analysis. Even so, our data shows that individuals with absent or low abundance of *Prevotella* in the intestinal microbiome may still present with high levels of *P. copri*-specific IgA in feces and plasma. This is in line with studies from Hapfelmeier et al. who showed that IgA plasma cells in the intestine of mice provide long-lasting immunity, with reactivity up to of 6–8 weeks after their respective target bacteria were completely cleared from the host (59). In this respect, we only collected feces at a single time point and changes in the abundance of *Prevotella* bacteria in the feces of volunteers could have taken place over the time of the study. It would have been interesting to be able to follow our cohort in order to determine qualitative (presence/absence) changes in the abundance of *Prevotella* and other bacterial genera over time.

In our volunteers, systemic (plasma) and intestinal IgA binding to *Bifidobacterium longum* was significantly lower than to other commensals such as *Prevotella copri* and *Bacteroides fragilis*. Bifidobacteria are one of the first bacteria to colonize the intestine after birth and are beneficial for human health by their ability to skew T-helper cell responses toward Th1 response (60, 61). Further, in a study in pigs, colonization with *Bifidobacterium* and *Lactobacillus* induced increased Rota-virus specific intestinal IgA responses after Rotavirus infection (62), though to our knowledge, we are the first to describe *Bifidobacterium*-specific IgA responses in humans.

In conclusion, we show that plasma IgA reactivity to 5 indicator microorganisms frequently found in the human intestine is higher than intestinal IgA reactivity. Systemic IgA reactivity to *B. longum* is reduced in individuals of Cluster 2 with high abundance of *Prevotella* in the intestinal microbiome. Whereas, no differences were observed in the blood B-cell compartment, Th17-, Th17.1- and Th22-cell numbers were significantly different between individuals with distinct intestinal

microbiota Cluster profiles (enterotypes). Still, we have studied a small study cohort and studies involving much larger cohorts, e.g., population-based cohorts with the potential to correct for confounding factors such as lifestyle or diet will be required to confirm our results.

DATA AVAILABILITY

This Targeted Locus Study project (BioProject PRJNA515953) has been deposited at DDBJ/EMBL/GenBank under the accession KCRL00000000. The version described in this paper is the first version, KCRL01000000.

AUTHOR CONTRIBUTIONS

CG-W and MvZ: designed research; CG-W, DR, JH, and MvZ: wrote the manuscript; CG-W, DR, HvdW, and KS: performed experiments; JH: supervised feces sample processing and storage; RK and JH: contributed to the study methodology and essential discussions of the paper. All authors critically read the manuscript and approved of the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2019.00341/full#supplementary-material>

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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