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Impact of age and sex on the composition and abundance of the intestinal microbiota in individuals with and without enteric infections

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Abstract

Purpose: The intestinal microbiome is critical for human health and preventing colonization by enteric pathogens. There are notable differences in the microbiota composition among individuals with and without enteric infections, though the impact that age and gender has on the composition and abundance of intestinal microbes is not known.

Methods: A comparative 16S rRNA gene sequencing study was performed on stool DNA from 200 patients with enteric infections and 75 healthy family members representing both sexes and multiple age groups.

Results: Microbial community profiles were affected by age and sex in patients with enteric infections and their healthy family members. Overall, we observed an increase in *Bacteroides* abundance and decrease in *Escherichia* abundance with age, though these differences were most apparent for patients with enteric infections. Genus *Bacteroides* was also higher in female communities while *Escherichia* predominated in males.

Conclusions: Because *Escherichia* abundance was previously linked to symptom severity, children with enteric infections may be most susceptible to severe disease outcomes due to high and low abundance of *Escherichia* and *Bacteroides*, respectively. Future studies should focus on classifying specific differences in the microbiome using metagenomics and identifying novel methods aimed at shifting the intestinal microbiome to a healthy state.

Keywords

Diarrhea; Microbiota; 16S rRNA

Introduction

The composition of the intestinal microbiota is imperative for health as it plays a critical role in metabolism, digestion, and nutrient absorption as well as immune system modulation and prevention of pathogen colonization [1,2]. Age-related changes in gut physiology and alterations in the composition and function of the microbiota have been described previously

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[1,3]. Such changes were suggested to be partly due to dietary and physiological factors that can alter the metabolic capacity of the gut and lead to an increased susceptibility to gastrointestinal infections [4]. One study, for example, demonstrated that children between 16 months and 7 years had a significantly greater proportion of Enterobacteria when compared with adults between 21 and 34 years [4]. Moreover, the lowest abundance of *Bifidobacteria*, which has been shown to be protective and associated with intestinal health, was observed in the elderly population. Other studies have also reported sex-dependent effects in the intestinal microbiota composition as a result of gastrointestinal physiology [5] and diet, which affected males differently than females *in vivo* using mouse and stickleback models [6]. Similarly, another study demonstrated that commensal microbial communities altered sex hormone levels and could subsequently regulate immune cell responses [7]. These prior studies, which are summarized in Table 1, demonstrate that both age and sex are important factors to consider when conducting studies aimed at characterizing the microbiome.

In our prior study of 200 patients with enteric infections caused by the four most common bacterial pathogens (*Salmonella*, Shiga toxin-producing *Escherichia coli*, *Campylobacter*, and *Shigella*) in Michigan and 75 healthy family members, we demonstrated that the intestinal microbial communities were variable across groups [11]. Specifically, patients with enteric infections had less diverse communities when compared with healthy family members as well as a significantly greater abundance of *Escherichia* that decreased after recovery. It is important to note that this increased abundance of *Escherichia* was observed among patients irrespective of the bacterial pathogen that caused each infection, suggesting that intestinal communities are altered in a similar way during the course of an enteric infection. After recovery from infection, patient *Escherichia* levels decreased in abundance and community diversity increased. As part of this prior study, however, we did not examine how the intestinal communities varied across age groups or other demographic characteristics including sex.

Examining intestinal microbial communities in a diverse population as in individuals with and without enteric infections, will enable the identification of microbial factors that are associated with disease susceptibility or more severe clinical symptoms. To better understand the impact of these demographics, we characterized the intestinal microbial communities of patients with enteric infections of varying age and sex by 16s rRNA sequencing, and made comparisons to intestinal communities from healthy family members. Analyzing the impact of epidemiologic factors on microbial communities of individuals with enteric infections may provide insight into disease susceptibilities in certain populations.

Material and methods

Sample collection and DNA isolation

Stool samples from patients with acute enteric infections caused by *Salmonella*, Shiga toxin-producing *E. coli*, *Campylobacter*, and *Shigella* ($n = 200$) and their otherwise healthy family members ($n = 75$) were collected via the Enteric Research Investigative Network at Michigan State University as described [11]. Family members represented individuals living in the same household who were related to the patients (e.g., parents, siblings,

grandparents). Stools were homogenized, centrifuged, and stored in triplicate at 80°C immediately after receipt from the Michigan Department of Health and Human Services (MDHHS). Community DNA was isolated using the QIAamp kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol with a modification to the denaturation step (95°C) and inclusion of bead beating to enhance DNA isolation of gram-positive bacteria [11].

Pyrosequencing and microbial community analyses

PCR amplification of the 16S rRNA gene (V5eV3 regions) was performed using universal barcoded primers (357F and 926R) and the AccuPrime Taq DNA polymerase (Invitrogen, Waltham, MA, USA) in triplicate. The PCR protocol included an initial denaturation step (95°C for 2 mins) followed by 30 cycles of 95°C for 20 seconds, 50°C for 30 seconds, and 72°C for 5 minutes as described in our prior study [11]. Individual amplicon libraries were purified using Agencourt AMPure XP (Beckman Coulter, Inc., Indianapolis, IN, USA) beads, quantified using the Quant-iT PicoGreen dsDNA quantitation kit (Invitrogen), and normalized for pooling. Amplicon libraries were sequenced using the 454 GS Junior Titanium (Roche, Indianapolis, IN, USA).

Sequencing analysis and epidemiologic associations

As described previously [11], sequencing reads were filtered and processed using the Quantitative Insights Into Microbial Ecology (QIIME) program [12] followed by trimming and filtering by noise reduction using default parameters. Quality sequences were aligned followed by chimeric and singleton removal using USEARCH and classified using the Greengenes 16S rRNA gene reference database [12,13]. Taxonomical data was also analyzed using Paleontological Statistics Software Package For Education and Data Analysis (PAST) with the multivariate tests, Analysis of Similarities (ADONIS) and Permutational Multivariate Analysis Of Variance (PERMANOVA); neighbor-joining trees were constructed in FigTree (http://tree.bio.ed.ac.uk/software/figtree/). Nonmetric Multidimensional Scaling (NMDS) plots were generated based on microbial abundance and composition using the BrayeCurtis and Jaccard similarity coefficients with 9999 permutations for a weighted and unweighted analysis, respectively [12].

The genera responsible for dissimilarities between groups were also identified by Similarity Percentages (SIMPER) based on the BrayeCurtis dissimilarity matrix [14], and linear discriminant analysis (LDA) effect size (LEfSe) was used to identify differentially abundant microbial features [15]. Data were analyzed in the following age groups: 0–10 years (n 91), 11–e18 years (n 32), 19–49 years (n = 84), 50–69 years (n = 45), and more than 70=years (n = 12; Table 2). With regard to sex, 141 intestinal communities originated from males and 126 originated from females. Age was missing from four healthy (uninfected) individuals, whereas sex was not available for eight individuals (three patients, five uninfected). Demographic characteristics were also examined after stratifying by the different microbial community clusters using the likelihood χ^2 test with odds ratios and 95% confidence intervals.

Results

Association between the microbiota and demographics

Analysis of 275 intestinal communities from patients with enteric infections and healthy, uninfected individuals revealed significant differences in the abundance and composition of taxa (PERMANOVA $P < .0001$) when stratified by sex and specific age groups. Overall, sex had a significant impact on the abundance of taxa based on the BrayeCurtis dissimilarity index (PERMANOVA $P = .008$). The average dissimilarity for the intestinal communities originating from males and females was 63.0 as calculated by SIMPER. Genera *Bacteroides* and *Escherichia* contributed most to the dissimilarity between sexes. On average, females had a slightly higher abundance of *Bacteroides* (37.9%) than males (32.5%), though the males had higher *Escherichia* levels (18.8%) compared with females (12.3%); neither proportion was significantly different.

When stratified by age groups, no differences were identified in community composition or abundance based on the Jaccard similarity coefficient ($P = .14$) or Bray–Curtis dissimilarity index ($P = .18$), and clustering was not observed in the NMDS analysis. A single factorial analysis among different age groups, however, identified significant differences in taxa abundance among individuals between 11 and 18 years relative to 50–69 year olds by Analysis of Similarity (ANOSIM) ($P = .04$) Similarly, abundance profiles were significantly different by PERMANOVA ($P = .04$) between children aged 0–10 years and adults between 19 and 49 years. The average dissimilarity for all five age groups was 65.9. The same taxa, *Bacteroides* and *Escherichia*, identified to be most dissimilar between sexes, contributed most to the differences by age group. The mean abundance of *Bacteroides* was 33.0% and 31.2% in children aged 0–10 and 11–18 years, respectively, but was slightly higher in adults aged 19–49 (36.3%) and older than or equal to 50 years (38.6%); the difference in proportions of *Bacteroides* was not significant between any of these age groups. Comparatively, the proportion of *Escherichia* was slightly higher in children younger than or equal to 18 years (19.5%) relative to adults between 19 and 49 years (12.0%), 50–69 years (16.8%), and adults older than or equal to 70 years (6.0%), although the differences were not statistically significant. Similar trends were observed for *Prevotella*, which also contributed to the dissimilarity in the groups, with a mean abundance of 3.6% (0–10 years), 1.7% (11–18 years), 4.3% (19–49 years), 1.9% (50–69 years), and 0.03% (≥70 years).

Effect of sex on the intestinal microbiota from infected and uninfected individuals

To determine whether the microbial communities from individuals of varying age and sex were impacted by intestinal health, the communities from uninfected and infected individuals were analyzed separately. Among the 70 healthy individuals of known sex, no difference was observed in abundance or composition. The average dissimilarity was 50.6 using SIMPER with genera *Bacteroides* (22.3%), *Prevotella* (11.3%), other *Rikenellaceae* (5.5%), *Parabacteroides* (5.0%), *Faecalibacterium*, and other *Ruminococcaceae* (4.4%) predominantly contributing to this difference. LEfSe identified genus *Paraprevotella* to be differentially abundant in healthy intestinal communities from males (LDA > 3), whereas *Dialister*, *Lactobacillus*, unclassified Lactobacillaceae, unclassified Comamonadaceae, and *Comamonas* were differentially abundant in females (LDA > 3). In addition, hierarchical

clustering based on the BrayeCurtis dissimilarity matrix demonstrated that the healthy intestinal communities clustered differently when analyzed separately without infected communities as was done previously [11]. Five clusters were defined among the uninfected communities, with the previously defined Cluster IV separating in to four groups (Clusters IVaeIVd; Fig. 1A). No significant differences were observed between clusters by sex.

When the 197 infected male and female communities were examined, stratifying by sex significantly affected the composition ($P = .04$) and abundance ($P = .002$) by PERMANOVA; only the abundance was affected ($P = .02$) by ANOSIM. The average dissimilarity between the 104 males and 93 females was 63.0 by SIMPER. In males, *Bacteroides* (30.9%) was most abundant followed by *Escherichia* (25.1%), unclassified Enterobacteriaceae (7.0%), and *Faecalibacterium* (2.8%). The same genera predominated in female communities and the abundance of *Bacteroides* (38.0%), unclassified Enterobacteriaceae (7.3%), and *Faecalibacterium* (3.5%) was similar when compared with males. The *Escherichia* (16.1%) abundance, however, was lower in infected females, although the difference in proportions was not statistically significant ($P = .12$). In addition, LEfSe detected 54 differentially abundant microbial features (LDA > 2.4) in the male and female patient communities, which is more than 10 times was detected in the healthy communities. Enterobacteriaceae predominated among the 11 differentially abundant features detected in males, whereas Bacteroidaceae predominated among the 43 differentially abundant features in females.

Unlike the hierarchical clustering, results for the healthy, uninfected communities, those communities from infected males and females more closely matched one of the three previously defined Clusters [11]. The few communities that previously classified as Cluster III were an exception as they were scattered throughout the neighbor-joining phylogeny after excluding the healthy communities (Fig. 1B). Infected males were significantly more likely to have intestinal communities representing Cluster I (odds ratio: 1.9; 95% confidence interval: 1.01–3.56) relative to females. On the other hand, infected females were more likely to have communities belonging to Cluster IV (Fisher's exact $P = .04$), though the sample size was low. An equal number of male and female communities comprised Clusters II and IV.

Effect of age on the intestinal microbiota of infected and uninfected individuals

Among the 72 communities from uninfected individuals with age data available, composition differences were identified between certain age groups, though some age groups had very low sample sizes. Intestinal communities of children between 0 and 10 years ($n = 26$), for example, were significantly different from 50 to 69eyear olds ($n = 8$) by PERMANOVA ($P = .03$), whereas children between 11 and 18 years ($n = 4$) were significantly different from 50 to 69eyear olds ($n = 8$) by PERMANOVA ($P = .03$). Among all uninfected communities, only genus *Lachnospira* was differentially abundant by LEfSe in individuals between 50 and 69 years (LDA = 4). Hierarchical clustering did not uncover any age-specific clusters in the neighbor-joining phylogeny (data not shown).

Within the 200 communities from patients, different age groups were also associated with specific community profiles. Pairwise comparison of the intestinal community composition

of infected children between 0 and 10 years ($n = 65$) detected significant differences relative to patients between 19 and 49 years ($n = 66$) by ANOSIM ($P = .02$) and PERMANOVA ($P = .01$). Community composition of 19–49 year olds also differed significantly from individuals older than 70 years ($n = 10$) by PERMANOVA ($P = .04$). The abundance profiles varied across age groups as well, with communities from patients between 11 and 18 years ($n = 21$) differing signifi cantly from communities of 50e69 year olds ($n = 37$) by ANOSIM ($P = .01$). Communities from infected children 0–10 years of age also differed relative to 70–90 year olds ($n = 10$) by PERMANOVA ($P = .01$). Additional comparisons using SIMPER resulted in an overall average dissimilarity of 62.6. *Bacteroides*, *Escherichia*, unclassified Enterobacteriaceae, *Faecalibacterium*, and *Prevotella* predominated and were responsible for the differences between the communities, although *Escherichia* and *Bacteroides* contributed most to the dissimilarity. *Bacteroides* increased slightly by age group and was found in 31.7% (0–10 years), 28.0% (11–18 years), 35.9% (19–49 years), 38.7% (50–69 years), and 38.4% (≥70 years) of infected communities (Fig. 2). In contrast, *Escherichia* abundance gradually decreased with age and was found in 27.6% (0–10 years), 20.8% (11–18 years), 17.1% (19–49 years), 20.0% (50–69 years), and 7.3% (70–90 years) of the infected communities. Nonetheless, no significant differences in the proportion of either *Bacteroides* or *Escherichia* were detected across groups, and similar to the uninfected communities, the intestinal communities from patients did not cluster together by age group in the neighbor-joining phylogeny.

Discussion

In this analysis, we have demonstrated that intestinal microbial communities vary across individuals and that intestinal health is the most important factor contributing to community composition differences by sex and age group. This result is consistent with prior studies that have detected variation in community composition across individuals [6,16] as well as our prior study, which observed greater diversity and richness in healthy versus infected intestinal communities [11].

Among the healthy individuals examined herein, *Lactobacillus* and *Comamonas* were differentially abundant in the female relative to male communities, which may be linked to diet and nutrition. *Comamonas* has been identified as a member of the microbiome comprising murine intestinal crypts, which are important for epithelial regeneration and health [17], whereas members of *Lactobacillus* have been linked to probiotic use. Such foods are likely to be differentially consumed by males and females. Because no difference in the abundance of either *Lactobacillus* or *Comamonas* was observed by sex in patients with enteric infections, the benefits are unclear. Indeed, the probiotic effects of lactobacilli were suggested to be strain specific and not broadly applicable to one genus [18]; hence, additional longitudinal studies using metagenomics are required to assess whether certain bacterial species are linked to disease susceptibility and may be differentially present in males and females. Similar findings were observed among healthy individuals representing different age groups, although only *Lachnospira* was differentially abundant in individuals between 50 and 69 years of age. The biological implications of these findings are not understood, though the small number of healthy communities examined in this study may

have prevented the identification of meaningful differences across healthy individuals of varying ages.

In contrast to the healthy, uninfected communities, more differences were identified in patients after stratifying by both sex and age. Interestingly, *Escherichia*, which we previously reported to be higher in patients than healthy individuals [11], was more abundant in the male communities than female communities. Communities from males were also overrepresented in Cluster I of the neighbor-joining phylogeny; Cluster I communities were associated with more severe symptoms in our prior analysis [11]. It is therefore possible that males are more susceptible to increases in *Escherichia* levels, which is correlated with decreases in *Bacteroides* and negatively impacts intestinal health. Although these data are consistent with prior studies demonstrating that sex-dependent differences are linked to hormones [7], physiology [5], and diet [6], additional studies are required to test the effect of sex on the abundance of specific microbial populations during an enteric infection and throughout the recovery period.

Similar findings were observed after stratifying by age group as children younger than 18 years had the highest abundance of *Escherichia* and lowest abundance of *Bacteroides*. Indeed, as age increased, the abundance of *Escherichia* decreased and the abundance of *Bacteroides* increased. These data suggest that children with enteric infections, particularly those younger than or equal to 10 years, may be more susceptible to increases in the *Escherichia* population and may partly explain why children are more prone to severe clinical outcomes. Prior studies have suggested that dietary factors combined with age-associated changes in gut physiology as well as the composition and function of the microbiota can impact susceptibility to enteric infections [3,4]. In one study, healthy children between 16 months and 7 years had a greater proportion of Enterobacteria when compared with young adults, whereas the lowest abundance of *Bifidobacteria* was detected in elderly subjects [4]. If children have more abundant populations of Enterobacteria (e.g., *Escherichia*) before an infection, then it is possible that increases in the resident *Escherichia* population will be more pronounced in infected children relative to infected adults with lower baseline levels. Given that community members belonging to Bacteroidetes (e.g., *Bacteroides*), which were less abundant in infected children, cannot use nitrate, then it is possible that over-growth of *Escherichia* in certain individuals is linked to nitrate respiration [19]. It is interesting to note that the abundance profiles observed in the elderly patients were opposite the profiles observed in children and suggests that other factors besides the microbiota composition may work in concert to enhance susceptibility to disease in the elderly.

Collectively, these data highlight the significant perturbations that occur in the intestinal microbial communities of patients with enteric infections, and further demonstrate that both age and sex can impact the abundance of specific taxa. Overgrowth of potentially harmful bacterial populations such as *Escherichia* and decreases in beneficial populations like *Bacteroides* may be important for both disease susceptibility and time to recovery. Although we previously found that decreases in *Escherichia* and increases in *Bacteroides* occurred in patients postrecovery [11], additional studies are needed to better understand

the factors that contribute to major shifts in these populations during infections, particularly among specific individuals at the risk of more severe disease.

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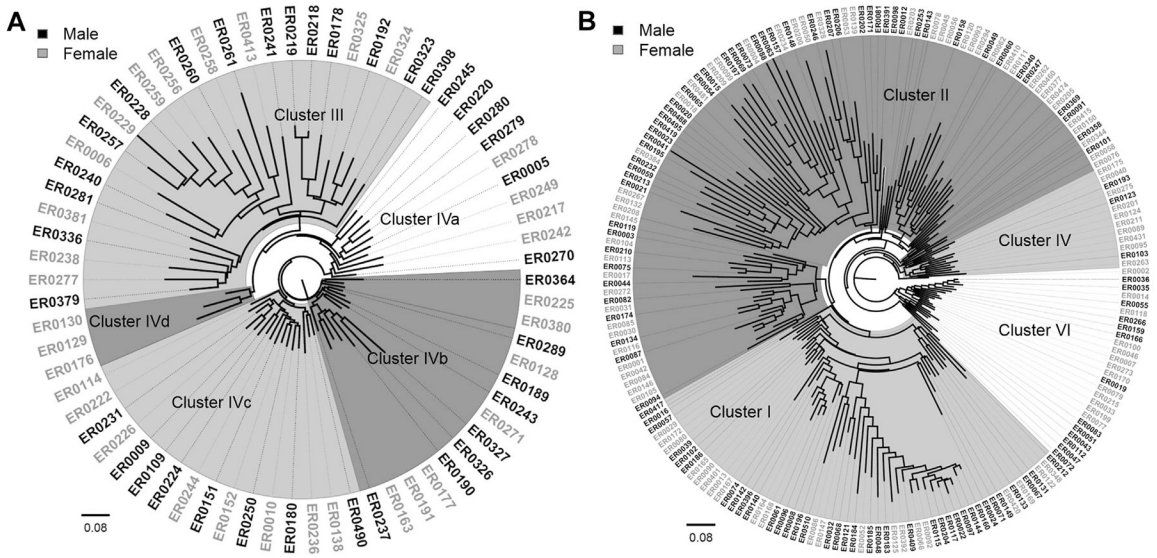

Fig. 1.

Phylogeny of intestinal microbiota profiles by sex. Hierarchical clustering identified three distinct clusters in (A) healthy family members of patients with enteric infections, and five clusters in (B) the patients. The Neighbor-Joining tree was constructed based on the BrayeCurtis dissimilarity index with 1000 bootstrap replications. Communities from males are labeled in black outside the phylogeny, whereas female communities are labeled in gray.

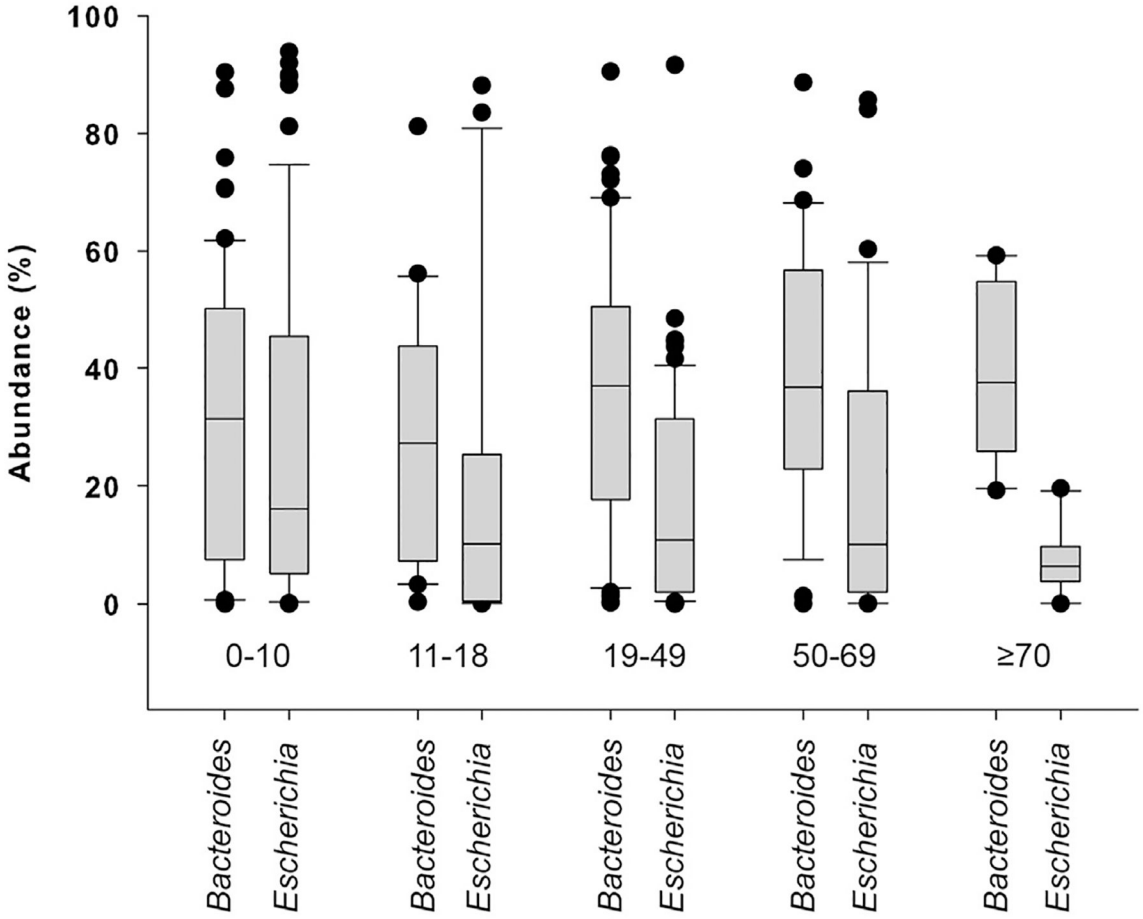

Fig. 2.

Abundance of *Escherichia* and *Bacteroides* in the intestinal communities of patients with enteric infections. The box plot represents the relative abundances of genera *Escherichia* and *Bacteroides* in infected patient communities across different age groups.

Table 1

Review of studies that have identified variation in microbiota profiles by sex or age group

Date	Study population	Method	Effect of sex	Effect of age	Reference
2000	Children 16 mos–7 years (n = 10), adults 21–34 years (n = 7), elderly ≥ 67 years (n = 5)	16S rRNA abundance	Not assessed	Greater abundance of Enterobacteria in children compared with adults	Hopkins et al. [4]
2006	Healthy adults between 20 and 50 years (n = 85), healthy adults, and elderly >60 years (n = 145)	Fluoresence *in situ* hybridization of 16S rRNA genes	Greater abundance of *Bacteroides* and *Prevotella* in males	Greater abundance of Enterobacteria in the elderly	Mueller et al. [8]
2007	Infants (n = 150), adults 25–35 years (n = 54), elderly 80–82 years (n = 45)	qPCR and fluorescence *in situ* hybridization of 16S rRNA genes specific for *A. muciniphila*	Not assessed	Decreased abundance of *A. muciniphila*, a type of mucin-degrading bacteria in the intestinal tract, in the elderly	Collado et al. [9]
2009	Adults 18–31 years (n = 17), institutionalized elderly 78–94 years (n = 17)	Denaturing gradient gel electrophoresis and qPCR	Not assessed	Greater abundance of *Bacteroides* and decreased abundance of *Bifidobacteria* in the elderly	Zwielehner et al. [10]
2013	Male (n = 123) and female (n = 132) nonobese diabetic germ-free and pathogen-free mice	16S rRNA gene amplicon sequencing	Intestinal communities were distinct among male and female mice	Sex-specific differences in the intestinal community were most apparent in adult mice	Markle et al. [7]

Table 2

Characteristics of the study population

Characteristics	Patients with diarrhea (*n* = 200)	Otherwise healthy family members (*n* = 75)
	No. (%) of individuals	No. (%) of individuals
Age group		
0–10 years (*n* = 91)	65 (32.5)	26 (34.7)
11–18 years (*n* = 32)	21 (10.5)	4 (5.3)
19–49 years (*n* = 84)	66 (33.0)	32 (42.7)
50–69 years (*n* = 45)	37 (18.5)	8 (10.7)
≥ 70 years (*n* = 12)	10 (5.0)	2 (2.7)
Gender		
Male (*n* = 141)	104 (52.0)	37 (49.3)
Female (*n* = 126)	93 (46.5)	33 (44.0)
Pathogen causing the infection*		
Campylobacter (*n* = 89)	71 (35.5)	18 (24.0)
Salmonella (*n* = 98)	66 (33.0)	32 (42.7)
Shiga toxin-producing *E. coli* (*n* = 50)	34 (17.0)	16 (21.3)
Shigella (*n* = 38)	29 (14.5)	9 (12.0)

Percentages represent the frequency of each characteristic out of the total number of individual per group. Denominators do not always add up to the total number of individuals in each column because of missing data.

*Numbers in the otherwise healthy column refer to the pathogen that infected their family members.