Among older adults, age-related changes in the stool microbiome differ by HIV-1 serostatus

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ABSTRACT

Background: HIV-1 infection and physiological aging are independently linked to elevated systemic inflammation and changes in enteric microbial communities (dysbiosis). However, knowledge of the direct effect of HIV infection on the aging microbiome and potential links to systemic inflammation is lacking.
Methods: In a cross-sectional study of older people living with HIV (PLWH) (median age 61.5 years, N = 14) and uninfected controls (median 58 years, n = 22) we compared stool microbiota, levels of microbial metabolites (short-chain fatty acid levels, SCFA) and systemic inflammatory biomarkers by HIV serostatus and age.
Findings: HIV and age were independently associated with distinct changes in the stool microbiome. For example, abundances of *Enterobacter* and *Paraprevotella* were higher and *Eggerthella* and *Roseburia* lower among PLWH compared to uninfected controls. Age-related microbiome changes also differed by HIV serostatus. Some bacteria with inflammatory potential (e.g. *Escherichia*) increased with age among PLWH, but not controls. Stool SCFA levels were similar between the two groups yet patterns of associations between individual microbial taxa and SCFA levels differed. Abundance of various genera including *Escherichia* and *Bifidobacterium* positively associated with inflammatory biomarkers (e.g. soluble Tumor Necrosis Factor Receptors) among PLWH, but not among controls.
Interpretation: The age effect on the gut microbiome and associations between microbiota and microbial metabolites or systemic inflammation differed based on HIV serostatus, raising important implications for the impact of therapeutic interventions, dependent on HIV serostatus or age.

1. Introduction

With the advent of effective antiretroviral therapy (ART), the life expectancy of people living with HIV-1 infection (PLWH) has dramatically improved, with recent estimates suggesting that 70% of PLWH will be age 50 or older by 2030 [1]. However, even with effective virologic suppression, chronic HIV infection is associated with a low level inflammatory state that has been linked to an increased risk of comorbidities, as well as geriatric syndromes including dementia and frailty [2–4]. Physiological aging is similarly associated with low levels of inflammation (inflammaging), as measured by increased circulating systemic inflammatory markers such as interleukin-6 (IL-6) and C-reactive protein (CRP) [5–8], and is linked to increased comorbidities [9] [10]. Thus, many PLWH will experience a double inflammatory "hit" that may together accelerate the aging process.

One source of inflammation underlying both HIV infection and aging is the human gut [11–13]. Intestinal epithelial barrier damage and associated microbial translocation has been observed with both HIV infection [14–19] and with physiological aging in animal models [20–24] and humans [25,26]. We previously demonstrated an age-related increase in plasma biomarkers of intestinal epithelial barrier damage (intestinal fatty acid binding protein; IFABP) and microbial translocation (lipopolysaccharide; LPS) in study participants without HIV as well as age-related increases in circulating markers of cellular immune activation (sCD14, sCD27) and inflammation (IL-6, CRP), linking gut barrier

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Research in context

Evidence before this study

The development of highly effective antiviral therapies have greatly increased the life expectancy of people living with HIV (PLWH). However, even with long-term effective treatment, a persistent, low-grade inflammation persists in PLWH which has been linked to increased risk for comorbidities such as diabetes and heart disease. The inflammatory profile and comorbid conditions associated with chronic HIV infection are similar to those reported with aging. Thus, many PLWH will experience a double inflammatory "hit" that may accelerate the aging process. Previous studies have highlighted that gut microbial communities (microbiome) are different in both PLWH and in older persons and this dysbiosis is independently associated with inflammation. However, knowledge of the direct effect of HIV infection on the aging microbiome and potential links to inflammation is lacking in older PLWH.

Added value of study

In this present study, we compared the profiles of bacteria in the stool of older PLWH with those of older persons that were not infected with HIV (controls). We also compared levels of short-chain fatty acids (SCFAs), products produced by bacteria that are important for gut health, and various indicators of inflammation between the two groups. HIV and age were independently associated with distinct changes in the stool microbiome, and age-related microbiome changes differed by HIV serostatus. Stool SCFA levels were similar between the two groups yet patterns of associations between microbiota and SCFA levels differed.

Implications of all the available evidence

To the best of our knowledge, this study represents the first description of the combined effects of HIV infection and aging on the stool microbiome. We show that not only are HIV and age independently associated with distinct changes in the stool microbiome in an older population, but HIV infection and age interact together to shape the microbiome. Our observations highlight that responses to microbial-based therapeutic interventions may differ by HIV serostatus and by age.

leakiness to systemic inflammation [26]. Changes in the composition of stool microbial communities (dysbiosis) have also been observed in both PLWH and with aging (reviewed in [27–29]). Although these studies varied by the age of the population, lifestyle (diet, physical activity), and geographic location, some commonalities exist between stool dysbiotic profiles with HIV and with aging. These include changes in diversity [30–44], increases in abundances of Proteobacteria [30,31,41,43,45–49] and/or alterations in *Prevotella/Bacteroides* ratio [39,41,43,48,50–52]. This dysbiosis has been linked to physiologic consequences: stool profiles characterized by increased abundance of taxa containing commensal bacteria with pathogenic potential, termed pathobionts [53,54] (e.g. Enterobacteriaceae) have been associated with markers of systemic immune activation and inflammation in both ART-treated PLWH [45] and with chronological aging [30,55]. Indicators of biological aging such as frailty have also been linked to increased abundance of pathobionts including Enterobacteriaceae [56], *Eggerthella* [57] and *Prevotella* [58].

Microbial metabolites, such as the short chain fatty acids (SCFA) butyrate, propionate and acetate, are primarily synthesized following fermentation of dietary fiber [59] and play important roles in gut function

including maintaining homeostasis [60]. For example, butyrate serves as a primary source of energy for intestinal epithelial cells and has immunomodulatory properties in the gut immune system [61,62]. Butyrate is produced primarily by bacterial species belonging to the *Roseburia*, *Faecalibacterium* and *Ruminococcus* genera and propionate by members of the Firmicutes (e.g. *Blautia*, *Dialister*) and Bacteroidetes (e.g. *Bacteroides*, *Prevotella*, *Alistipes*) phyla, while acetate is produced by a wide variety of enteric bacteria [63–65]. We and others have demonstrated lower abundance of butyrate-producing bacterial taxa (in gut or stool) among both ART-treated and untreated PLWH [32,34,35,48,51,52,66]. Furthermore, a lower abundance of genera containing butyrate-producing species (*Faecalibacterium*, *Roseburia*) in the gut mucosa is associated with higher systemic markers of immune activation and microbial translocation [34,51,66]. A lower abundance of butyrate-producing bacteria species has also been seen with both chronological age [30,42,46,47,67] and with measures of frailty [56,57,68,69].

Although these studies suggest similarities in parameters of gut dysfunction in both HIV and in aging, a direct understanding of the collective effect in an individual aging with HIV is an understudied area of research. Therefore, the goals of this pilot study were to investigate the combined effect of age and HIV infection on the stool microbiome, and explore the functional impact of microbiome differences through SCFA production and markers of systemic inflammation.

2. Materials and methods

2.1. Participants

Participants were recruited from two clinical studies. Eight participants with and twenty-two without HIV were recruited from the "Exercise in Healthy Aging" study, a randomized controlled trial examining the effect of an exercise intervention on markers of inflammation and physical function (Clinical Trials #: NCT02404792) [70]. Six additional PLWH were recruited from "Assessing Tenofovir Pharmacology in Older HIV-infected Individuals Receiving Tenofovir-based Antiretroviral Therapy", an observational study analyzing the pharmacokinetics of tenofovir in older vs younger individuals with HIV (Clinical Trials #: NCT02304263). All participants were between the ages of 50 and 75 years old; PLWH were on ART for ≥2 years, had an HIV-1 RNA <200 copies/mL, and a CD4 count of >200 cells/μL. Key exclusion criteria included active diarrhea, antibiotic use within the last two weeks, active hepatitis C infection, diabetes requiring insulin, BMI <20 or > 40 kg/m², and chronic steroid usage. All participants provided written, informed consent, and both studies were approved by the Colorado Multiple Institutional Review Board.

2.2. Microbiome analysis

A self-collected stool sample was obtained after consent and prior to any intervention and immediately stored in the participant's home freezer until transfer to a −80C freezer. All samples were processed within 6 months of storage. Stool bacterial profiles were generated by broad-range amplification and sequence analysis of bacterial 16S rRNA genes following established methods [51,71–75]. DNA was extracted using the QIAamp Power Fecal DNA Isolation Kit (QIAGEN, Venlo, Netherlands) and 16S rRNA gene [51,73] amplicons were generated using oligonucleotide primers that target the V3 V4 variable region of the 16S rRNA gene (primers 338F and 805R) and included barcode [76] and Illumina adapter sequences. Illumina paired-end sequencing was performed on the Miseq platform using a 600-cycle (v.3) reagent kit. Paired-end reads were quality-filtered, demultiplexed, merged, and classified using SINA(1.3.0-r23838) [77,78] as previously described [51,71–75]. Operational taxonomic units (OTUs) were produced by clustering sequences with identical taxonomic assignments.

2.3. SCFA extraction and analysis

Stool SCFA was measured using gas chromatography as previously described [79] with the following modification: seven grams of stool taken from disparate sections of each sample were dissolved in purified water (1:5, w/v) and homogenized by vortexing.

2.4. Dietary analysis

Each participant completed a 3-day dietary survey designed to quantify micro and macronutrient intake during the period of stool collection. Raw dietary data was converted to caloric intake by the Nutrition Core at the Colorado Clinical and Translational Sciences Institute [80]. Dietary measurements were averaged over 3 days.

2.5. Soluble biomarker analysis

Plasma levels of soluble CD14 (sCD14), sCD163, IFABP, interleukin-10 (IL-10) and serum levels of tumor necrosis factor alpha (TNFα) and soluble tumor necrosis factor receptor 1 and 2 (sTNFR1 and sTNFR2) were measured by ELISA (R&D Systems; Minneapolis, MN, USA). Plasma CRP and IL-6 were quantitated by ELISA in the Colorado Clinical and Translational Research Center.

2.6. Statistical analysis

Participant characteristics were summarized with frequencies (%) for categorical variables and means (standard deviation) or median (interquartile range [IQR]) for continuous variables. Fisher's exact tests were used to test for differences between PLWH or uninfected controls in categorical variables, and two-sample *t*-tests with unequal variances were used to test for differences in means among continuous variables. Linear regression was used to evaluate differences in dietary intake or SCFA by HIV serostatus.

Relative abundance (RA) was calculated as the number of sequences for a specific taxa standardized to the total number of sequences. Taxa which were present in <5% of the population were collapsed into a single "other" category. Stacked bar plots visually displayed mean RA by HIV serostatus or predicted RA at ages 50 or 70. Low abundance taxa at <2% were collapsed into a single "other" category for visualization purposes on the stacked bar plots. To obtain predicted RA for ages 50 and 70, the intercept and age estimate regression coefficients from the negative binomial regression models (described below) were used to calculate the expected counts for each taxa, given the specified age. The expected counts were calculated using an assumed offset of 10,000 sequences, and the RA was then calculated by dividing the counts by 10,000. Alpha-diversity measures (Sobs, Shannon diversity [H], and Shannon evenness [H/H_{max}]) were compared by HIV serostatus using a linear regression models. Beta-diversity was compared using a permutation-based multiple analysis of variance (PERMANOVA) test and the Bray-Curtis diversity measures calculated at the phylum, family, and genus taxonomic levels. Relationships between HIV serostatus or age with individual taxa were evaluated using generalized linear models assuming a negative binomial distribution and log link function. Interactions between HIV and age were evaluated for each taxa to determine whether the HIV effect differed with age; interaction terms meeting an false discovery rate (FDR) *p*-value threshold <0.20 were included in final models. A FDR correction was applied to control for multiple comparisons, with significance defined as FDR p-value <.05. Explicet [81] (v2.8.3) was used to store/export data and calculate diversity measures, and statistical comparisons of bacterial communities were performed with R software (v 3.4.4) packages *vegan*, *MASS* and *gplots* [82–84].

3. Results

3.1. Study demographics

The 14 PLWH and 22 uninfected controls included in the study were similar by age, body mass index, race, and alcohol and tobacco use (Table 1). All but 1 study participant identified as male. Male PLWH were more likely to report sex with men compared to the male uninfected controls (p < .001). Among the PLWH, the median CD4 count was 570 (IQR: 364) cells/μl and all had a HIV-1 RNA <50 copies/ml.

3.2. Effect of HIV serostatus on the stool microbiome

Stool microbiota profiling was successful in all samples (median of 115,568 sequences/sample, median Goods coverage of 99.97%). Measures of microbial alpha diversity (richness, diversity, evenness) were similar between PLWH and controls (Supplemental Fig. 1). PERMANOVA tests of beta diversity to determine overall compositional differences in microbiota between PLWH and controls at the phylum, family and genus levels are shown in Fig. 1A. No significant differences were seen at the phylum level although the profile of PLWH generally reflected higher RA of Fusobacteria and Proteobacteria in conjunction with decreased Firmicutes. Comparisons at the family and genus levels indicated non-significant overall differences in microbiota between PLWH and controls (p = .059 and p = .096 respectively), most prominently reflected by higher RA of *Prevotellaceae/Prevotella*, *Fusobacteriaceae/Fusobacterium* and lower RA of *Bacteroidaceae/Bacteroides*, *Faecalibacterium* and *Roseburia* among PLWH.

HIV-related effects on the RA of specific taxa between PLWH and controls were explored with negative binomial regression models. No significant differences by HIV serostatus were detected in the RA of any phylum, including the five most abundant phyla: Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria and Proteobacteria (Fig. 1B, Supplemental Table 1). At the family level, Leptotrichaceae (fold change (effect size) 184, p = .04) and Desulfovibrionaceae (fold change 2.94, p = .047) were significantly higher among PLWH compared to controls, while Lachnospiraceaea (fold change 0.72, p = .03) and Oxalobacteraceae (fold change 0.44, p < .0001) were significantly lower (Fig. 1C, Supplemental Table 1). At the genus level (Fig. 1D, Supplemental Table 1), *Eggerthella* (Actinobacteria phylum) was significantly lower (0.17 fold, p = .047) in PLWH. Within the phylum Bacteroidetes, lower RA of *Barnesiella* (0.09 fold, p = .001) and *Odoribacter* (0.70 fold, p < .0001) were noted while *Paraprevotella* was higher (1.53 fold, p < .0001) in PLWH. Numerous genera belonging to the phylum Firmicutes were also higher in PLWH including *Allisonella* (2.85 fold, p < .0001), *Anaerovibrio* (422 fold, p < .0001) and *Howardella* (1.74 fold, p < .0001) with lower RA of *Oscillospira* (0.01 fold, p = .001). Within the phylum Proteobacteria, *Enterobacter* was significantly higher (73.7 fold, p = .001) while *Oxalobacter* was significantly lower (0.43 fold, p < .0001) in PLWH compared to controls.

Table 1
Study participant characteristics.

Characteristic	PLWH (n = 14)	Controls (n = 22)	P value
Male	14 (100)[a]	21 (95)[a]	1.0
Age (years)	61.5 (13.5)[b]	58 (8.8)[b]	0.81
White Race	10 (71)[a]	17 (77)[a]	0.33
Men who have sex with other men	13 (93)[a]	6 (27)[a]	<0.001
Body mass index (kg/m²)	27.0 (4)[b]	27.5 (6)[b]	0.82
Alcohol intake			1.0
>2 drinks/day	1 (7)[a]	2 (9)[a]	
≤2 drinks/day	13 (93)[a]	20 (91)[a]	
Current tobacco smoker	3 (21)[a]	2 (9)[a]	0.11
HIV-1 RNA < 200 copies/mL	14 (100)[a]	NA	NA
CD4 count, cells/μL	570 (364)[b]	NA	NA

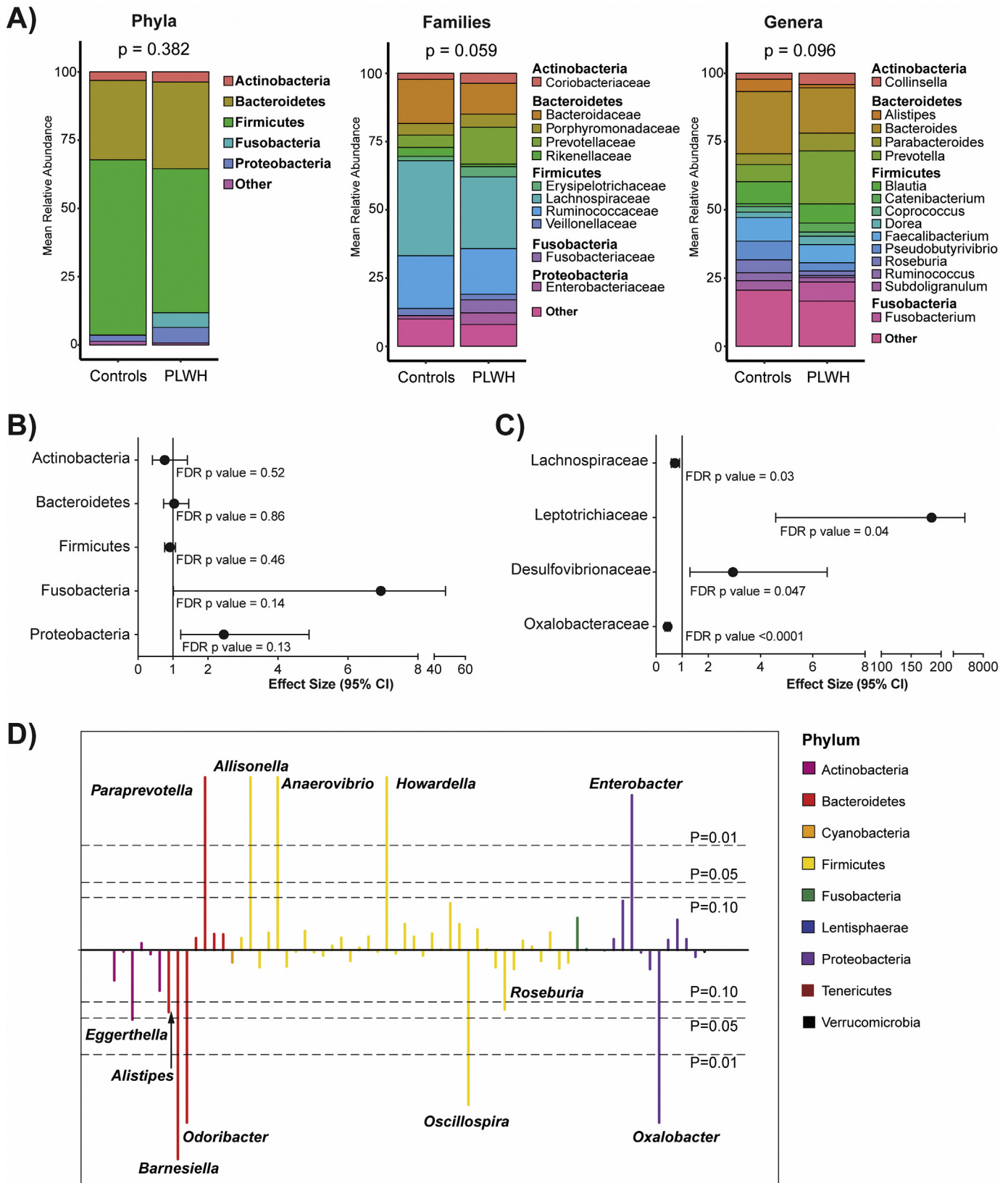Results presented as frequency (%)[a] or median (interquartile range)[b].

Fig. 1. Effect of HIV status on stool microbiota. (A) Stacked bar charts representing the mean relative abundance at the phylum, family, and genus levels. P-values represent beta-diversity differences between groups at each taxonomic level, compared using a PERMANOVA test of Bray-Curtis diversity measures. Taxa with relative abundance <2% were collapsed into a single "Other" category. (B, C) Forest plots were created displaying effect size and 95% confidence interval (CI) for (B) top 5 most abundant phyla and (C) specific families that were significantly different based on FDR p values <.05 (all data can be found in Supplemental Table 1). Effect size >1 indicates taxa relative abundance is higher with HIV infection. (D) Manhattan plot indicating alterations in genera using negative binomial modeling with genus-level taxa as outcomes and HIV status as the predictor. FDR p-values above the center line represent taxa that are higher in PLWH compared to controls. The colors of each genus bar corresponds with the phylum from which the genus originates (legend).
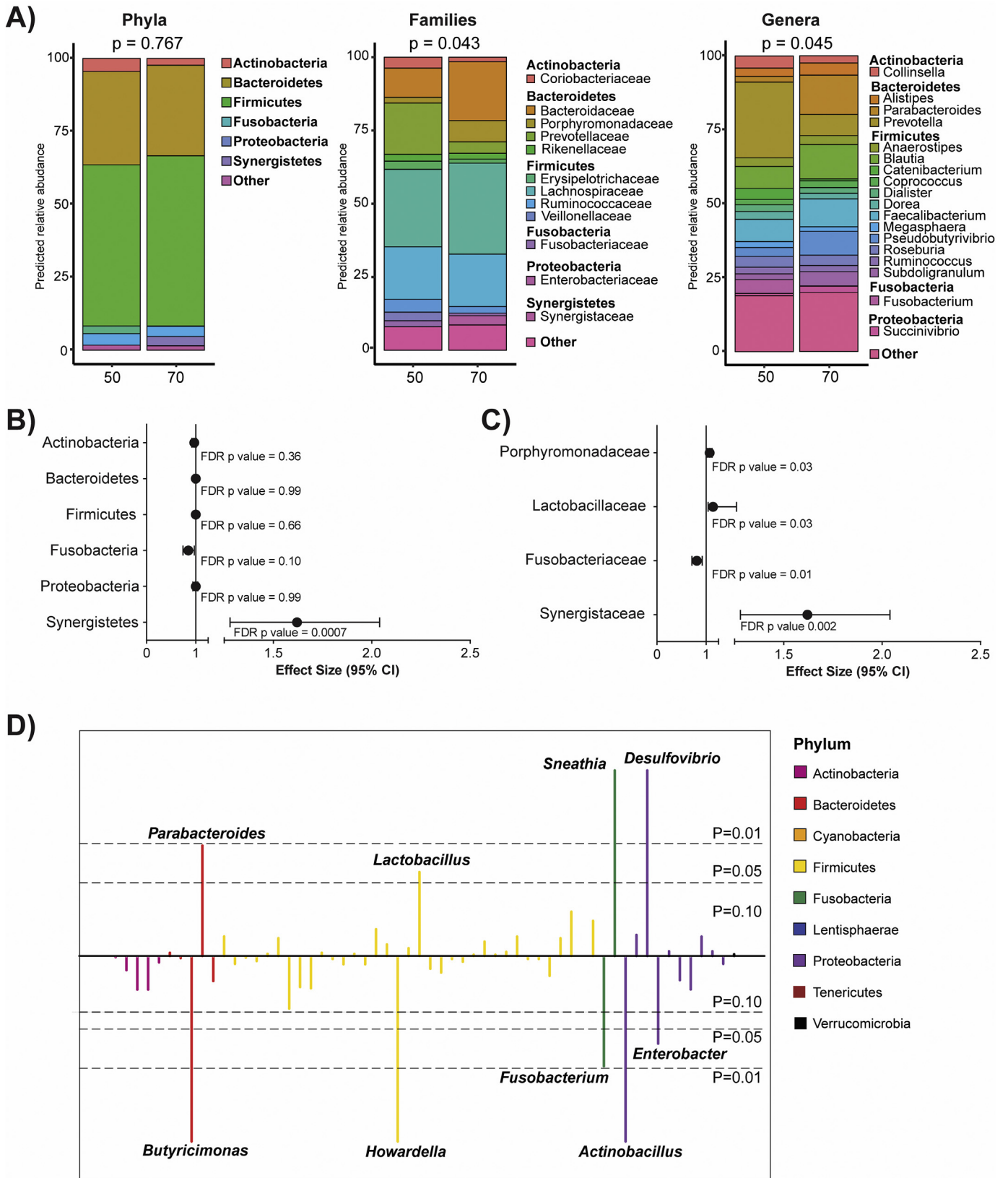
Fig. 2. Effect of age on stool microbiota. (A) Stacked bar charts representing the predicted relative abundance for an individual at age 50 and 70 at the phylum, family and genus taxonomic levels. P-values represent beta-diversity differences between groups at each taxonomic level, compared using a PERMANOVA test of Bray-Curtis diversity measures. Due to the continuous nature of the age variable, a predicted relative abundance was estimated using coefficients from the negative binomial regression models for each taxa. Taxa with relative abundance <2% were collapsed into a single "Other" category. (B, C) Forest plots were created displaying effect size and 95% confidence interval for (B) top 6 most abundant phyla and (C) families that were significantly different based on FDR p values <.05 (all data can be found in Supplemental Table 1). Effect size >1 indicates when the relative abundance increases per year of increasing age. (D) Manhattan plot indicating alterations in genera using negative binomial modeling with genus-level taxa as outcomes and age as the predictor. FDR p-values above the center line represent taxa that significantly increase with age. The colors of each genus bar corresponds with the phylum from which the genus originates (legend).

Among genera known to contain SCFA-producing species [63–65], the RA of *Roseburia* (contains butyrate- and propionate-producing species) and *Alistipes* (contains propionate-producing species) were lower (*Roseburia*: 0.44 fold, p = .07; *Alistipes*: 0.32 fold, p = .06) in PLWH versus controls (Fig. 1D, Supplementary Table 1) but did not reach statistical significance. No significant differences were observed between PLWH and controls in the RA of other genera containing butyrate-producing species (*Butyrivibrio*, *Faecalibacterium*, *Anaerostipes*, *Subdoligranulum*), propionate-producing bacteria (*Bacteroides*, *Blautia*, *Akkermansia*, *Prevotella*, *Dialister* and *Phascolarctobacterium*) or genera that include species that produce either butyrate or propionate (*Coprococcus*) (Supplementary Table 1).

3.3. Effect of age on the stool microbiome

To assess the overall impact of age on the stool microbiome independent of HIV serostatus, the predicted RAs of an individual at age 50 versus age 70 were calculated using data from both groups (Fig. 2A). The predicted profiles suggested that by age 70, microbial communities were different to those at age 50, most notably at family and genus taxonomic levels. Using negative binomial regression models, only the RA of the phylum Synergistetes was found to significantly increase per year of increasing age (1.62 fold; p = .0007) (Fig. 2B, Supplementary Table 2). At the family level (Fig. 2C, Supplemental Table 2), significant increases in the RA of Porphyromonadaceae (1.07 fold; p = .03), Lactobacilliaceae (1.14, p = .03) and Synergistaceae (1.62 fold p = .002) were observed with increasing age, whereas Fusobacteriaceae decreased (0.81, p = .01) (Fig. 2C, Supplemental Table 2). For each year of increasing age, significant increases in a number of genera were also noted including *Parabacteroides* (1.09 fold, p = .01), *Lactobacillus* (1.15 fold, p = .03), *Sneathia* (1.44, p < .0001) and *Desulfovibrio* (1.04, p < .0001) (Fig. 2D, Supplementary Table 2). In contrast, *Butyricimonas* (0.96 fold, p < .0001), *Howardella* (0.96 fold, p < .0001), *Fusobacterium* (0.81, p = .01), *Actinobacillus* (0.10, p < .0001) and *Enterobacter* (0.75 fold, p = .03) decreased in RA for each year of increasing age (Fig. 2D, Supplementary Table 2). No significant age effects were evident among the butyrate- or propionate-producing genera (all p > .1) (Supplementary Table 2).

3.4. Interacting effect of HIV serostatus and age on stool microbiome composition

To understand whether the age effect on the gut microbiome differed in the presence versus absence of HIV infection, we tested an interaction between age and HIV serostatus. The age effect differed between PLWH and controls across multiple taxa (interaction *p*-value <.20) (Table 2, Table 3, Supplementary Fig. 2). For example, while Proteobacteria was lower with increasing age among controls, it was not in PLWH (Table 2). Conversely, a number of bacterial families (e.g. Bifidobacteriaceae, Bacteroidaceae, Peptostreptococcaceae) and genera (e.g. *Bifidobacterium*, *Bacteroides*) were significantly higher with increasing age in PLWH, but not in controls (Tables 2, Table 3, Supplementary Fig. 2). In some instances, significant changes were observed in both PLWH and controls, but were opposite in direction. For example, Leptotrichiaceae and the genera *Butyricimonas*, *Escherichia* and *Oxalobacter* significantly increased with age in PLWH, but were significantly lower with age in controls (Table 2, Table 3, Supplementary Fig. 2). Significant age and HIV interactions were also observed for some genera known to contain butyrate- or propionate-producing bacteria. *Alistipes* (containing propionate-producing spp.) increased with age in PLWH, but was not significantly different in controls; *Butyrivibrio* (butyrate-producers) decreased with age in both PLWH and controls, but to a significantly greater degree in controls (Table 3, Supplementary Fig. 2).

3.5. Stool SCFA levels, dietary intake and associations with stool microbiota

Levels of stool SCFAs and dietary intake were compared between PLWH and controls using linear regression with HIV serostatus as the predictor (Table 4). Stool propionate levels were lower among PLWH (mean 10.2 mmol/g) compared to controls (14.0 mmol/g), although this did not reach statistical significance (p = .075). Acetate (PLWH: 37.7 mmol/g, Controls: 42.0 mmol/g; p = .35) and butyrate levels (PLWH: 7.7 mmol/g, Controls: 7.3 mmol/g; p = .87) were not significantly different by HIV serostatus. Dietary intake was generally similar between the two groups with the exception of soluble fiber intake which was slightly higher in PLWH compared to controls (9.5 g vs 7.1 g; p = .073).

Next, we explored associations between stool SCFA levels and dietary intake measures with the RA of the top 25 bacterial genera (based on all study participants) and noted differences in patterns of significantly strong associations, based on HIV status (Fig. 3). For instance, the RA of *Prevotella* was strongly positively correlated with stool levels of all 3 SCFAs while *Subdoligranulum* and *Alistipes* were inversely correlated with acetate and propionate levels in PLWH, but not controls. Furthermore, in PLWH, but not controls, *Bifidobacterium* and *Dialister* RA were positively correlated with dietary soluble fiber intake. *Fusobacterium* RA was positively correlated with fat/fiber consumption

Table 2
Age Effect Among People Living with HIV (PLWH) and Controls, at Phylum and Family Taxonomic Ranks.

	Age effect among PLWH			Age effect among Controls			Age and HIV Interaction
	Effect Size	95% CI	P value	Effect Size	95% CI	P value	P value*
Phylum							
Cyanobacteria	0.74	0.60, 0.90	0.022	0.98	0.84, 1.12	0.82	0.087
Proteobacteria	1.02	0.95, 1.09	0.68	0.90	0.84, 0.96	0.016	0.032
Family							
Phylum Actinobacteria							
Bifidobacteriaceae	1.16	1.02, 1.30	0.043	0.99	0.88, 1.10	0.91	0.10
Phylum Bacteroidetes							
Bacteroidaceae	1.15	1.08, 1.22	0.0001	1.00	0.94, 1.06	0.99	0.005
Phylum Firmicutes							
Peptostreptococcaceae	1.28	1.16, 1.41	<0.0001	1.00	0.91, 1.09	0.99	0.001
Phylum Fusobacteria							
Leptotrichiaceae	1.36	1.33, 1.38	<0.0001	0.35	0.14, 0.84	0.043	0.008
Phylum Proteobacteria							
Alcaligenaceae	1.04	0.94, 1.13	0.53	0.93	0.85, 1.01	0.14	0.13
Enterobacteriaceae	0.97	0.84, 1.12	0.80	0.82	0.72, 0.93	0.012	0.13
Oxalobacteraceae	1.12	1.08, 1.15	<0.0001	0.95	0.93, 0.96	<0.0001	<0.0001

* Taxa with an HIV-age interaction of FDR p value of ≤0.20 are included, with findings suggesting that the age effect differs by HIV serostatus. The effect size can be interpreted as the per year increase (if >1) or decrease (if <1) in relative abundance of each taxa. CI = confidence interval.

Table 3
Age Effects Among People Living with HIV (PLWH) and Controls, by Genus Taxonomic Rank.

	Age effect among PLWH			Age effect among Controls			Age and HIV
	Effect Size	95% CI	P value	Effect Size	95% CI	P value	Interaction[*]
Phylum Actinobacteria							
Bifidobacterium	1.16	1.02, 1.31	0.044	0.99	0.88, 1.23	0.92	0.10
Eggerthella	1.04	0.90, 1.18	0.67	0.89	0.78, 1.00	0.10	0.14
Phylum Bacteroidetes							
Alistipes	1.15	1.05, 1.25	0.0058	1.00	0.92, 1.08	0.98	0.046
Bacteroides	1.15	1.08, 1.22	0.0001	1.00	0.94, 1.06	0.99	0.004
Barnesiella	1.14	1.02, 1.27	0.038	0.99	0.89, 1.09	0.92	0.096
Butyricimonas	1.02	1.00, 1.02	0.0025	0.95	0.93, 0.95	<0.0001	<0.0001
Paraprevotella	0.99	0.98, 1.00	0.30	0.88	0.86, 0.89	<0.0001	<0.0001
Phylum Firmicutes							
Anaerostipes	1.05	0.97, 1.13	0.30	0.96	0.89, 1.03	0.37	0.15
Butyrivibrio	0.95	0.94, 0.96	<0.0001	0.75	0.74, 0.76	<0.0001	<0.0001
Dorea	0.95	0.95, 0.95	<0.0001	1.01	1.01, 1.01	<0.0001	<0.0001
Flavonifractor	1.23	1.08, 1.40	0.0041	0.99	0.87, 1.11	0.92	0.035
Holdemania	1.11	1.01, 1.21	0.044	0.98	0.90, 1.06	0.69	0.074
Howardella	0.94	0.92, 0.94	<0.0001	0.99	0.97, 0.99	0.059	<0.0001
Pseudobutyrivibrio	0.96	0.87, 1.05	0.43	1.09	0.99, 1.18	0.096	0.087
Phylum Proteobacteria							
Escherichia	1.37	1.19, 1.56	<0.0001	0.82	0.73, 0.93	0.005	<0.0001
Oxalobacter	1.12	1.08, 1.15	<0.0001	0.95	0.93, 0.96	<0.0001	<0.0001

[*] Taxa with an HIV-age interaction of FDR p value of <0.20 are listed, with findings suggesting that the age effect differs by HIV serostatus. The effect size can be interpreted as the per year increase (if >1) or decrease (if <1) in relative abundance of each taxa. CI = confidence interval.

whereas *Ruminococcus* was inversely associated with this dietary measure. Few correlations between bacterial genera and stool SCFA or dietary intake were observed in controls. The RA of *Dorea* was strongly correlated with fat intake in controls, but not in PLWH.

3.6. Impact of HIV infection on systemic biomarkers and associations with stool microbiota

PLWH had higher levels of sCD163, sCD14, and sTNFR2 compared to uninfected controls (Table 5). Levels of IL-10, IFABP, TNFα, sTNFR1, CRP, and IL-6 were not statistically different (Table 5). Again, major differences were noted in the patterns of associations of microbiota and inflammatory biomarkers between PLWH and controls. For example, among PLWH, *Escherichia* and *Subdoligranulum* were positively correlated with sTNFR1 and sTNFR2, *Bifidobacterium* with TNFR1 and IL-6, and *Prevotella* inversely correlated with sTNFR1 levels (Fig. 4). Among controls, but not PLWH, *Faecalibacterium* positively correlated with levels of CRP, sTNFR1 and IL-10.

Table 4
Stool Short Chain Fatty Acid (SCFA) and dietary intake differences between People Living with HIV (PLWH) and controls.

	PLWH (n = 14)	Controls (n = 22)	P value
SCFA Total (mmol/g)	55.6 (22.3)	63.4 (21.9)	0.31
Acetate (mmol/g)	37.7 (14.1)	42.0 (11.7)	0.35
Propionate (mmol/g)	10.2 (4.9)	14.0 (7.6)	0.075
Butyrate (mmol/g)	7.7 (6.1)	7.3 (5.0)	0.87
	PLWH (n = 13)	Controls (n = 22)	P value
Average daily energy intake (kcal)	2229 (629)	2034 (647)	0.39
% Carbohydrates	43.3 (6.6)	44.80 (10.9)	0.67
% Fat	39.6 (6.0)	36.5 (8.5)	0.25
Fiber (g)	23.0 (8.8)	20.5 (7.5)	0.36
Soluble fiber (g)	9.5 (5.2)	7.1 (2.3)	0.073
Insoluble fiber (g)	13.2 (4.5)	12.8 (6.3)	0.86
Fat/fiber ratio	4.9 (2.0)	4.7 (2.1)	0.82

Levels of SCFAs or dietary intake measurements were compared between PLWH and controls using linear regressions with HIV status as the predictor. Data are presented as mean (standard deviation). Dietary intake is missing for one PLWH.

4. Discussion

An emerging body of evidence suggests that gut dysbiosis occurs in both the normal aging process and among PLWH, and that these alterations in gut microbial communities may contribute to the low grade chronic inflammatory state that underlies age-associated co-morbidities [11,27,85]. However, evaluation of the effect of HIV infection on the aging microbiome, and the potential links to systemic inflammation is lacking. To the best of our knowledge, this study represents the first description of the combined effects of aging and HIV infection on the stool microbiome and demonstrates that, not only are HIV and age independently associated with distinct changes in the stool microbiome in an older population, but HIV infection and age interact to shape the microbiome. Furthermore, within this older population, associations between microbes and stool metabolites, dietary intake characteristics, and inflammatory biomarkers differed based on HIV serostatus.

Interaction analyses highlighted that numerous bacterial taxa increased with age in PLWH, but were either decreased or not significantly changed among controls. Many of these bacteria have been linked to gut inflammatory diseases. For example, *Escherichia* belongs to the family Enterobacteriaceae, facultative anaerobes that are commonly increased in abundance in inflammatory bowel disease (IBD) and colorectal cancer [86–88]. Similarly, Peptostreptococcaceae was reported to be overrepresented in persons with colorectal cancer [86,89]. Intriguingly, *Bacteroides* spp. also increased with age in PLWH, but not in controls. This genus typically plays an important role in immune regulation including the induction of regulatory T cells [90]; however certain strains of *B. fragilis* have also been linked to gut diseases including colorectal cancer [86]. Notably, a number of the bacterial species that increased with age among PLWH, but not in controls, such as *Oxalobacter*, *Alistipes* and *Bifidobacterium*, potentially have health-promoting properties. *Oxalobacter* spp. are important in degrading oxalate, excessive amounts of which are associated with pathological conditions including kidney stone formation [91,92]. *Alistipes* contains propionate-producing organisms and *Bifidobacterium*, a frequent component of probiotic mixtures, has anti-inflammatory properties [93]. Some of these findings may be related to dietary differences. For example, PLWH in our study tended to have higher fiber intake; *Bifidobacterium* utilize dietary fiber as a growth substrate [94], and, indeed, *Bifidobacterium* RA was associated with greater fiber intake
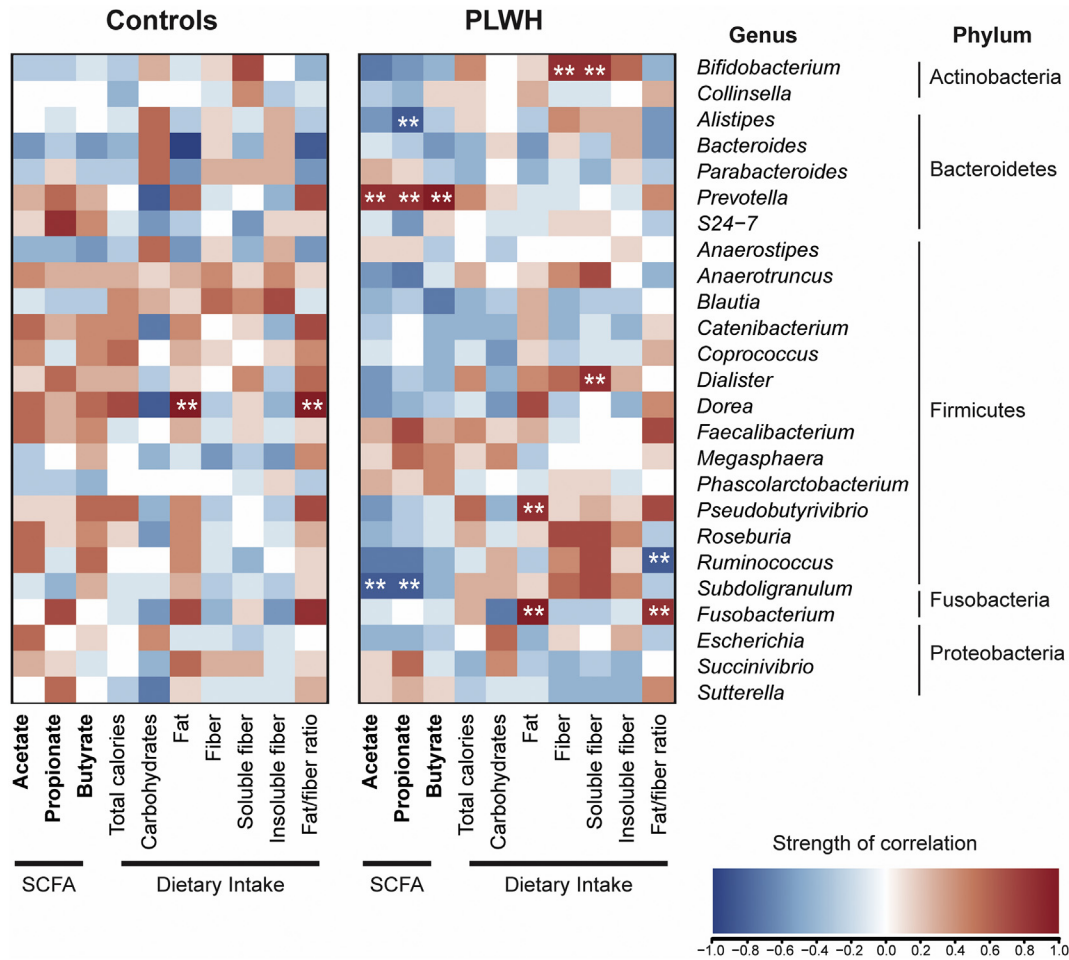
Fig. 3. Associations between stool SCFA levels and dietary intake with stool microbiota. Associations between the top 25 most abundant genera based on all study participants with levels of stool SCFA levels (bolded text) and dietary intake in controls (N = 22) and in PLWH (N = 13; dietary intake was unavailable from one study participant). Genera are grouped by phylum. Pearson correlations were determined with red shading representing positive association and blue shading indicating a negative association. Correlations that were both significant (p < .05) and strong correlations based on r > 0.6 or < −0.6 are indicated with white asterisks (**). Units for SCFA measurements are mmol/g. Macronutrients are calculated relative to total energy intake: Carbohydrates (%), Fat (%), Soluble and Insoluble fiber (g).

among PLWH. Increased abundance may also be a direct result of probiotic use; however probiotic use was not routinely collected in this study.

Numerous studies have demonstrated that PLWH manifest stool dysbiosis, despite effective viral suppression with ART (reviewed in [85]). Indeed, recent studies have shown that ART drugs themselves may contribute to dysbiosis, although potential mechanisms by which this occurs remain to be fully elucidated (reviewed in [95]). Therefore, in addition to determining if the impact of age differed among PLWH and controls, the design of our study also permitted investigation into

Table 5
HIV effect on systemic biomarker levels.

Biomarker	HIV Effect	95% CI	P value
IL-6 (pg/ml)	0.64	−0.49, 1.77	0.28
TNFα (pg/ml)	0.21	−0.08, 0.5	0.17
sTNFR1 (pg/ml)	199.7	−11.3, 410.7	0.079
sTNFR2 (pg/ml)	744.2	283.1, 1205.2	0.0034
CRP (mg/L)	1.50	−0.44, 3.43	0.14
sCD163 (ng/ml)	134.0	27.6, 240.3	0.019
sCD14 (ng/ml)	469.7	226.1, 713.3	0.0006
IFABP (pg/ml)	555.9	−1596.0, 2707.7	0.62
IL-10 (pg/ml)	0.17	−1.98, 2.32	0.88

Levels of biomarkers were compared between PLWH and controls using linear regressions with HIV status as the predictor. Effect size and confidence (CI) intervals are shown with effect size >1 indicating higher levels in PLWH.

the interrelationships between HIV, the gut microbiome, and functional outcomes (SCFA production and systemic inflammation) in this older cohort of PLWH. Similar to a number of previous studies of stool microbiota of ART-treated PLWH, we observed higher RA of bacteria belonging to the Enterobacteriaceae (*Enterobacter*) and Prevotellaceae (*Paraprevotella*) families and lower relative abundance of *Barnesiella*, *Alistipes*, and *Roseburia* in our participants with HIV [45,48,49,52,96]. The latter two genera are of particular relevance given that they contain species with the capacity to produce butyrate or propionate [63–65]. Indeed, PLWH tended to have lower levels of stool propionate than controls potentially due to lower abundances of the propionate-producing species *Alistipes putredinis* or *Roseburia inulinivorans* [63–65]. However, in PLWH, the RA of the genus *Alistipes* was inversely correlated with levels of stool propionate; thus additional studies with species identification are required to fully delineate the role decreased *Alistipes* may play in HIV-related pathogenesis. While *Roseburia* contains a number of species with butyrate-producing ability, levels of butyrate were similar between the two groups. However, it is important to note that butyrate is produced following fermentation of soluble dietary fibers [94], and the higher soluble fiber intake among PLWH may account for the lack of difference in butyrate levels. Although not significantly different between PLWH and controls, *Subdoligranulum*, known to contain the butyrate-producing bacteria *S. variabile* [63,64], inversely correlated with levels of acetate and propionate in PLWH. This observation may
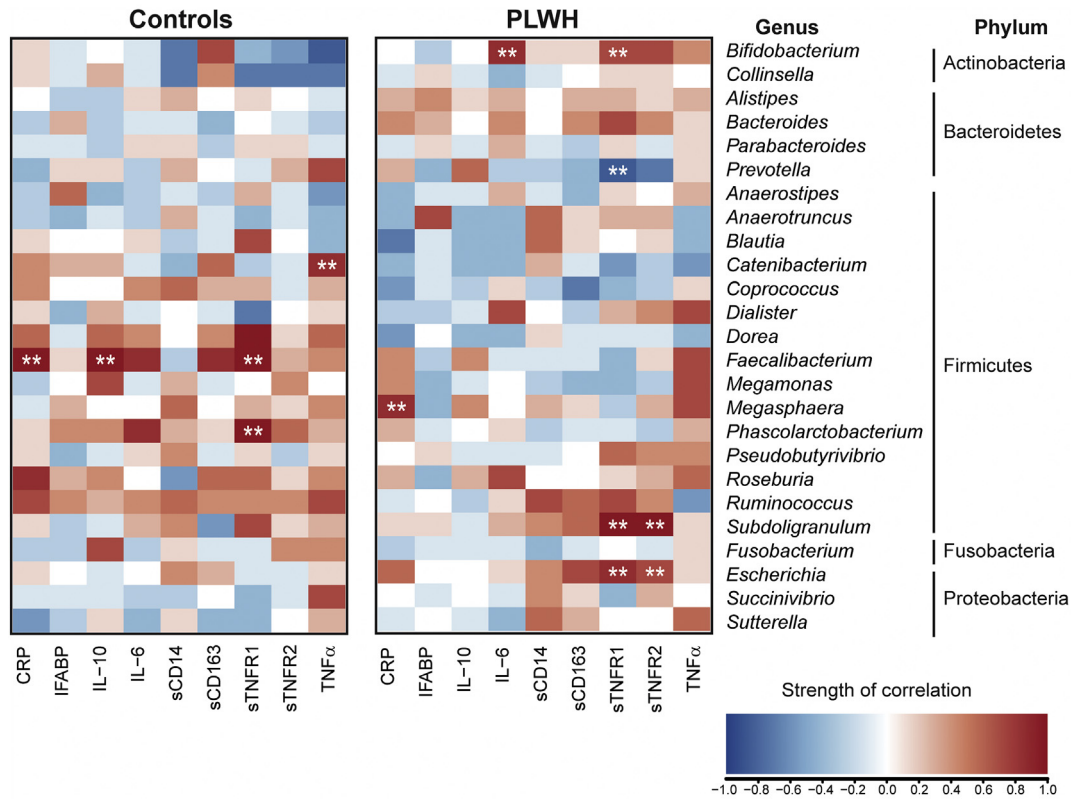
Fig. 4. Associations between systemic inflammatory and immune activation biomarkers with stool microbiota. Associations between the top 25 most abundant genera based on all study participants with levels of systemic biomarkers indicative of inflammation and immune activation in controls (N = 22) and in PLWH (N = 14). Genera are grouped by phylum. Pearson correlations were determined with red shading representing positive association and blue shading indicating a negative association. Correlations that were both significant (p < .05) and strong correlations based on r > 0.6 or < −0.6 are indicated with white asterisks (**). CRP = C reactive protein; IFABP = intestinal fatty acid binding protein; IL-10 = interleukin 10; IL-6 = interleukin 6; sCD14 = soluble CD14, sCD163 = soluble CD163, sTNFR = soluble tumor necrosis factor receptor [1] and [2]; TNFα = tumor necrosis factor alpha.

reflect the process of bacterial cross-feeding whereby the metabolic end products produced by one bacteria are utilized by another as has been reported for other known butyrate-producing bacteria and the utilization of acetate for butyrate formation [97].

Few studies similarly investigating stool microbiomes in ART-treated PLWH have detailed alterations in the remaining genera that we observed to be lower (*Odoribacter*, *Oscillospira*, *Oxalobacter*, *Eggerthella*) or higher (*Allisonella*, *Anaerovibrio*, *Howardella*) in PLWH versus controls. Lozupone and colleagues noted lower abundance of *Odoribacter* in ART-treated PLWH relative to uninfected controls [96]. *Eggerthella* was higher among PLWH receiving protease inhibitors in combination with nucleoside/tide reverse transcriptase inhibitors (NRTI) relative to uninfected controls, but was not different among those on other NRTI combination therapies, suggesting associations with both ART regimen and HIV [38]. It is also important to note that *Allisonella*, *Anaerovibrio* and *Howardella* were enriched in MSM versus non-MSM from prior studies [44]; given that our controls were not matched for sexual behavior, the overall higher abundance of these specific genera may reflect sexual behavior rather than HIV infection itself.

As expected, we observed that a number of biomarkers of systemic immune activation and inflammation were higher among PLWH. Some biomarkers did not differ significantly by HIV serostatus (e.g. IL-6, CRP): as age is a major driver of both IL-6 and CRP, any HIV-related differences may have been attenuated by the age effects. Furthermore, we identified expected and unexpected associations between the stool microbiome and systemic inflammation in PLWH, with similar associations not observed in controls. In keeping with its role as a potentially inflammatory bacterium, *Escherichia* RA was associated with levels of sTNFR1 and sTNFR2 in PLWH whereas *Prevotella*, a genus also known to contain species linked to inflammatory diseases (reviewed in [98]),

inversely correlated with sTNFR1 in PLWH. Moreover, *Bifidobacterium* RA positively associated with sTNFR1 and IL-6 levels, an additional unexpected finding given its previously described anti-inflammatory capabilities and the prominent use of *Bifidobacterium* strains in probiotics. In older persons without HIV infection, few associations between microbiota taxa and inflammatory biomarkers were noted with the exception of *Faecalibacterium* which positively correlated with IL-10, CRP and sTNFR1. The association with IL-10 is congruent with the reported ability of strains of *F. prausnitzii* to induce IL-10 production from human peripheral blood immune cells and myeloid dendritic cells in vitro [99,100], yet positive correlations with CRP and sTNFR1, indicators of systemic inflammation, contrast with the generally accepted anti-inflammatory role of *F. prausnitzii* [101,102]. Taken together, these observations suggest some intriguing links between microbiota and systemic inflammation in older persons both with and without HIV infection; however, it is essential to emphasize that these associations do not indicate causality. Further studies will be required to determine if the association holds in a larger study population and if they are direct associations driven by individual species or indirectly driven by alterations in networks of bacterial communities. It will also be important to evaluate if, like enterotoxigenic strains of *E. coli* and *B. fragilis*, certain species traditionally considered to reflect a healthy microbiome, have undergone adaptive evolution due to environmental pressures (i.e., bacterial community changes or interactions with host immune system) resulting in positive selection of virulence genes [103–105].

A number of study limitations should be noted: the sample size was small, which limited our ability to adjust for numerous confounders, including sexual behaviors [44,106,107] and prebiotic/probiotic use, that may influence the microbiome. Few women or minorities were enrolled therefore generalizability is limited. Lastly, bacterial classification was

limited to genus-level identification due to short read sequencing. Overall, our findings are preliminary and need to be confirmed in larger, more diverse participant populations.

In summary, this study has demonstrated that not only are alterations in the gut microbiome present in older PLWH, but HIV influenced age-associated effects on the gut microbiome. Furthermore, associations between the stool microbiome and microbial metabolite levels, dietary factors and inflammatory biomarkers differed by HIV serostatus. A 'one-bug-fits-all' interpretation may therefore not be applicable, because microbiome changes in one population may not have the same implications as in another population. This also raises important implications for therapeutic interventions: targeted replacement of certain microbial taxa may have different effects depending on the host. Further research is needed to confirm our findings in a larger sample size with a wider age range, and to test whether the response to therapeutic interventions such as prebiotics, probiotics or fecal microbial transplantation, differ by HIV serostatus and by age.

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Author contributions

KME, JL, SS, DF, CW, and MK contributed to the study design. JL, KME, and SS contributed to data collection. RJ and MK led the statistical analysis, with input from JL, CW, and KME. JL, DF, CR, DI, YT, XZ, and BH analyzed samples for the microbiome and/or SCFA outcomes; JH analyzed samples for the dietary outcomes. JL prepared the initial draft with substantial input from SD, CW, and KME. All authors provided input on the manuscript and have reviewed and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2019.01.033.

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