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Gut *Lactobacillales* are associated with higher CD4 and less microbial translocation during HIV infection

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Abstract

Objective—Early HIV infection is characterized by a dramatic depletion of CD4 T cells in the gastrointestinal tract and translocation of bacterial products from the gut into the blood. In this study, we evaluated if gut bacterial profiles were associated with immune status before and after starting antiretroviral therapy (ART).

Design—We evaluated the gut microbiota of men recently infected with HIV (n = 13) who were participating in a randomized, double-blind controlled trial of combination ART and maraviroc versus placebo and who were followed for 48 weeks.

Methods—To evaluate the gut microbiota of participants, we pyrosequenced the bacterial populations from anal swabs collected before and longitudinally after the initiation of ART. Associations of the gut flora with clinical variables (lymphocyte profiles and viral loads), activation and proliferation markers in peripheral blood mononuclear cells and gut biopsies (measured by flow cytometry) and markers of microbial translocation (lipopolysaccharide and soluble CD14) were performed by regression analyses using R statistical software.

Results—Using pyrosequencing, we identified that higher proportions of *Lactobacillales* in the distal gut of recently HIV-infected individuals were associated with lower markers of microbial translocation, higher CD4% and lower viral loads before ART was started. Similarly, during ART, higher proportions of gut *Lactobacillales* were associated with higher CD4%, less microbial translocation, less systemic immune activation, less gut T lymphocyte proliferation, and higher CD4% in the gut.

Conclusion—Shaping the gut microbiome, especially proportions of *Lactobacillales*, could help to preserve immune function during HIV infection.

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Conflicts of interest J.P.S., S.G., M.M., C.A.S., M.Y.K., S.R.V., D.P., P.S.J., J.A.Y. and S.J.L. do not have any commercial or other associations that might pose a conflict of interest. D.D.R. has served as a consultant for Bristol-Myers Squibb, Gilead Sciences, Merck & Co, Monogram Biosciences, Biota, Chimerix, Tobira, and Gen-Probe. D.M.S. has received grant support from ViiV Pharmaceuticals and consultant fees from Gen-Probe.

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Keywords

gut-associated lymphoid tissue; gut microbiome; immune activation; microbial translocation; pyrosequencing

Introduction

The gut-associated lymphoid tissue (GALT) is a crucial part of the immunological network that maintains the integrity of the gastrointestinal (GI) tract against gut microbes [1]. Early HIV infection results in substantial depletion of CD4 T cells, preferentially in the GALT [2–4]. Consequences of this depletion include mucosal immune dysfunction, increased permeability of the gut and ultimately translocation of bacterial products [5], which contribute to chronic immune activation [6–8]. Immune activation is one of the strongest predictors of HIV disease progression [9–11]. Antiretroviral therapy (ART) seems to at least partially restore gut integrity [12–14] with marginal reduction in microbial translocation, but not to the levels seen in HIV-uninfected persons [6,7].

The GI tract is colonized by numerous commensal microorganisms, which can be identified by next-generation sequencing (NGS). NGS of the gut microbiome has revealed that certain disease states, such as inflammatory bowel disease, HIV, and others may be associated with modified gut flora [15–17]. Certain microbes may interact with the GALT to preserve gut integrity [18], thereby decreasing the likelihood of translocation of microbial products [19]. For example, consumption of probiotics and specifically *Lactobacillales* may modulate inflammatory responses, eradicate potential pathogens, and reduce gut permeability [19–24]. Manipulation of the gut flora may therefore benefit immune recovery during HIV infection. In this study, we conducted a metagenomics analysis to longitudinally characterize the changes of the gut microbiome during acute and early HIV infection and examined the effects of ART on this microbiome by associating clinical and immunological factors before starting and during ART.

Material and methods

Study cohort

Eligible participants were men who had sex with men (MSM) co-enrolled in the San Diego Primary Infection Cohort (n = 13) and a randomized, double-blind controlled trial of combination ART and maraviroc versus placebo. The Institutional Review Board of our center approved this study and all participants provided written informed consent. All patients initiated ART within 2 weeks of study enrollment with a combination of tenofovir, emtricitabine and ritonavir-boosted atazanavir, with or without maraviroc. The double-blind clinical trial is ongoing with all patients remaining blinded to maraviroc use. Anal swabs, blood, semen, peripheral lymphocyte profiles, and HIV levels (Amplicor, Roche) were collected at baseline (within a week before the initiation of ART) and approximately every 4 weeks thereafter for 48 weeks. A proportion of participants consented to repeat colonoscopies to obtain mucosal biopsies of the rectosigmoid colon and terminal ileum. Epidemiological, behavioral risk and HIV-related data were also collected from the participants. We determined estimated duration of infection (EDI) using results of serologic and virologic tests as described previously [25].

DNA extraction and viral quantification from peripheral blood mononuclear cells, stool, and semen

Genomic DNA was extracted from 5 million peripheral blood mononuclear cells (PBMCs) for each timepoint using QIAamp DNA Mini Kit (Qiagen) per manufacturer's protocol. Extracted DNA was eluted in 100 μl elution buffer and total proviral HIV-1 DNA was quantified by real-time PCR in an ABI 7900HT thermocycler (Applied Biosystems) with virus-specific PCR primers and two DNA-locked nucleic acids (LNA) detection probes as previously published [26]. Cellular input was normalized with beta-actin PCR as previously described [27] and results were expressed in HIV DNA copies per 1 million actin cells equivalents.

Stool DNA from anal swabs was extracted using the QIAamp Stool DNA kit (Qiagen) per manufacturer's protocol except that the elution was performed in 200 μl incubated for 5 min before the final spin. DNA extraction and quantification of cytomegalovirus (CMV) in seminal plasma and stool DNA was done as previously published [28].

Amplification of bacterial DNA and pyrosequencing

Amplification of the V6 hypervariable region of the 16 s rDNA gene was carried out in a 50 μl reaction using the highly purified Amplitaq Gold Low DNA polymerase (Applied Biosystem) to reduce bacterial contamination as manufacturer's protocol with primers previously described [15]. The cycling conditions followed were: initial activation at 93°C for 15 min; 30 cycles of 95°C for 30 s, 57°C for 30 s and 72°C for 1 min; followed by a final extension at 72°C for 10 min. Samples were run in duplicates and a 1% agarose gel electrophoresis was used to confirm the ~110 bp size of product. Duplicate samples were combined and purified immediately after reaction (Qiagen PCR Purification Kit). We used the Agilent 2100 BioAnalyzer to quantify and assess purity of DNA. Amplicon pyrosequencing (Roche 454 FLX Titanium) was performed using standard protocols [29].

Classification of bacteria

We considered bacterial sequences with at least 90 continuous base pairs, which contained a quality score of at least 20 [30–32] for metagenomics analyses. We classified sequences to the order level using the Ribosomal Database Project [33]. We used the tool ESPRIT [34] to assign operational taxonomic units (OTU) based on genetic distance to unclassified sequences. With a cut-off of 10%, we built a consensus sequence for each OTU and classified it using small subunit rRNA taxonomy and alignment pipeline (STAP) [35]. We evaluated orders of bacteria in common across all samples and sequences that were unique to an individual were categorized as 'Other'.

Microbial translocation markers

Limulus Amebocyte Lysate QCL-1000 and Quantikine ELISA Human sCD14 Immunoassay were used to measure plasma lipopolysaccharide (LPS) and soluble CD14 (sCD14), respectively, following manufacturer's protocols.

Flow cytometry

Fresh whole blood was collected at weeks 0,12, 24, and 48 and processed using density gradient centrifugation to obtain PBMCs. Cells were incubated with antibodies for surface marker staining, before fixation and permeabilization for intracellular assays (eBioscience). We used the following antibody combinations to evaluate immune activation and proliferation in T cells: HLA-DR–FITC, CD45RO–PE, CD38–PE-Cy7, CD27–APC, CD3–APC-Cy7, CD4–PerCP-Cy5.5 and CD8–Pacific Blue, and Ki67–FITC, CD45RO–PE, CD27–APC, CD3–APC-Cy7, CD4–PerCP-Cy5.5 and CD8–Pacific Blue, (BD Biosciences).

These measures were used to assess naïve ($CD45RO^{-}CD27^{+}$), central memory ($CD45RO^{+}CD27^{+}$) and effector memory ($CD45RO^{+}CD27^{-}$) in CD4 and CD8 T-cell subsets.

Rectosigmoid junction and the terminal ileum gut biopsies were available for a subgroup of nine patients at weeks 0 and 48 and interval biopsies at weeks 12 or 24 for a proportion of the subgroup. Tissue samples were incubated with collagenase and DNAse before passage through a cellular strainer (PGC Scientifics) and viably stored at −140°C. We assayed proliferation of collected tissue on viably thawed mucosal mononuclear cells using the following combination of antibodies: Ki67–FITC, CD45RO–PE, CD27–APC, CD3–APC–Cy7, CD4–PerCP-Cy5.5, CD8–Pacific Blue (BD Biosciences) and Aqua-L-D (Invitrogen) All samples were run on the BD FACSCanto (BD Biosciences) and data were analyzed with FlowJo software (Tree Star Inc.).

Statistical analyses

Methods of unsupervised clustering, statistical tests and regression analyses were implemented utilizing R statistical software. A two-tailed Mann–Whitney test was used to assess statistical difference between groups and comparison of bacterial proportions between groups was performed using Fisher exact test.

Normality of each order of bacteria and clinical variables was tested using a Shapiro test with a significance of $P < 0.05$. We evaluated cross-sectional associations between gut bacterial profiles (GBP) and clinical and immunological variables using fixed effects linear models. We utilized mixed effects linear models for analysis of longitudinal data to adjust for repeated measurements (packages *lme4* and *languageR*).

We modeled each participant's GBP as a vector $\overrightarrow{x} = (x_1, x_2, \ldots, x_{13})$, where x_i corresponds to every order of bacteria classified, excluding the category 'Other'. We calculated the sample variance of a GBP generalizing the sample variance formula for vector calculations. We measured intra-patient variability calculating the sample variance of the GBP at all timepoints for that particular patient. We computed inter-patient variability taking all the samples at each timepoint available.

Results

Study cohort

Study participants were 10 Caucasians and three Asians with an average age of 33 years and EDI of 6.5 weeks. All participants started ARTwithin 1 week of enrollment and were followed for 48 weeks. A summary of the clinical variables measured at baseline is provided in Table 1. We excluded two participants (L and M) from the baseline analysis because of antibiotic use shortly before the collection of their stool samples.

Classifications of gut bacterial profiles

The study classified proportions of distal gut bacterial flora at the order level (13 orders) using the V6 region of the bacterial 16s rDNA. The overall median intra-patient variability in GBP was significantly lower than inter-patient variability ($P = 0.006$), as reported previously [36]. An unsupervised clustering analysis of baseline orders of bacteria revealed two distinct GBP (Fig. 1). The first GBP cluster (Group 1) showed significantly lower proportions of Lactobacillales, Enterobacteriales, Pseudomonadales, Xanthomonadales, Aeromonadales, Rhizobiales, and Neisseriales ($P = 0.006$ for all) and higher proportions of *Bacteroidales* ($P = 0.01$) and *Clostridiales* ($P = 0.04$) compared with the second GBP cluster (Group 2) (Fig. 1 and Supplementary Fig. 1, http://links.lww.com/QAD/A335).

Interestingly, participants in these two groups also differed significantly by the percentage of CD4 (CD4%) and viral load at baseline, but there were no differences in markers of microbial translocation, LPS and sCD14 (Supplementary Table 1, http://links.lww.com/QAD/A336). Although Group 1 had low CD4% (median = 9.5%) and high viral load (median = 6.90 HIV RNA $\log_{10}$ copies/ml), Group 2 had high CD4% (median = 33%) and low viral load (median = 4.39 HIV RNA $\log_{10}$ copies/ml, $P = 0.01$, for both CD4% and viral load) (Fig. 2 and Supplementary Table 1, http://links.lww.com/QAD/A336). We observed the most profound differences between groups in the proportion of *Lactobacillales*. Group 1 with low CD4% and high viral load had low *Lactobacillales*, while Group 2 with high CD4% and low viral load had high *Lactobacillales* (median 10.6 vs. 46.5%) (Supplementary Fig. 1, http://links.lww.com/QAD/A335). As the proportion of gut *Lactobacillales* has been associated with mode of delivery at birth [37], we also examined reported differences between vaginal versus cesarean delivery of participants and found no differences between groups ($P = 0.48$).

Immune and clinical correlates of *Lactobacillales* before antiretroviral therapy

Before initiating ART, proportions of gut *Lactobacillales* were significantly correlated with CD4% ($P = 2.8 \times 10^{-5}$), CD4/CD8 T-cell ratio ($P = 0.0003$) and CD4 cell count ($P = 0.03$), and negatively associated with CD8% ($P = 0.002$), but only a negative trend with CD8 cell count ($P = 0.09$, Fig. 3a–e). Proportions of *Lactobacillales* were also negatively correlated with viral load ($P = 0.03$) and sCD14 ($P = 0.04$, Fig. 3f–g), but there was no association with LPS ($P = 0.88$, Fig. 3h). Despite an association with a marker of microbial translocation (sCD14), there was no observed relationship between *Lactobacillales* and either CD4 and CD8 lymphocyte activation (measured as HLA-DR$^+$ or CD38$^+$ T cells) or gut CD4 lymphocyte proliferation (percentage of Ki67$^+$ of CD4 T cells, data not shown). There was, however, a negative association between higher proportion of gut *Lactobacillales* and lower gut CD8 lymphocyte proliferation in the central memory subset (percentage of Ki67$^+$ of CD8$^+$CD45RO$^+$CD27$^+$, $P = 0.04$, Fig. 3i). Although other orders of bacteria also showed associations with clinical and immunological variables, most consistent associations were found with *Lactobacillales* (Supplementary Table 2, http://links.lww.com/QAD/A336).

Since duration of HIV infection is associated with immune activation [38] and participants had variable EDI at baseline, we evaluated if EDI influenced the associations between baseline proportions of *Lactobacillales* and lymphocyte activation in the blood. As might be expected, participants with a more recent EDI (≤4 weeks or acute phase of the infection, $n = 8$) at baseline showed a higher T-cell activation and proliferation than participants with longer EDI at baseline (>4 weeks, $n = 3$, Supplementary Fig. 2, http://links.lww.com/QAD/A335); however, baseline proportions of *Lactobacillales* were not associated with EDI (data not shown).

Immune and clinical correlates of *Lactobacillales* during antiretroviral therapy

To determine the impact of ART on the associations between proportions of *Lactobacillales*, clinical variables (viral load, CD4%, CD4 cell count, CD8% and CD8 cell count), markers of translocation (sCD14 and LPS), and lymphocyte activation (HLA-DR, CD38) and proliferation (Ki67) markers, we performed regression analyses for all variables at each study timepoint, longitudinally including all time-points and cross-sectionally at weeks 24 and 48. As expected, ART was very effective at suppressing viral load, and only one participant (Patient H) had detectable viral load at weeks 24 and 48 (2.70 and 1.91 HIV RNA $\log_{10}$ copies/ml, respectively). Similarly, all participants increased their CD4% during ART (median +9%, range 4–25%, $P = 0.001$) (Supplementary Fig. 3A, http://links.lww.com/QAD/A335), and there was a trend for proportions of *Lactobacillales* to be positively associated with CD4% gains ($P = 0.07$) (Supplementary Fig. 3A, http://links.lww.com/

QAD/A335). Although all participants demonstrated an increase in CD4% and a decrease in viral load during ART, those who started with low CD4% (Group 1), maintained lower CD4% compared with Group 2 participants and could still be identified by their GBP (Supplementary Fig. 4, http://links.lww.com/QAD/A335). Further, the relationships between *Lactobacillales* and CD4%, CD4/CD8 ratio and CD8% remained consistent with baseline results at week 24 but not at week 48 ($P = 0.01$, $P = 0.01$, $P = 0.05$, respectively, Fig. 4a–c); however, there were no associations between proportions of *Lactobacillales* and CD4 or CD8 T-cell count at either week 24 or 48 (data not shown). After 48 weeks of ART, increased proportions of *Lactobacillales* were positively correlated with increased CD4% in the gut ($P = 0.04$, Fig. 4d). As detectable viral load may have confounded the analysis, we also excluded Patient H from cross-sectional analysis, and *Lactobacillales* became positively associated with CD4 cell count at weeks 24 and 48 ($r = 0.78$, $P = 0.04$ and $r = 0.82$, $P = 0.04$, respectively). All other correlations maintained significant levels except with CD4% at week 48 of the gut, likely due to a power issue (data not shown).

Evaluation of lymphocyte activation demonstrated a strong negative correlation between the proportions of *Lactobacillales* and CD8 T-cell activation in the blood after 24 weeks of ART ($CD45RO^+HLA\text{-}DR^+$, $P = 0.002$, $CD45RO^+CD38^+$, $P = 0.01$, Fig. 4e–f). After 48 weeks of ART, the proportions of *Lactobacillales* were still negatively associated with $CD38^+$ lymphocyte activation ($P = 0.04$), but only a trend remained for $HLA\text{-}DR^+$ ($P = 0.09$) in CD8 T cells (Fig. 4e–f). The proliferation of central memory CD4 T cells in the gut was negatively associated with *Lactobacillales* at week 24 of ART (percentage of $Ki67^+$ of $CD4^+CD45RO^+CD27^+$, $P = 0.05$), but not after 48 weeks ($P = 0.22$, Fig. 4g). Regarding microbial translocation, the negative association between sCD14 and *Lactobacillales* observed at baseline was lost at week 24 but regained after 48 weeks of ART ($P = 3.7 \times 10^{-4}$, Fig. 4h). Throughout the study, sCD14 was inversely correlated with *Lactobacillales* ($P = 0.02$) and time on ART ($P = 0.04$) (Supplementary Fig. 3B, http://links.lww.com/QAD/A335). In contrast, LPS showed an isolated negative association at week 24 ($P = 0.03$, Fig. 4i). Other orders of bacteria also showed significant associations with these factors at week 24 but most were not significant by week 48 (Supplementary Table 3, http://links.lww.com/QAD/A336). Similar to above, exclusion of Patient H only decreased the significance of correlations with activation markers (HLA-DR and CD38) at week 48, possibly a power limitation (data not shown).

HIV latent reservoir and cytomegalovirus shedding

As changes in immune activation may impact the HIV latent reservoir [39], we also evaluated HIV DNA levels in PBMCs, but we did not observe any associations between HIV proviral DNA and *Lactobacillales* after 24 or 48 weeks of ART. Proviral DNA did not correlate with CD8 T-cell activation (Supplementary Fig. 5A-B, http://links.lww.com/QAD/A335). There was a strong negative correlation, however between proviral DNA levels and activation of CD4 lymphocytes ($CD45RO^+HLA\text{-}CD38^+$, $P = 0.007$) at week 24 of ART, but not at week 48 (Supplementary Fig. 5C-D, http://links.lww.com/QAD/A335). We also investigated the relationship between CMV shedding in the gut and the genital tract and *Lactobacillales*, as the presence and magnitude of CMV shedding may influence immune activation [28] and all participants were CMV antibody positive. Only two patients (D and H) had detectable levels of CMV in rectal swabs. Patient D had 2.41 and 2.14 $\log_{10}$ copies/swab at weeks 16 and 24 and patient H had 1.46 $\log_{10}$ copies/swab at week 4. Due to limited sample availability, only nine semen samples were screened for CMV shedding and Patient D exhibited high levels of CMV (4.49 $\log_{10}$ copies/swab). The eight remaining samples showed no evidence of CMV, though no samples were available for Patient H. Interestingly, both patients (D and H) exhibited the lowest overall proportion of *Lactobacillales* (<10%)

throughout infection and Patient D in particular had the lowest CD4% after 48 weeks of ART.

Discussion

This is the first study to identify associations between specific GBP and higher CD4 cell count and CD4%, lower viral load, less CD4 T-cell proliferation in the gut and less evidence of microbial translocation in untreated HIV infection. All of these factors have been previously associated with better HIV disease outcomes [40,41]. The associations between GBP, CD4%, immune activation, and markers of bacterial translocation continued, albeit weakly, during ART that suppressed viral load. The important caveat of this study is that the associations between changes in the GBP and the HIV disease markers cannot determine causality.

The human gut prevents the translocation of commensal bacteria through physical barriers (e.g. epithelial tight junctions), biochemical agents (e.g. antibacterial peptides and mucus), and immune mechanisms (e.g., secretory IgA and Toll-like receptor mediated sensing, oxidative bursts) [1,42–45]. During early HIV infection, gut populations of *Bifidobacteria* and *Lactobacillus* species are reduced [46], the GALT is depleted [7], the gut barrier is compromised and translocation of bacterial products can occur. The translocation of these products is associated with HIV disease progression, most likely through persistent immune activation [6,7]. This study aimed to further evaluate these connections by observing multiple factors during acute and early HIV infection and by treating HIV infection at the earliest stages possible, and identifying correlates associated with optimal immune recovery and preservation. The current study extends previous observations [47] by linking the constitution of the gut microbiome itself to immunologic and virologic dynamics during recent HIV infection and subsequent ART, specifically identifying that higher proportions of gut *Lactobacillales* are associated with markers that are predicative of better HIV outcomes including higher CD4 percentage, lower viral load, and less evidence of microbial translocation. Moreover, the higher proliferation of central memory cells in GALT, as a surrogate marker of antigen stimulation, was less likely to occur in participants with higher proportions of *Lactobacillales* suggesting a favorable gut immune health. As *Lactobacillales* can modulate anti-inflammatory responses and immune cells (e.g., T-regulatory cells), improve gut integrity and reduce gut permeability in other conditions [20–24,48], it is interesting that this bacterial order would be identified as the main component of GBP associated with markers of improved HIV outcome. However, it still needs to be investigated whether these GBP are metabolically associated or represent only a biomarker of a favorable state.

There are a number of limitations that should be considered. First, we conducted this investigation in the setting of a randomized double-blind controlled trial of maraviroc versus placebo combined with standard ART, but the overall study remains blinded to maraviroc use because participant enrollment continues. Maraviroc use is not thought to have contributed significantly to the study observations as associations between GBP and clinical and immunological variables were identified at baseline, before the start of ART for all participants. In general, these associations persisted during follow-up, and unblinding or modification of the parent study is thought to be unnecessary. However, as maraviroc inhibits CCR5, it could theoretically alter the composition of T cells in the GALT, influencing our observations after the start of ART, and this will need to be assessed after the unblinding of the study. Second, although NGS allowed us to conduct large-scale metagenomic analyses of the distal gut microbiome, the analysis is limited by the classification of sequences. To focus our study on the main drivers of gut bacteria across all participants, we only considered bacterial populations shared across individuals. Bacterial

sequences that were not identified in all participants were classified as 'Other', and these 'Other' populations may be very informative and warrant further investigation. Along these lines, the study only considered classification at the order level and a more granular classification may provide additional insight. To this end, all bacterial sequences have been publically deposited at http://mepac.ucsd.edu. Third, the observation that sCD14 is more highly correlated with measures of microbial translocation than LPS has been previously reported [7,49], and could be related to the EDI. Microbial translocation occurs in early phases of infection, thus levels of LPS do not increase until later HIV stages, while levels of sCD14 increase earlier during the course of infection [7]. Alternatively, plasma inhibitors [50] were observed that might have interfered with LPS assay measurements. Fourth, in limited number of samples, we investigated if CMV shedding in the distal GI tract or semen demonstrated similar associations to classified GBP. Although, the associations between rectal CMV shedding and lower CD4 T-cell count and percentage were provocative in two of the participants, the numbers are too small to draw conclusions for a role of CMV to impact gut microbiota, but should provide testable hypotheses for future investigations.

It is increasingly evident that the human immune system is linked to the GI system [48], but guidance regarding how this information should influence clinical care is lacking, especially for HIV infection. Although the gut microbiome is influenced by methods of birth delivery, host genetics, household contacts, age, geography, and so on [51], it is unknown if the microbiome can be shaped with agents like prebiotics, probiotics, or targeted antibiotics for beneficial outcomes. This study determined that increasing *Lactobacillales* in the gut could be important for recovering and preserving immune system function during HIV infection and a promising clinical target may be HIV-infected individuals who are able to suppress their viral load with ART, but are unable to sufficiently recover their CD4 T-cell count.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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J.P.S. performed the amplification and sequencing of bacterial DNA experiments, participated in the data analyses for this study, performed the statistical analyses, and wrote the primary version of the article. S.G. performed the CMV and HIV proviral DNA experiments and participated in the data analyses. M.M. analyzed the flow cytometry, and other clinical and immunological data. C.A.S. and M.Y.K. designed the antibody panel for flow cytometry and participated in the data analyses. S.R.V. participated in the data analyses. D.P. obtained the gut biopsies. J.A.Y. obtained the demographic, epidemiological and HIV-related data of participants. P.S.J. performed microbial translocation markers experiments. S.J.L. and D.M.S. enrolled participants. D.D.R., S.J.L., and D.M.S. designed the present study, participated in data analysis. All authors read and approved the final article.

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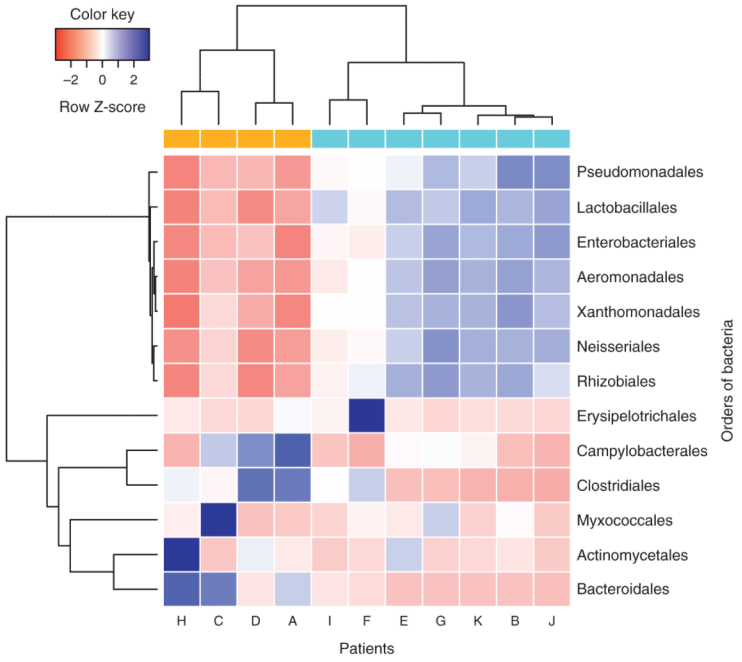

Fig. 1. Unsupervised clustering before antiretroviral therapy (ART)

Gut bacterial profiles separated our participants in two main groups colored as gold and blue representing participants with low and high CD4% (Group 1 vs. Group 2), respectively. Orders of bacteria are separated into two main groups clustered by correlation. Overall, bacteria in the same cluster are positively correlated, whereas bacteria in different clusters are negatively correlated. Blue and red correspond to enrichment or depletion on proportions of bacteria, respectively.

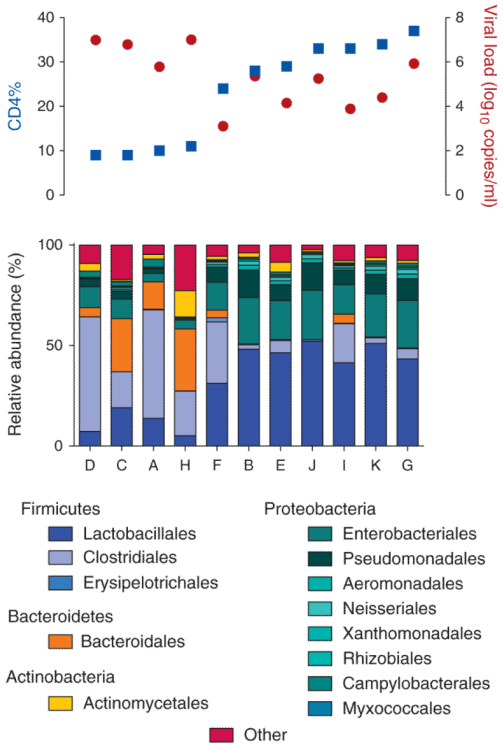

Fig. 2. Overview of participants' CD4%, viral load (VL) and gut bacterial profiles (GBP) at baseline

Participants' CD4% and VL are colored blue and red, respectively, and their corresponding GBP is in the bottom. Participants with lower CD4% and higher VL exhibit lower proportions of *Lactobacillales*.

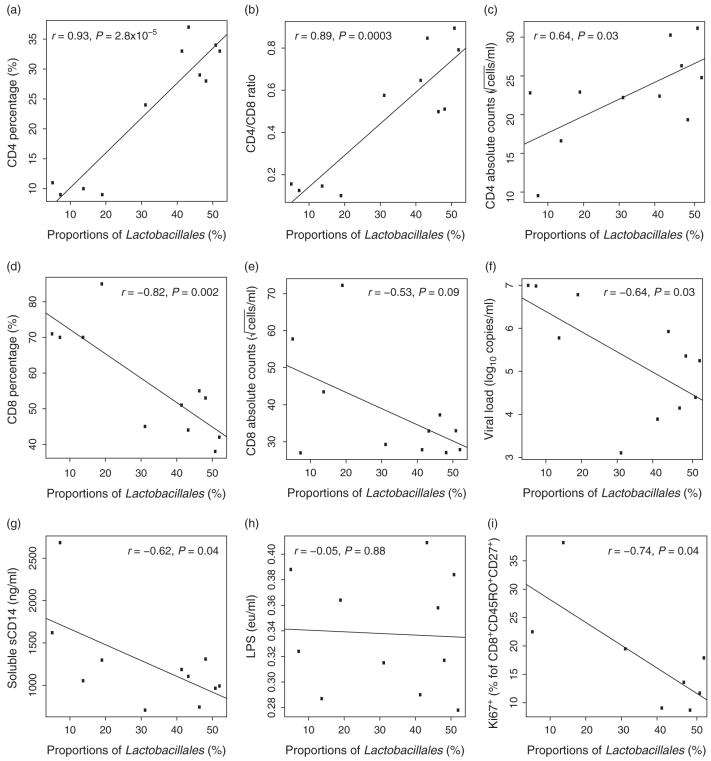

Fig. 3. Associations of *Lactobacillales* with clinical and immunological variables at baseline and before antiretroviral therapy (ART)

There is a positive association with (a) CD4%, (b) CD4/CD8 ratio, (c) CD4 cell count and a negative correlation (or trend) with (d) CD8%, (e) CD8 cell count, (f) viral load, (g) soluble CD14, and (i) Gut $CD4^+$ T-cell proliferation. (h) There was no correlation between *Lactobacillales* and lipopolysaccharide (LPS). All these correlations suggest that higher proportions of *Lactobacillales* are beneficial for the host in the absence of ART.

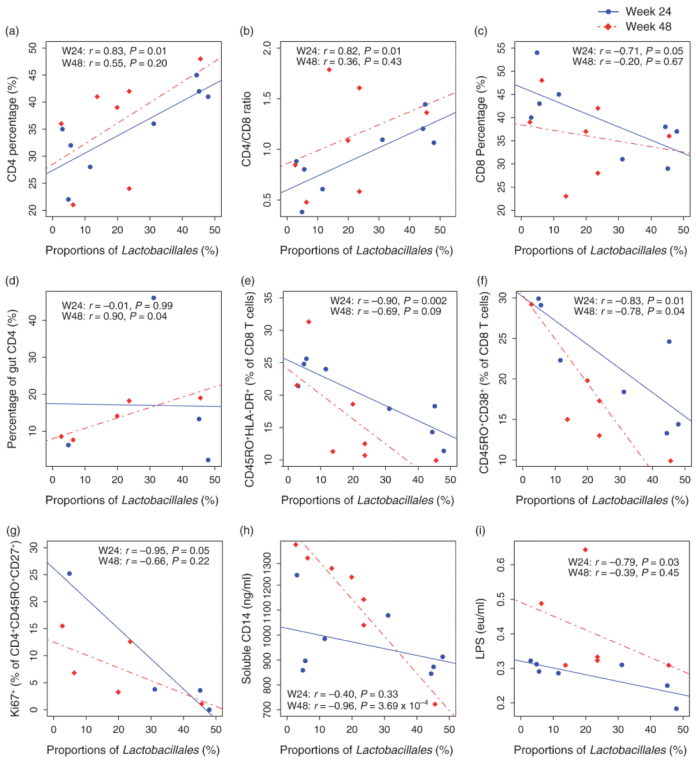

Fig. 4. Associations of *Lactobacillales* with clinical and immunological variables after ART

Week 24 is represented by solid dots and lines, and week 48 by empty dots and dotted lines. At week 24, there is a positive association with (a) CD4%, (b) CD4/CD8 ratio and a negative correlation with (c) CD8%, as observed at baseline. Additionally, higher gut *Lactobacillales* are associated with higher CD4% in the gut (d), less $CD8^+$ T-cell activation (e, f), less $CD4^+$ T-cell proliferation (g), and less microbial translocation (h, i). Associations were independent of ART and suggest that higher proportions of *Lactobacillales* are associated with better immune health.

Table 1

Demographics and clinical variables at baseline.

PID	Race[a]	Ethnicity[b]	Age	EDI (weeks)	Viral load (log_{10} copies/ml)	CD4 absolute (cells/μl)	CD4%	CD8 absolute (cells/μl)	CD8%	CD4/CD8 ratio
A	A	NH	23	2	5.78	276	10	1890	70	0.15
B	A	NH	28	2	3.10	493	24	856	45	0.58
C	C	NH	35	3	6.78	525	9	5216	85	0.10
D	C	H	22	3	6.99	91	9	728	70	0.13
E	A/C	NH	39	3	4.15	692	29	1387	55	0.50
F	C	NH	52	3	5.36	374	28	732	53	0.51
G	C	NH	21	3	5.93	916	37	1082	44	0.85
H	C	NH	28	4	7.00	520	11	3334	71	0.16
I	C	H	28	10	3.89	501	33	775	51	0.65
J	C	NH	26	11	5.25	614	33	776	42	0.79
K	C	H	33	12	4.40	971	34	1086	38	0.89
L	C	NH	40	14	4.15	383	30	558	42	0.69
M	C	NH	55	14	3.77	913	40	714	31	1.28
		Average	33.1	6.46	5.12	559.15	25.15	1471.85	53.62	0.56

[a] A, Asian; C, Caucasian.

[b] H, Hispanic; NH, non-Hispanic. Participants are ordered according to estimated duration of infection (EDI).

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