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Microbial translocation revisited: targeting the endotoxic potential of gut microbes in HIV-infected individuals

Short title: Endotoxic potential of the gut microbiome

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Objective: Translocation of microbial products such as lipopolysaccharides (LPS) from the gut may contribute to chronic inflammation in HIV-infected individuals. Recent studies indicate that differences in degree of acylation of gut-bacterial derived LPS may explain variable immune effects, with hexa-acylated rather than penta-acylated LPS having pro-inflammatory capacity. We investigated whether the degree of acylation of gut-derived LPS associates with systemic inflammation, and the potential effect of probiotic intervention.

Methods: Gut microbiota profiles from a probiotics intervention were investigated, and validated in a cohort of HIV-infected individuals commencing ART. The PiCRUSt software was used to predict overall functional capacity of the microbiota, and in-house bioinformatics to distinguish between bacteria producing hexa- and penta-acylated LPS.

Results and conclusion: HIV-infected individuals with the highest ratio of pro-inflammatory hexa-acylated LPS- to non-inflammatory penta-acylated LPS-producing bacteria exhibited increased levels of systemic inflammation (neopterin, $P < 0.001$) and tryptophan catabolism (kynurenine/tryptophan-ratio, $P = 0.01$), indicating a link between pro-inflammatory LPS, tryptophan catabolism and inflammation. After probiotics for eight weeks, there was a decrease in gram-negative bacteria ($P = 0.01$), related primarily to a reduction in bacteria producing penta-acylated LPS ($P = 0.01$), but not hexa-acylated LPS. The reduction in gram-negative bacteria correlated positively with decreased plasma LPS ($r = 0.72$), mainly related to a reduction in bacteria producing non-inflammatory penta-acylated LPS ($r = 0.58$). Notably, gut bacteria producing hexa-acylated LPS were outnumbered by penta-acylated LPS with a factor of 25 in HIV-infected individuals. Further studies are warranted to determine whether microbes producing hexa-acylated LPS might be a more relevant trigger of systemic inflammation compared to plasma LPS captured by the existing limulus assay.

Keywords: HIV, probiotics, endotoxins, LPS, gastrointestinal microbiome, inflammation, limulus test

Background

The widespread access to antiretroviral therapy (ART) for HIV-1 patients has changed the disease from a lethal to a chronic condition. However, the chronically infected patients exhibit an increased risk of non-AIDS morbidities such as cardiovascular, hepatic, renal or neurologic diseases.(1, 2) This phenomenon has been linked to the persistent low-grade inflammation observed in patients successfully treated with ART.(3-5)

The initial HIV infection causes a massive loss of Th17 positive CD4⁺ T-cells in the gut-associated lymphoid tissue (GALT). The depletion of these cells is associated with inflammation in the mucosa, apoptosis of epithelial cells and breakdown of tight junctions.(6, 7) It has been suggested that the loss of integrity of the gut and blood barrier may allow gut microbial products, such as lipopolysaccharide (LPS), to translocate into the portal and systemic circulation. LPS, a component of the outer wall of gram-negative bacteria, is a potent immune activator via interaction with the Toll-like receptor (TLR)-4 complex through recognition by MD-2 and CD14 co-receptors.(8, 9)

In HIV-infected individuals, plasma LPS is elevated and potentially linked to systemic inflammation, activated T cells and cardiovascular risk factors.(6, 10-12) Furthermore, activated T cells produce interferon (IFN)- γ , which together with other cytokines activates tryptophan catabolism through upregulation of indolamine 2,3-dioxygenase (IDO-1).(7, 13) Moreover, microbial translocation and gut microbiota alterations associate with increased kynurenine to tryptophan ratio (KT-ratio), a measure of tryptophan degradation which is linked to systemic inflammation, disease progression and increased mortality in HIV-infected individuals.(7, 14, 15) The main pathway of tryptophan catabolism is to kynurenine, which has immunosuppressive properties, in part by inhibiting T-cell proliferation and depleting Th17 cells, which in turn weakens the mucosal barrier and promotes low grade endotoxemia, inflammation and T cell activation.(7) Activated T cells produce IFN- γ , which induce tryptophan catabolism through upregulation of IDO-1, and contributes to neopterin release by activated monocytes/macrophages. (13) (13)(13) Recent studies have explored the metagenomic heterogeneity of LPS and its capacity to activate a downstream immune response

after binding to the TLR4 receptor complex.(16-20) The endotoxic activity of LPS depends on the degree of acylation of the lipid A moiety of the molecule. Some gram-negative bacteria produce a penta-acylated form, which antagonizes activation of human TLR4, whereas others produce a TLR4 activating hexa-acylated form of LPS.(21) Currently available biochemical assays capture all types of LPS independent of their pro-inflammatory effects, and to our knowledge no biochemical assay exists to determine the levels of the two LPS variants in blood. Revealing the relative abundances of, and ratio between bacteria producing penta-acylated and hexa-acylated LPS within the gut microbiota would therefore be a measure of the endotoxic pro-inflammatory potential of LPS, and has been proposed as a driver for asthma and type 1 diabetes.(20, 22)

Several strategies have been applied to reduce systemic inflammation in HIV by targeting microbial translocation, including LPS-binding agents and antibiotics, but with mostly negative results.(23, 24) In a previous study we reported a reduction in levels of D-dimer, and a tendency to reduced levels of CRP and IL-6 after probiotic intervention in HIV-infected patients on stable ART.(25) However, no changes in markers of microbial translocation were observed.

In the present study we aimed to expand the analyses of microbial translocation in the same patient cohort by focusing on the gut metagenome, specifically addressing the biosynthesis of LPS and the ratio of hexa:penta-acylated LPS-producing bacteria. We hypothesized that i) alterations of gut microbial genes related to LPS biosynthesis and degree of acylation are related to markers of inflammation and tryptophan degradation in HIV-infected patients, ii) the effects of probiotic intervention in HIV-infected are associated with alterations of gut microbial genes related to LPS biosynthesis, in particular genes related to hexa-acylated LPS.

Methods

Study participants

We investigated a subgroup with available gut microbiota profiles (n=22) from a previously published probiotics intervention. For a complete methodology and baseline characteristics, we refer to supplementary table 1, <http://links.lww.com/QAD/B402> and the initial study.(25) In brief, HIV-

infected >18 years old with HIV-RNA <50 copies per milliliter for at least 6 months and CD4 count <500 cells per microliter and with available 16S ribosomal RNA (rRNA)-based gut microbiota profiles were included (n=10 in the probiotic and n=12 in the control group). In the intervention group, the patients received 250mL fermented skimmed milk supplemented with *Lactobacillus rhamnosus* (10⁸ cfu/mL), *Bifidobacterium animalis subsp. Lactis* (10⁸ cfu/mL) and *Lactobacillus acidophilus La-5* (10⁷ cfu/mL) for eight weeks, as previously described. In addition, we included a validation cohort consisting of HIV-infected individuals commencing ART, including available microbiota samples from HIV-infected individuals sampled 10 (4-15) months after introduction of ART (n=16), non-infected controls (household members/partners of HIV-infected individuals, consisting mainly of MSM, n=9) and three HIV-infected elite controllers, which was sampled and sequenced using the same pipeline as in the probiotics intervention.(26)

Soluble factors

As previously described, total plasma LPS was analyzed by limulus amebocyte lysate colorimetric assay (Lonza, Walkersville, MD).(27) Soluble CD14 (sCD14) was analyzed by Quantikine ELISA kits (R&D Systems Europe, Abingdon, United Kingdom).(25) Intestinal fatty acid binding protein (I-FABP) was analyzed by ELISA kit (Hycult biotech, Uden, The Netherlands). Extracted DNA was sequenced on the Illumina MiSeq platform, targeting the V3-V4 region of the 16s rRNA. Reads were subsampled/rarefied to 9442 reads per sample and mapped using default values in “closed reference operational taxonomic unit (OTU) clustering” in QIIME 1.8.0 against the Greengenes database version 1308.(28)

Microbial genes analysis

Two different methods were used to determine microbial genes related to biosynthesis of LPS. The software PICRUSt was used to predict the genetic content of the metagenome based on the 16s rRNA sequencing data, which was subsequently assigned to KEGG (Kyoto Encyclopedia of Genes and Genome) pathways.(29) Available genera from the 16s rRNA sequencing were classified as gram-negative, and further separated into hexa-acylated, penta-acylated or tetra-acylated lipid A producing

bacteria, or as non-LPS producing bacteria involving all gram-positive species, with the exception of *Veillonella*. Classification of bacteria into hexa-, penta- or tetra-acylated LPS producers was based on genomic information from all whole genome-sequenced deposited at the nucleotide database NCBI (www.ncbi.nlm.nih.gov), and then coupled to the taxonomic identification based on 16s rRNA gene sequences. Bacteria carrying the *LpxL* gene, along with the remaining lipid A biosynthesis enzymes, produce the penta-acylated LPS variant, while carriers of both the *LpxL* and *LpxM* genes generally produce the proinflammatory hexa-acylated LPS. Bacteria carrying lipid A biosynthesis enzymes but neither *LpxL* or *LpxM* were classified as a tetra-acylated LPS producer.

Statistical analysis

All continuous variables are presented as median and interquartile range (IQR). Nonparametric statistics were applied, using Wilcoxon matched pairs test, Mann–Whitney U test and Spearman's rho test, as appropriate. Statistical correction for multiple testing were not applied because of the low sample size and the exploratory focus of the study. A two-tailed significance level of 0.05 was used. Statistical analyses were performed in SPSS Statistics v24.0 (IBM Corporation, Armonk, NY, USA). Graphical presentations were made using Prism V7.0d software (GraphPad, San Diego, CA).

Results

Penta-acylated bacteria outnumber hexa-acylated bacteria in HIV-infected individuals

In the total study population at baseline in the probiotic intervention cohort (n=22), the median of the ratio of hexa:penta-acylated LPS-producing bacteria was 0.04 (0.01-0.11, fig. 1A), hence penta-acylated bacteria outnumbered hexa-acylated bacteria with a factor of 25 in HIV-infected individuals.

To further evaluate the magnitude of this ratio, we externally validated our results in a previously published cohort of HIV-infected individuals commencing ART, finding the same fraction of bacteria producing hexa and penta-acylated LPS (median ratio 0.03 (0.01-0.21), P=0.69). This validation cohort also consisted of non-infected controls (n=9) and three HIV-infected elite

controllers. Although the hexa:penta-ratio was not significantly lower in non-infected controls (0.02 (0.01-0.10), $P=0.39$), the elite controllers had substantially lower levels of hexa:penta-ratio compared to HIV-infected individuals on ART (median 0.001, $P=0.002$, Fig.1A).

As some microbes can express several degrees of LPS-acylation, we also aimed to examine tetra-acylated LPS. However, no microbes (as defined by the genus level resolution available from 16s rRNA sequencing) expressing tetra-acylated LPS were found in the gut microbiota in any of the included patients in the present study.

Ratio of hexa:penta-acylated LPS-producing bacteria is related to neopterin and tryptophan catabolism in HIV-infected individuals

Since increased relative levels of pro-inflammatory hexa-acylated LPS-producing species may be an indicator of enhanced gut inflammatory status, we next explored whether the ratio of hexa:penta-acylated LPS-producing bacteria in the gut (hexa:penta-ratio), at baseline in the probiotic intervention cohort, could be related to markers of systemic inflammation. We identified a significant correlation between plasma neopterin and the hexa:penta-ratio ($r=0.59$, $P=0.01$, fig. 1B), suggesting that a more pro-inflammatory milieu within the gram-negative bacteria in the gut is linked with systemic neopterin, which is released from activated monocytes mainly after IFN- γ stimulation.(13)

Furthermore, when dichotomizing the dataset according to the median ratio of hexa:penta-ratio, the group with the highest ratio not only had significantly higher levels of neopterin ($P<0.001$, fig. 1C), but also an increased KT-ratio as a measure of tryptophan catabolism ($P=0.01$, fig. 1D). Furthermore, plasma levels of neopterin were strongly correlated with KT-ratio ($r=0.67$, $P=0.001$). No significant differences between the high and low hexa:penta-ratio groups were observed for CRP, D-dimer, IL-6, IFABP, total plasma LPS or sCD14 (Supplementary table 2 and 3, <http://links.lww.com/QAD/B402>), or any of the HIV-related factors detailed in Supplementary table

1, <http://links.lww.com/QAD/B402> (MSM vs non-MSM, current CD4 count, nadir CD4 count, duration of ART).

We did not find any positive correlation between KT-ratio and hexa:penta-ratio in the non-infected controls (n=9), in fact there was a non-significant negative correlation ($r=-0.40$, $P=0.29$) and a tendency to lower KT-ratio in individuals with the highest hexa:penta-ratio ($P=0.19$, Fig. 1E) in the control group.

Probiotic intervention in HIV-infected individuals is associated with decrease in overall LPS biosynthesis related primarily to non-inflammatory penta-acylated LPS-producing gram-negative bacteria

We next explored the effect of probiotics on LPS biosynthesis, and in line with the original study of the complete set of participants (25), no changes were observed after the intervention in relation to plasma LPS levels in the subgroup with available microbiota profiles ($P=0.65$, table 1). Furthermore, we performed genome-based bioinformatics analysis of the LPS-producing capacity within the gut microbiota, finding a significant reduction after probiotics intervention in median gene counts related to the KEGG pathway involved in overall LPS biosynthesis: 13894 (5256-19683) to 9683 (4827-14200) (-30%, $P=0.01$, fig. 2A). This reduction was paralleled by a change in relative abundance of gram-negative bacteria (decreased from 0.27 (0.11-0.55) to 0.12 (0.05-0.18), $P=0.01$, table 2), and a strong correlation between the two ($r=0.88$, $P=0.001$, fig. 2B).

The decrease in gram-negative bacteria was mirrored by a reduction in the relative abundance of gram-negative bacteria with capacity for penta-acylated LPS production (from a median of 0.38 (0.11-0.59) to 0.18 (0.07-0.27) ($P=0.01$, fig. 2C, table 2)), while no significant changes were seen for bacteria producing the pro-inflammatory hexa-acylated LPS (Fig. 2D, table 2). Notably, the abundances of hexa-acylated LPS-producing gut bacteria were generally very low (from 0.01 (0.002-0.01) to 0.002 (<0.001-0.02), Fig. 2D). Likewise, no significant changes were seen in the ratio of hexa:penta-acylated LPS-producing bacteria (0.01 (0.01-0.04) to 0.03 (0.01-0.08), $P=0.29$) after probiotics administration (Fig. 2E).

When focusing on which penta-acylated LPS-producing bacteria were reduced, we found a statistically significant reduction of *Bacteroides* (from relative abundance of 0.19 (0.04-0.39) to 0.03 (0.002-0.09), $P=0.01$, table 2), to be counter-regulated by increased abundances of total gram-positives after the probiotics intervention (Table 2). In this regard we identified a negative correlation between the decrease of gram-negative bacteria and the increase of the supplemented *Bifidobacterium* ($r=-0.72$, $P=0.02$) and *Lactobacillus* ($r=-0.68$, $P=0.03$). No changes were observed in the control group (Table 2).

Reduction in gut bacteria producing penta-acylated LPS during probiotic intervention correlate with changes in plasma levels of LPS

When focusing on changes in the relative abundance of gram-negative bacteria in relation to systemic markers of microbial translocation and inflammation, we found that the decrease in gram-negative bacteria correlated positively with decreased plasma LPS ($r=0.72$, $P=0.02$, fig. 3A) during the intervention, but not with changes in levels of CRP, IL-6, sCD14, D-dimer, neopterin or KT-ratio (Supplementary table 4, <http://links.lww.com/QAD/B402>). Of note, the reduction in relative abundance of bacteria producing penta-acylated LPS showed the same trend for correlation with changes in total plasma LPS ($r=0.58$, $P=0.08$, fig. 3B), whereas no such correlation was seen for bacteria producing hexa-acylated LPS (Supplementary table 4, <http://links.lww.com/QAD/B402>).

Discussion

In the present study we focused on the biosynthesis and acylation degree of LPS by the gut microbiome, aiming to investigate whether the degree of acylation of gut-derived LPS associates with markers of systemic inflammation, and the potential effect of probiotic intervention in HIV-infected individuals. The main findings can be summarized as follows: 1. A higher ratio of bacteria producing hexa:penta-acylated LPS at baseline in the probiotic intervention cohort was associated with elevated markers of tryptophan degradation and the pro-inflammatory marker neopterin, reflecting an

association between the LPS type ratio in the gut and the systemic inflammatory status. 2. A reduction in gut microbial gene counts related to biosynthesis of LPS was observed after probiotic intervention, mainly driven by a reduction in bacteria producing penta-acylated, but not hexa-acylated LPS. 3. Changes in penta-acylated but not hexa-acylated LPS correlated closely with changes in plasma LPS, suggesting that LPS levels captured by the limulus assay to a large degree reflect non-inflammatory LPS.

It should be noted that hexa-acylated LPS producing bacteria had a very low abundance with a ratio of 1 to 25 as compared to penta-acylated LPS in HIV-infected individuals. To our knowledge, no such data are available from other HIV cohorts, and future studies should assess the magnitude of this ratio in a healthy background population. Possibly, low frequency microbes including bacteria producing hexa-acylated LPS might be relevant for systemic inflammation and metabolism, as suggested by the association between ratio of bacteria producing hexa:penta-acylated LPS and tryptophan catabolism, as well as to plasma neopterin.

Taken together, our observations could indicate that the ratio of bacterial genes related to hexa:penta-acylated LPS is a relevant measure of the microbial stimulatory capacity related to systemic inflammation in chronic HIV-infection. However, this ratio is a simplified measure of the overall interplay between microbes expressing different forms of LPS and the immune system, and acylation of LPS may not be the sole determinant of TLR4-responsiveness. In fact, some microbes such as *Porhyromonas* can produce and exploit multiple acylated forms of LPS, including tetra-, penta- and hexa-acylation to evade the innate immune system(30). However, in the present study cohorts, we could not identify any microbes expressing tetra-acylated LPS. It should also be noted that some strains of *Bacteroides* have the capacity to produce penta-acylated LPS with highly divergent potency(31). These variations are not captured by our bioinformatic approach taking advantage of 16s data. Hence, direct quantification and functional analyses of differentially acylated forms of LPS would add valuable information in future studies.

In several studies of the gut microbiome in HIV-infected individuals, significant differences are mainly reported among highly abundant bacterial taxa, such as increased abundance of *Prevotella* and reduced abundance of *Bacteroides*.⁽³²⁾ Recently, MSM-status was identified as an important confounder of the HIV-associated *Prevotella*-rich enterotype as opposed to higher abundance of *Bacteroides* in non-MSM, irrespective of HIV status.⁽³³⁾ Of note, *Prevotella* and *Bacteroides* both produce penta-acylated LPS, and apparently, hexa:penta-ratio was not associated with MSM status in the present study. In the non-infected individuals from the validation cohort, consisting of household members/partners of HIV-infected individuals, mostly MSM, we did not find significantly lower ratio of bacterial genes related to hexa:penta-acylated LPS, although this ratio was much lower in three elite controllers. However, the study cohorts are limited by small sample size, and we acknowledge that our findings, although intriguing, need to be replicated in larger, properly designed cohorts of HIV-infected individuals of various stages of immune status (including elite controllers and immunological non-responders) and appropriate controls (non-infected MSM and non MSM). Another limitation is that the genetic microbial potential of LPS biosynthesis genes may not necessarily translate into circulating levels of hexa- and penta-acylated LPS, which could be affected by probiotic intervention. Although no assay currently exists that can distinguish inhibitory from inflammatory LPS in the periphery⁽³⁴⁾, there is clearly a need for such a kit in future work of this emerging field of research.

Probiotic intervention did not alter the abundance of hexa-acylated LPS-producing bacteria, corresponding with no effect on plasma levels of neopterin, KT-ratio or sCD14. Previous studies aiming to reduce microbial translocation have not shown effects on systemic total LPS levels in HIV-infected individuals.^(23, 24) Rifaximin, a non-absorbed oral antibiotic, decreased total plasma levels of LPS by 50% in patients with alcoholic cirrhosis, however no effects were seen when rifaximin was given to ART-treated HIV patients.^(24, 35) Sevelamer carbonate, a phosphate-lowering drug, decreases circulating LPS levels in patients with renal insufficiency.^(36, 37) Yet, no such effects were seen when administered to HIV-infected individuals.⁽²³⁾ Our results indicate that although we found a reduction in the total abundance of LPS-producing gut bacteria, this reduction was mainly explained

by a decrease in the genus of *Bacteroides*, which produces penta-acylated LPS. Of note, no changes were seen in the ratio of hexa:penta-acylated LPS-producing bacteria after probiotic intervention, suggesting no effect on the overall endotoxic potential of the gut microbiome.

A large number of studies focusing on microbial translocation in HIV have been published since Brenchley et al. first described the phenomenon in 2006.(6) To our knowledge, none of these studies have investigated differences in the endotoxic potential of LPS. Importantly, measuring LPS in plasma would be a biomarker of microbial translocation, but in order to study LPS as an immune activator, there is a need to distinguish between the different forms and the inflammatory potential of this toxin. In our data, the ratio between genes related to hexa-acylated, pro-inflammatory LPS, and inhibitory penta-acylated LPS in the gut microbiota varied greatly between individuals; with numbers ranging from 1/500 to 1/10. In addition, the close correlation between reduction in microbes producing penta-acylated LPS and changes in systemic total LPS levels, suggest that measurements of total plasma LPS reflect mainly penta-acylated LPS, which has a much lower TLR4 stimulatory potential than hexa-acylated LPS. This point is of relevance since the most frequently used limulus assay (Lonza) used for measurement of plasma LPS cannot distinguish between LPS variants with different degrees of acylation.(38) This could explain the low to moderate correlations between total plasma LPS levels and inflammatory markers in the literature (6, 39, 40); which was also replicated in this study. In light of our findings, one could question whether plasma LPS measured by the limulus assay is a relevant biomarker in trials targeting the gut microbiome and/or microbial translocation.

In conclusion, we identified an association between increased ratio of hexa:penta-acylated LPS-producing bacteria and systemic inflammation measured as neopterin and tryptophan catabolism in HIV-infected individuals. The reduction in penta-acylated but not pro-inflammatory hexa-acylated LPS after probiotic intervention correlated closely with changes in plasma LPS suggesting that LPS levels captured by the limulus assay to a large degree reflects non-inflammatory LPS. Notably, gut bacteria producing hexa-acylated LPS varied substantially between individuals, and were vastly outnumbered by penta-acylated LPS. Further studies are warranted to determine whether the ratio of hexa:penta-acylated LPS-producing bacteria might be a more relevant measure for trigger of systemic

inflammation than the currently existing limulus assay, and whether the hexa:penta-ratio could relate to disease progression and clinical outcome in HIV-infected individuals.

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Figures legends

Figure 1. Ratio of hexa:penta-acylated LPS producers in the gut is related to systemic inflammatory markers in HIV-infected individuals.

A The ratio of gut bacteria producing hexa:penta-acylated LPS at baseline before probiotic intervention (n=22, black), HIV+ from a validation cohort after commencing ART(n=16, red), non-infected household-members of HIV+ (n=9, green) and elite controllers (n=3, blue). **B** Hexa:penta-acylated LPS producers correlates with plasma levels of neopterin in HIV-infected. When dichotomizing, the patients with the highest ratio of gut bacteria producing hexa:penta-acylated LPS showed elevated levels of neopterin (**C**) and the KT-ratio (**D**). **E** In healthy controls there was a trend of lower levels of KT-ratio with higher hexa:penta-ratio.

KT-ratio: kynurenine-tryptophan-ratio, a measure of tryptophan degradation.

Mann–Whitney U test (a, c-e) and Spearman's rho test (b).

Figure 2. Effect of probiotic administration to HIV-infected individuals on type of LPS-producing gut bacteria.

A A reduction in gut microbial genes related to LPS biosynthesis was seen in the probiotics group after 8 weeks of intervention. **B** The change in the predicted metagenome of genes related to LPS biosynthesis correlated to the change in relative abundance of gram-negative bacteria. The relative abundance of gut bacteria producing penta-acylated (**C**) and hexa-acylated LPS (**D**) before and after the probiotics intervention. **E** The ratio of hexa:penta-acylated LPS producers within the gut metagenome before and after the probiotics intervention.

Wilcoxon matched pairs test (a, c-e), Spearman's rho test (b).

Figure 3. Correlations between gram-negative gut bacteria and bacteria producing penta-acylated LPS and plasma LPS levels

The change in the relative abundance of gram-negative gut bacteria correlated to the change in total plasma levels of LPS in the probiotics group (A). Likewise, the change in the relative abundance of bacteria producing non-inflammatory penta-acylated LPS correlated to changes in total plasma levels of LPS in the probiotics group (B). Spearman's rho test.

Table 1 Characteristics of soluble markers in HIV-infected individuals in the probiotic intervention cohort.

	Probiotics group (n=10)			Control group (n=12)			Between groups	
	Baseline	8. week	P-value	Baseline	8. week	P-value	Baseline, P=	8. week, P=
sCD14 (µg/ml)	1691 (1320-2124)	1601 (1302-2074)	>0.10	1692 (1423-1928)	1687 (1348-1978)	>0.10	>0.10	>0.10
LPS (EU/mL)	83 (81-95)	82 (74-97)	>0.10	93 (83-104)	95 (75-103)	>0.10	>0.10	>0.10
IL-6 (pg/mL)	1.2 (0.9-2.4)	1 (0.9-1.3)	0.09	1.4 (0.9-3)	1.4 (1-1.8)	>0.10	>0.10	>0.10
D-dimer (ng/mL)	341 (230-505)	239 (149-417)	0.06	293 (200-329)	285 (184-369)	>0.10	>0.10	>0.10
Neopterin (nmol/L)	18.7 (15.3-22.5)	19.2 (16.1-22.4)	>0.10	18.8 (16.6-25.5)	19.1 (16.2-23.1)	>0.10	>0.10	>0.10
KT-ratio	0.03 (0.02-0.03)	0.02 (0.02-0.04)	>0.10	0.03 (0.02-0.05)	0.03 (0.02-0.04)	>0.10	>0.10	>0.10
CRP (mg/L)	1.9 (0.9-5)	0.8 (0.6-5.2)	0.05	1.2 (1-5.7)	2.1 (0.8-7.4)	>0.10	>0.10	>0.10
I-FABP (ng/mL)	1231 (925-1817)	1840 (817-2188)	>0.10	1247 (814-2322)	1137 (855-2110)	>0.10	>0.10	>0.10

KT-ratio: kynurenine-tryptophan-ratio, a measure of tryptophan degradation.

Data are presented as median [interquartile range (IQR)] values. Wilcoxon matched pairs test in longitudinal analysis, Mann-Whitney U test between groups, not corrected for multiple testing.

Table 2 Relative abundance of bacterial taxa in the gut of HIV-infected individuals.

	Probiotics group (n=10)			Control group (n=12)			Between groups	
	Baseline	8. week	P-value	Baseline	8. week	P-value	Baseline, P=	8. week, P=
Gram-negative	0.27 (0.11-0.55)	0.12 (0.05-0.18)	0.01	0.22 (0.11-0.34)	0.24 (0.08-0.35)	0.70	0.63	0.31
Penta-acylated	0.38 (0.11-0.59)	0.18 (0.07-0.27)	0.01	0.21 (0.1-0.37)	0.24 (0.1-0.35)	0.39	0.46	0.54
Hexa-acylated	0.005 (0.002-0.01)	0.002 (<0.001 -0.02)	0.76	0.02 (0.01-0.07)	0.007 (0.002-0.032)	0.05	0.07	0.28
<i>Bifidobacterium</i>	0.01 (0.001-0.02)	0.02 (0.005-0.06)	0.04	0.02 (0-0.04)	0.012 (0.003-0.021)	0.14	0.35	0.74
<i>Lactobacillus</i>	0 (0-0.005)	<0.001 (<0.001 -0.002)	0.06	<0.001 (<0.001 -0.003)	<0.001 (<0.001 -0.001)	0.18	0.71	0.02
<i>Bacteroides</i>	0.19 (0.04-0.39)	0.03 (0.002-0.09)	0.01	0.04 (0.01-0.08)	0.07 (0.01-0.1)	0.69	0.09	0.54
<i>Parabacteroides</i>	0.03 (0.006-0.07)	0.003 (0.002-0.02)	0.01	0.01 (0-0.01)	0.005 (0.002-0.02)	0.31	0.01	0.88
Gram-positive	0.70 (0.42-0.86)	0.81 (0.80-0.93)	0.01	0.78 (0.66-0.89)	0.75 (0.65-0.90)	0.70	0.28	0.42

Data are presented as median [interquartile range (IQR)] values. Wilcoxon matched pairs test in longitudinal analysis, Mann-Whitney U test between groups, not corrected for multiple testing.