

Reduced Levels of D-dimer and Changes in Gut Microbiota Composition After Probiotic Intervention in HIV-Infected Individuals on Stable ART

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Background: Microbial translocation and chronic inflammation may contribute to non-AIDS morbidity in patients with HIV. This study assessed the impact of probiotic intervention on microbial translocation and inflammation in patients on antiretroviral therapy with viral suppression and subnormal CD4 count.

Methods: Thirty-two patients receiving antiretroviral therapy (CD4 <500 cells/μL) were randomized in a double-blind fashion to multistrain daily probiotics (n = 15), placebo (n = 9), or controls (n = 8) for 8 weeks. Soluble inflammation markers, D-dimer, lipopolysaccharide (LPS), sCD14, T-cell activation, tryptophan metabolites, and gut microbiota composition were analyzed at baseline and end of study. Nonparametric statistics were applied.

Results: Twenty-four participants completed the study and were included in as-treated analyses. In patients receiving probiotics, there was a significant reduction in D-dimer levels

(median change 33%, $P = 0.03$) and a tendency to reduced levels of C-reactive protein (CRP) ($P = 0.05$) and interleukin (IL)-6 ($P = 0.06$). The changes in CRP and IL-6 were highly correlated ($r = 0.95$, $P < 0.01$), whereas changes in D-dimer did not correlate with changes in CRP or IL-6. Increases in *Bifidobacteria* ($P = 0.04$) and *Lactobacilli* ($P = 0.06$) were observed in the probiotic group, whereas the relative abundance of *Bacteroides* decreased ($P \leq 0.01$). No significant changes were seen in markers of microbial translocation or T-cell activation. However, the expansion of *Bifidobacteria* correlated negatively with differences in LPS ($r = -0.77$, $P = 0.01$), whereas the reduction in *Bacteroides* correlated positively with changes in LPS during the study period ($r = 0.72$, $P = 0.02$).

Conclusions: Probiotic intervention seemed to reduce markers of coagulation and inflammation without overt changes in microbial translocation. These findings warrant further studies in larger cohorts with long-term follow-up.

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M.T. designed the study, included the patients, performed the laboratory experiments, and drafted the manuscript. B.S. planned and performed the laboratory experiments, analyzed the results, and wrote the manuscript. D.K., A.M.D.-R., P.N., and A.S. designed the study and facilitated the enrollment of the patients. P.N. and A.T. included the patients at Karolinska University hospital. A.M.D.-R. took part in the flow cytometry experiments. F.C.N., K.R., K.H., and J.R.H. established and conducted the microbiota analyses. P.M.U., A.E.A.D., and P.M.S. contributed to the tryptophan and coagulation analyses, respectively. S.-E.B. coordinated the production, quality control and supply of probiotics. All authors have critically revised the manuscript.

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INTRODUCTION

The widespread access to antiretroviral therapy (ART) during the last decades has changed HIV infection from a lethal disease to a chronic condition. In the same period, the relative burden of non-AIDS related chronic disorders such as cardiovascular disease and malignancy have increased in ART treated patients, possibly driven by residual chronic immune activation and inflammation.^{1,2} Despite suppressive ART, levels of inflammation and coagulation markers remain elevated^{3–5} and are associated with increased risk of non-AIDS clinical complications^{6,7} and all-cause mortality.^{4,8–10}

Microbial translocation across a damaged gut mucosal barrier has been proposed as an important contributor to persistent systemic immune activation in untreated HIV infection, and is reduced, but not normalized after ART.^{11–13} The selective depletion of the interleukin (IL)-17 producing CD4⁺ T-cells (Th17 cells) in the gut mucosa early in the HIV infection is believed to play a crucial role in this process,^{14,15} eventually leading to breakdown of the gut barrier and increased leakage of microbial products into the circulation.^{1,14,16}

Several studies have shown that the gut microbiota composition is changed in a nonfavorable direction during chronic HIV infection, with higher levels of opportunistic bacteria and lower levels of *Bifidobacteria* and *Lactobacilli* compared with a healthy population.^{17–21} This dysbiosis has been associated with systemic inflammation,^{18,20} tryptophan catabolism,¹⁸ and microbial translocation.^{19,20} In contrast, higher proportions of Lactobacillales in the gut microbiota were associated with a higher fraction of CD4⁺ T-cells and less microbial translocation during HIV-1 infection.²² Of note, we and others have recently shown that HIV-associated changes in gut microbiota are not restored on suppressive ART, providing the rationale for targeting the gut microbiota in treated HIV infection.^{18,20,23} Moreover, prebiotic supplementation seemed to increase the percentage of *Bifidobacterium* and to reduce soluble (s)CD14 levels in HIV-infected patients.²⁴ Serrano-Villar et al²⁵ recently reported partly reversed HIV-associated dysbiosis after intervention with prebiotics and glutamine. In simian immunodeficiency virus (SIV)-infected macaques on ART, a combination of prebiotic and probiotic supplementation increased gastrointestinal immune reconstitution and reduced fibrosis,²⁶ indicating that probiotic supplementation could have a beneficial effect also in treated HIV infection.

The aim of the study was to investigate the safety and efficacy of multistrain probiotics containing *Lactobacilli* and *Bifidobacteria*, upon markers of microbial translocation, T-cell activation, and systemic inflammation. We selected patients receiving ART with viral suppression but without a normalized CD4 count, as this patient group may benefit most from additional intervention.²⁷

METHODS

Patients

Thirty-two HIV-infected patients receiving ART were recruited between October, 2011 and March, 2013 from 2 study sites; 24 patients from Oslo University Hospital, Norway and 8 patients from Karolinska University Hospital, Sweden. Patients >18 years old with HIV-RNA <50 copies per milliliter for at least 6 months and CD4 count <500 cells per microliter were included. Exclusion criteria were the use of antibiotics or probiotics during the last 2 months, inflammatory bowel disease, episodes of infectious diarrhea, immune modulating therapy, or immigration from Asia, Africa, or Latin America during the last 6 months.

All participants provided written informed consent. The study was approved by the Regional Ethics Committees at both Oslo and Karolinska University Hospitals (2011/1449 C and 2012/459-31/3, respectively).

Study Design

Participants were randomized in a double-blind 2:1:1 fashion to the following study arms: (1) probiotics (n = 15), (2) placebo (n = 9), and (3) controls (n = 8). The intervention period was 8 weeks. The probiotics were self-administered and consisted of 250 mL/d fermented skimmed milk supplemented with *Lactobacillus rhamnosus* GG (10⁸ cfu/mL), *Bifidobacterium animalis* subsp. *lactis* B-12 (10⁸ cfu/mL), and *Lactobacillus acidophilus* La-5 (10⁷ cfu/mL). Heat-treated fermented skimmed milk without added probiotics served as placebo. The reason for including a control group in addition to the placebo group was the potential for microbial products having biological effects despite heat treatment.²⁸ Both the probiotics and placebo were produced every second week, with equal tastes and packed in neutral packaging. Every production was both bacteriological and sensory controlled to ensure appropriate concentration of and balance between the probiotic strains before transportation to the study sites. All products were stored in lockable refrigerators until supply. The participants were told to maintain their diet and particularly not to ingest any other probiotics. Furthermore, they underwent clinical examination at baseline and at end of intervention, and both blood specimens and fecal sampling were obtained at these visits. Potential adverse events were also recorded after 2 and 4 weeks. Both adverse events and changes in CD4 count during the study period were defined safety issues. Compliance was assessed by questioning at study visits and by deep sequencing of gut microbiota at end of study.

Blood Sampling and Peripheral Blood Mononuclear Cell Isolation

Citrate and EDTA plasma were snap frozen in pyrogen-free tubes, serum was centrifuged after coagulation, and both samples were stored at –80°C until further analyses. Peripheral blood mononuclear cells were isolated in cell preparation tubes (Becton Dickinson, Franklin Lakes, NJ) with sodium heparin and cryopreserved in 65% fetal calf serum (Sigma)/20% dimethyl sulfoxide/25% RPMI and stored at –150°C until analyses.

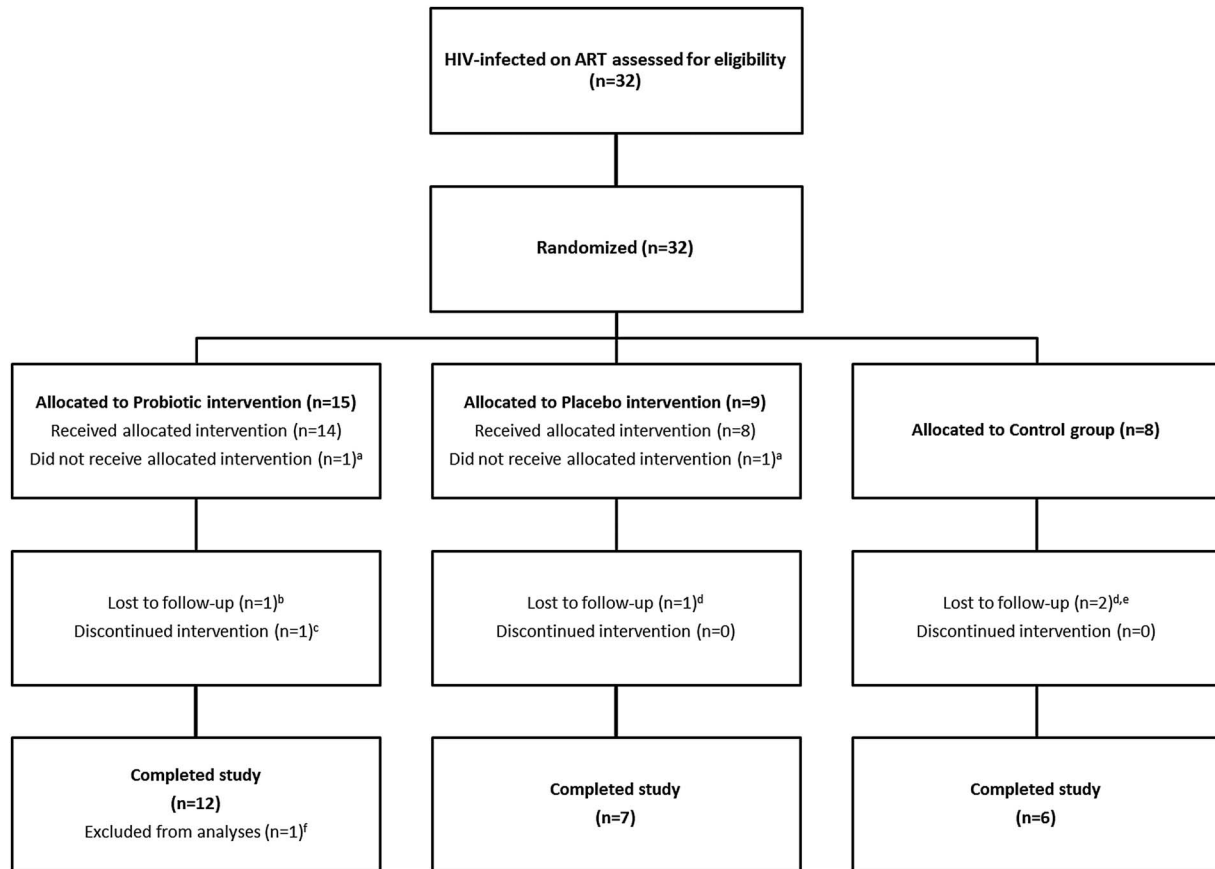


FIGURE 1. Study enrollment. Inclusion, randomization and study completion. ^aDid not meet at study start and was not included. ^bDid not start allocated treatment. ^cStopped after 3 doses due to mild gastrointestinal symptoms. ^dLost to follow-up after 4 weeks. ^eDid not meet after study start. ^fExcluded from analyses due to acute infection at week 8.

Analyses of Soluble Markers

Soluble biomarkers were analyzed in plasma except IP-10, which was measured in serum. Lipopolysaccharide (LPS) was analyzed by limulus amebocyte lysate colorimetric assay (Lonza, Walkersville, MD) as previously described.²⁹ IP-10, hsIL-6, and sCD14 were analyzed by Quantikine ELISA kits (R&D Systems Europe, Abingdon, United Kingdom). Samples were run with dilution 1:300 for sCD14 and 1:5 for IP-10. D-dimer was assessed by Asserachrom kit (Diagnostica Stago, Asnières, France). Thrombin generation was analyzed by calibrated automated thrombin (CAT) generation assay (Diagnostica Stago) as described in detail elsewhere.³⁰ 5pM tissue factor was used as trigger to initiate thrombin generation (PPP reagent), and the CAT parameters were normalized to a plasma pool of healthy controls and presented in percent. Patients receiving anticoagulation therapy with warfarin were excluded from the thrombin generation analyses (n = 2). All samples were run in batches and duplicates.

Plasma concentrations of tryptophan (Trp) and the Trp metabolite kynurenine (Kyn) were analyzed by liquid chromatography-tandem mass spectrometry by Bevitel A/S (www.bevital.no).³¹ The kynurenine/tryptophan ratio (KTR) was calculated by dividing the plasma concentration of Kyn (in nmol/L) by the concentration of Trp (in nmol/L).

The CRP analysis was part of the clinical routine, analyzed in fresh serum, and only accessible from the patients recruited at Oslo University Hospital (n = 17).

Immunophenotyping and Flow Cytometry

Details concerning flow cytometry analyses, the gating strategy, and information about selected antibodies are provided in Figure S1 (see Supplemental Digital Content, <http://links.lww.com/QAI/A724>).

Microbiota Analyses and Bioinformatics

Stool samples were collected without preservation and stored within 24 hours at -80°C until further use. Extracted DNA was polymerase chain reaction amplified targeting the V3-V4 region of the 16S rRNA gene using universal primers modified with addition of TruSeq Illumina adapters, as previously described.³² Sequencing was performed on the Illumina MiSeq platform using the 300 bp paired-end protocol (Illumina, San Diego, CA) at Norwegian Sequencing Centre (Oslo, Norway). Reads were preprocessed and mapped using default values in “closed reference operational taxonomic unit (OTU) clustering” in QIIME 1.8.0³³ against the Greengenes database version 1308.³⁴ OTUs containing

TABLE 1. Characteristics of the Study Cohort at Baseline

Total Study Population	Probiotic (n = 14)	Placebo (n = 8)	Control (n = 8)
Age, yrs (IQR)	50.3 (45.3–54.9)	50.1 (41.2–62.5)	52.5 (40.3–66.6)
Male gender, n (%)	10 (71.4)	4 (50)	8 (100)
Ethnicity, n (%)			
White	10 (71.4)	6 (75)	6 (75)
Risk group, n (%)			
MSM	6 (42.9)	4 (50)	6 (75)
IDU	1 (7.1)	1 (12.5)	0 (0)
No risk	7 (50)	3 (37.5)	2 (25)
Comorbidities, n (%)			
Cardiovascular/hypertension	3 (21.4)	2 (25)	4 (50)
Diabetes	1 (7.1)	0 (0)	1 (12.5)
Renal	3 (21.4)	0 (0)	0 (0)
Hepatitis C virus	1 (7.1)	1 (12.5)	0 (0)
Hepatitis B virus	0 (0)	0 (0)	0 (0)
HIV characteristics, (IQR)			
Years since HIV diagnosis	6.1 (3.3–12.7)	8 (3.9–16.4)	8.4 (4.3–13.2)
Years of effective ART	1.9 (1.4–3.5)	3.0 (1.7–5.5)	4.7 (3.6–6.6)
CD4 count nadir, cells/ μ L	145 (50–210)	130 (87–175)	109 (60–163)
Years since nadir	3.4 (1.7–6.2)	3.1 (1.8–11.4)	4.6 (2.6–9.7)
History of AIDS defining illness	3 (21.4)	1 (12.5)	4 (50)
ART regimen, NNRTI based	6 (42.9)	2 (25)	2 (25)
ART regimen, PI based	8 (57.1)	4 (50)	4 (50)
ART regimen, other	0 (0)	2 (25)	2 (25)
CD4 count, cells/ μ L	347 (244–420)	319 (252–412)	291 (257–433)
CD8 count, cells/ μ L	704 (552–1051)	735 (500–996)	645 (571–826)
CD4/CD8 ratio	0.4 (0.3–0.8)	0.4 (0.4–0.7)	0.5 (0.3–0.7)

Data are presented as n (%) of patients or median [interquartile range (IQR)] values. There were no significant differences between the groups (Kruskal–Wallis and Fisher's exact tests).

IDU, intravenous drug abuse; MSM, men who have sex with men; NNRTI, nonnucleoside reverse transcriptase inhibitors; PI, protease inhibitors.

less than 2 reads were filtered out, and the resulting OTU table was subsampled to 9442 reads per sample. Taxa summary plots (individual samples and groups), alpha diversity, and beta diversity analyses were based on this OTU table, using QIIME software.

Statistical Analyses

This trial was considered an exploratory study with unknown outcome estimates and variations, and consequently no formal sample size calculations were performed. However, based on published studies in the field,^{24,35} we aimed to include 50 patients. To evaluate the biological effect of probiotics, as-treated analyses were limited to subjects who had available data from both baseline and week 8. The initial analyses of the main variables showed no sign of potential

biological effects of the placebo product, and there were no significant differences between the placebo and control groups. Furthermore, the final number of patients was considerably lower than intended because of a demanding production and distribution pipeline of diary-based probiotics. Hence, the placebo and control groups became too small for meaningful comparisons and were for these reasons combined a posteriori in a category denoted nonprobiotic.

All continuous variables are presented as median and interquartile range. Nonparametric statistics were generally applied, using Wilcoxon matched pairs test, Mann–Whitney *U* test, Kruskal–Wallis test, Fisher's Exact test, and Spearman's rho test, as appropriate. A 2-tailed significance level of 0.05 was used. Statistical analyses were performed in Statistica v 7.0 (Statsoft, Tulsa, OK). Graphical presentations were made using Prism V6.03 software (GraphPad, San Diego, CA).

RESULTS

Study Participants and Baseline Characteristics

Thirty-two HIV-infected patients receiving ART were randomized. One participant withdrew consent a few days after inclusion and one did not attend at baseline to receive allocated treatment. These were not included in the baseline characteristics. Four participants were lost to follow-up. One patient in the probiotic group discontinued treatment because of mild gastrointestinal symptoms, and one patient was excluded from effect evaluation at week 8 because of acute infection at this time point. Hence, 24 participants completed the study and were included in as-treated analyses: 11 patients allocated to probiotics, 7 to placebo, and 6 controls (Fig. 1). Patient characteristics at baseline are given in Table 1. There were no significant differences between the groups, neither when cases were restricted to those who completed the study nor if the patients were grouped according to probiotic or nonprobiotic intervention (data not shown). Furthermore, there were no significant differences at baseline in any of the effect variables between the probiotic and nonprobiotic groups (see Table S1, Supplemental Digital Content, <http://links.lww.com/QAI/A724>).

Reduced Levels of D-dimer and Soluble Inflammatory Markers by Probiotic Intervention

In patients receiving probiotics, there was a significant reduction in levels of D-dimer, from a median of 320 ng/mL (interquartile range: 240–475) to 214 ng/mL (142–393) ($P = 0.03$) during the study period (Fig. 2). Furthermore, median CRP-levels decreased from 1.90 mg/L (0.90–4.15) to 0.75 mg/L (0.60–4.15) ($P = 0.05$) and IL-6 levels decreased from median 1.29 pg/mL (0.89–2.63) to 1.06 pg/mL (0.93–1.39) ($P = 0.06$) (Fig. 2). The changes in CRP and IL-6 were highly correlated ($r = 0.95$, $P < 0.01$). IL-6 and D-dimer were moderately intercorrelated at baseline ($r = 0.40$, $P = 0.03$), but the reduction in D-dimer did not correlate with the reduction

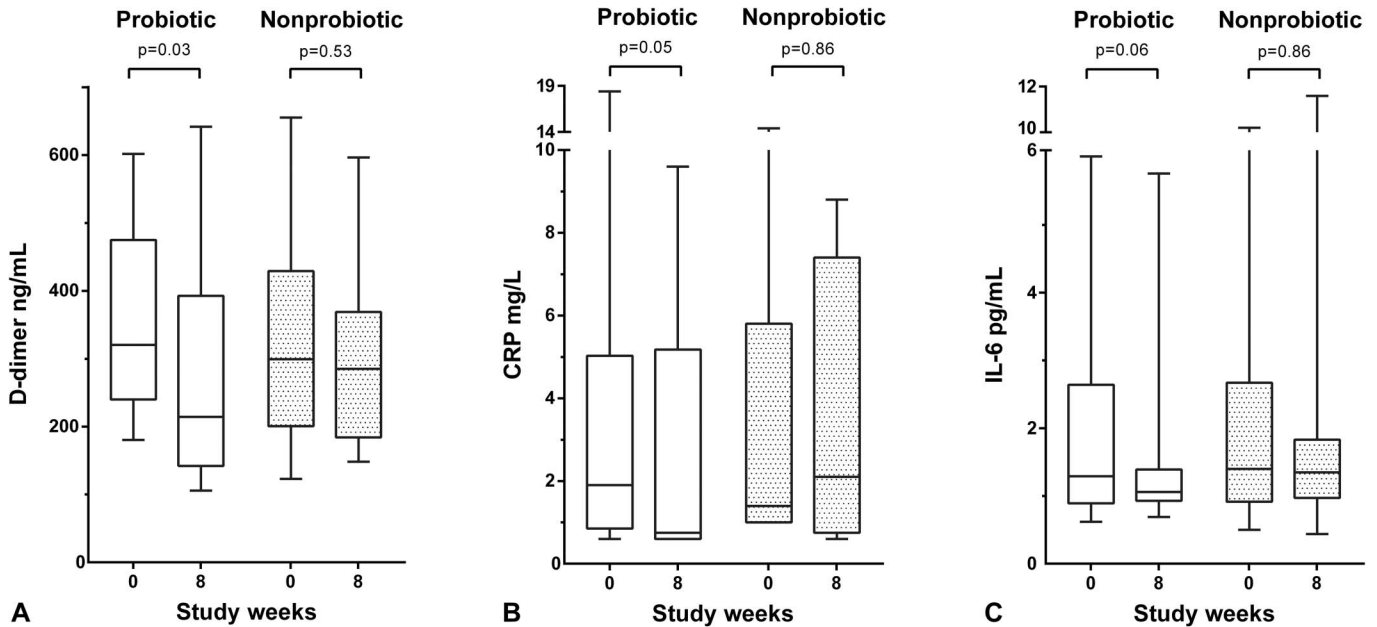


FIGURE 2. Reduction in inflammation markers after probiotic supplement. After 8 weeks of probiotic supplement, D-dimer was significantly reduced (A) and there was a tendency toward decreased levels of CRP (B) and IL-6 (C). These changes were not seen in the placebo group or among the controls (combined in analyses, denoted, “nonprobiotic”). The CRP analysis was only performed in participants from Oslo University Hospital (n = 17). Wilcoxon matched paired test.

in IL-6 or CRP, respectively. In the nonprobiotic group, the levels of D-dimer, CRP, and IL-6 remained stable. There were no significant differences in the changes in these variables between baseline and week 8 when comparing those allocated to the probiotic or to the nonprobiotic intervention ($P = 0.26$, $P = 0.32$, and $P = 0.36$, respectively).

To investigate whether the reduction in D-dimer could be a result of decreased activation of the coagulation system, we analyzed parameters of ex vivo thrombin generation, which is a functional test of the hemostatic–thrombotic system.³⁶ There were no significant changes in endogenous thrombin potential (%) or other measures of thrombin generation (lagtime % and peak thrombin %) during the intervention period in any of the allocated groups (see Table S1, Supplemental Digital Content, <http://links.lww.com/QAI/A724>).

Markers of Microbial Translocation or Tryptophan Catabolism Are Not Affected by Probiotic Intervention

Microbial translocation and subsequent monocyte activation were measured by circulating levels of LPS and sCD14, respectively. At 8 weeks, these levels did not differ from baseline, neither among those who received probiotics nor in the patients randomized to the nonprobiotic group (see Table S1, Supplemental Digital Content, <http://links.lww.com/QAI/A724>).

At baseline, tryptophan catabolism, as measured by KTR, correlated with the levels of D-dimer, IL-6, IP-10, sCD14, and LPS (see Figure S2, Supplemental Digital Content, <http://links.lww.com/QAI/A724>). There were no significant changes in the levels of tryptophan, kynurenine,

or KTR in any of the study groups during the intervention period (see Table S1, Supplemental Digital Content, <http://links.lww.com/QAI/A724>).

Peripheral Blood T-cell Activation and Regulation are Stable During Probiotic Intervention

The fraction of activated T-cells (CD38⁺HLA-DR⁺) remained unchanged in patients allocated to probiotic intervention, whereas in the nonprobiotic group, the percentages of activated CD4⁺ T-cells increased slightly from a median of 3.3% to a median of 3.5% ($P = 0.03$) after 8 weeks. However, the changes were not significantly different from those supplemented with probiotics ($P = 0.81$) and were not related to variations in the other soluble or cellular biomarkers. The fractions of Th17 cells (CD4⁺CD45RA-CD161⁺), Tc17 cells/mucosal-associated invariant T-cells (CD8⁺CD161⁺⁺), and Tregs (CD4⁺CD25^{high}CD127^{low}) did not change in any of the groups during the study period.

Gut Microbiota Analyses

The most common bacterial phyla were Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria accounting for more than 90% of the overall microbes both at baseline and at the end of the study (see Figure S3, Supplemental Digital Content, <http://links.lww.com/QAI/A724>). On phylum level, both the relative abundance of Actinobacteria and Firmicutes increased significantly in the probiotic group (both $P = 0.02$) (Fig. 3). On genus level, the changes in Actinobacteria were largely driven by an expansion of the genus

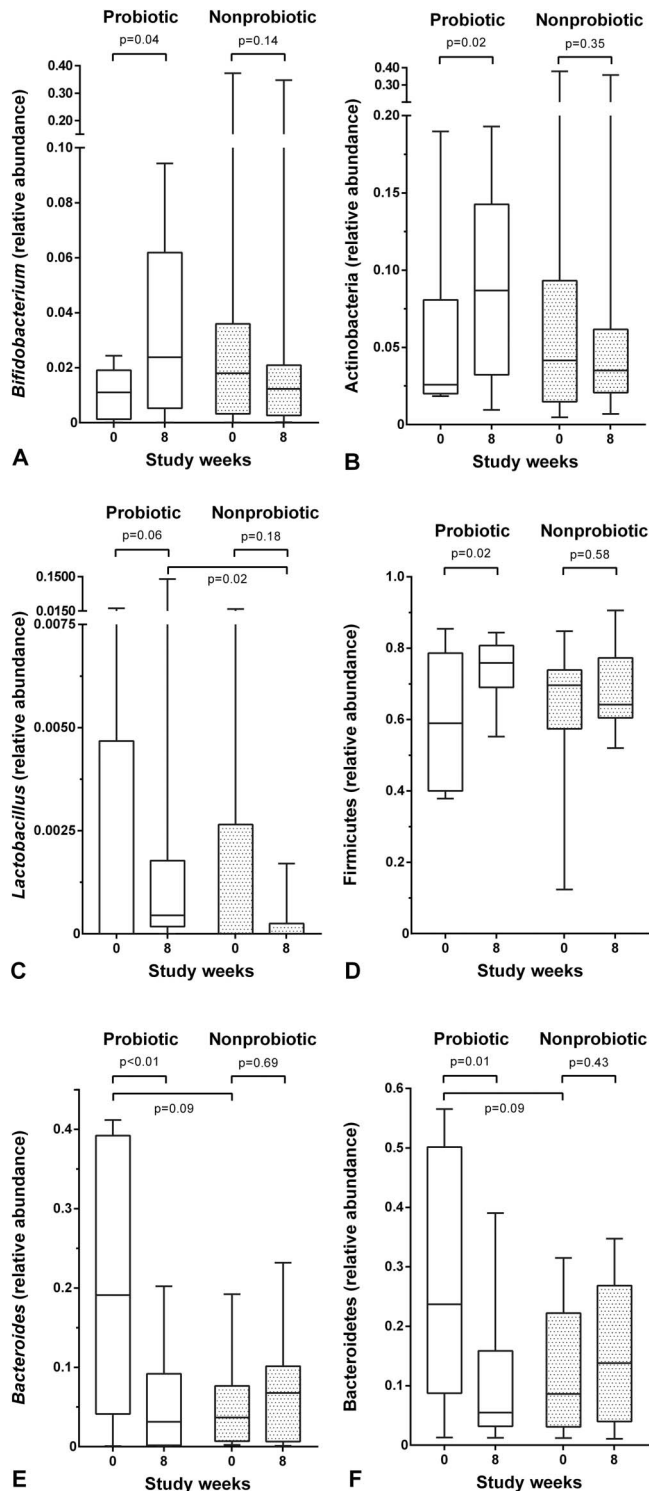


FIGURE 3. Changes in gut microbiota composition during the study period at genus (A, C, and E) and phylum level (B, D, and F). In those who received probiotics, we registered increased relative abundance of the genera *Bifidobacterium* (A) and *Lactobacillus* (C) and a subsequent expansion of the Actinobacteria (B) and the Firmicutes (D) phyla, respectively. We observed no such shift in the gut microbiota among the participants in the nonprobiotic group. The relative

abundance of *Bifidobacterium* ($P = 0.04$) (Fig. 3). Compared with the nonprobiotic group, those allocated to probiotic supplement had both a larger increase in the fraction of *Lactobacillus* ($P < 0.01$) between the time points and a higher relative abundance of *Lactobacillus* at the end of intervention ($P = 0.02$) (Fig. 3).

Furthermore, there was a significant decrease in the probiotic group of Bacteroidetes on phylum level ($P = 0.01$), which seemed mainly to be induced by a decrease in the *Bacteroides* genus ($P < 0.01$) (Fig. 3). There were no significant shifts in the proportion of the *Prevotella* genus (see Table S1, Supplemental Digital Content, <http://links.lww.com/QAI/A724>). The changes in *Bacteroides* were significantly different from the nonprobiotic group ($P = 0.01$ and $P < 0.01$). However, there was a tendency to higher relative abundance of Bacteroidetes ($P = 0.09$) and *Bacteroides* ($P = 0.09$) in those randomized to probiotics at baseline (Fig. 3).

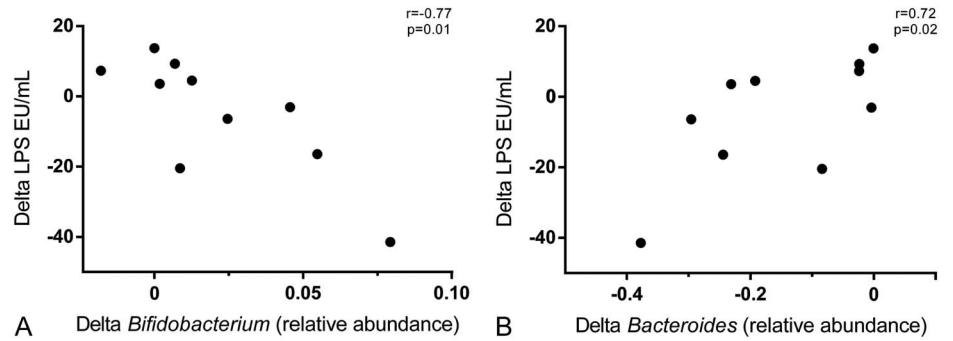
In addition, the increase in *Bifidobacteria* correlated negatively with shifts in LPS, whereas the reduction in *Bacteroides* was positively correlated with changes in LPS during the study period (Fig. 4). The same pattern was seen on phylum level (Actinobacteria and Bacteroidetes, respectively). Finally, the increase in Firmicutes were negatively correlated with changes in LPS levels ($r = -0.72$, $P = 0.02$), although this association was not significant on genus level (*Lactobacillus*). The expansion of *Bifidobacteria* or *Lactobacillus* genera did, however, not correlate with the degree of decline in D-dimer, IL-6, or CRP.

Studies have shown that ART naive HIV-infected patients have reduced levels of several butyrate-producing bacteria belonging to the Firmicutes phylum.^{19,37} There was a tendency to an increase in the main class Clostridia ($P = 0.09$) and a significant expansion in the most abundant family Ruminococcaceae ($P = 0.04$) in the probiotic intervention arm (see Figure S4, Supplemental Digital Content, <http://links.lww.com/QAI/A724>), which probably contributed to the expansion seen in Firmicutes. The relative abundances of the families Lachnospiraceae, Veillonellaceae, and Clostridiaceae remained unchanged in both groups (see Table S1, Supplemental Digital Content, <http://links.lww.com/QAI/A724>).

With regard the phylum Proteobacteria, there was a trend toward reduced proportions at week 8 in both groups (see Table S1, Supplemental Digital Content, <http://links.lww.com/QAI/A724>). No differences were observed in alpha diversity measures (see Table S1, Supplemental Digital Content, <http://links.lww.com/QAI/A724>).

abundance of *Lactobacillus* at week 8 was significantly higher among those allocated to probiotic compared with nonprobiotic supplementation (C). The relative abundances of *Bacteroides* (E) and Bacteroidetes (F) decreased significantly in the probiotic group. It should be noted that baseline levels of both *Bacteroides* and Bacteroidetes tended to be higher in those randomized to probiotic compared with nonprobiotic intervention. Wilcoxon Matched paired test and Mann-Whitney U test.

FIGURE 4. Correlations between changes in microbiota and LPS. Significant correlations between differences (delta) in plasma LPS (end of study vs baseline) and differences in relative abundances of *Bifidobacterium* (A) and *Bacteroides* (B), respectively. The changes in LPS levels were negatively correlated with difference in *Bifidobacterium* and positively correlated with the alteration in *Bacteroides* during the study period. Spearman's rank order correlations.



Compliance and Safety Measures

The compliance was considered acceptable, underscored by the fact that all participants allocated to probiotics increased in either *Bifidobacterium* or *Lactobacilli* in their gut microbiota during the intervention period. No serious adverse events were recorded. The CD4 count and CD4/CD8 ratio remained unchanged in the probiotic group after 8 weeks of intervention, whereas the CD4 count increased significantly in the nonprobiotic group ($P = 0.03$) (see Table S1, Supplemental Digital Content, <http://links.lww.com/QAI/A724>). However, the difference in the CD4 level between the groups at week 8 was not significant ($P = 0.36$) and the latter seemed to be a temporary increase, as the CD4 count rebounded to baseline levels at follow-up 3–6 months later (data not shown). Three patients in the nonprobiotic group (2 placebo and 1 control) had minor blips (HIV RNA >50 copies/mL) during the intervention period. In those receiving probiotics, one had a minor blip at the end of study.

DISCUSSION

The main findings in this study were reduced levels of D-dimer and the inflammatory markers IL-6 and CRP after 8 weeks of probiotic intervention in HIV-infected subjects on stable ART. Of note, the inflammation and coagulation systems remain more activated in HIV-infected than in HIV-negative patients despite suppressive ART.^{3–5} Furthermore, markers of inflammation and coagulation are associated with adverse outcomes, and these pathways have been suggested as potential therapeutic targets in HIV infection.^{1,4,6}

Whether this probiotic intervention exerted its main effect on the coagulation system or indirectly through inflammation is uncertain. The first step in D-dimer formation is thrombin generation with fibrin formation.³⁸ Although the ex vivo CAT assay used in our study did not confirm any impact on thrombin generation, minor changes in the endogenous tissue factor expression would probably not be detectable. Coagulation and inflammation are closely related processes,³⁹ and although we did not demonstrate any correlation between changes in D-dimer and IL-6 or CRP, respectively, the tissue factor expression might be attenuated as a result of decreased inflammation or through Toll like receptors modeling.⁴⁰ Several studies have specifically identified D-dimer, CRP, and IL-6 as independent markers of non-AIDS related morbidity and mortality in patients on

ART.^{4,6,8,41,42} Hence, our results could imply a beneficial effect of probiotics by attenuating inflammation and perhaps also coagulation activity.

Probiotics including *L. rhamnosus* have been shown to reduce inflammation in several disease models, possibly because of an interaction with various pattern recognition receptors.^{43,44} In SIV-infected macaques, intervention with the probiotic strain VSL#3 combined with *L. rhamnosus GG* and prebiotics resulted in improved gut immunity and a trend toward reduced plasma levels of D-dimer.²⁶ Furthermore, in a pilot trial investigating prebiotic, probiotic, or synbiotic therapy (inulin, *L. rhamnosus* and *B. lactis*) in ART naive patients, the synbiotic therapy seemed to reduce IL-6 and 16S rRNA levels (a measure of bacterial load), whereas probiotics affected levels of 16S rRNA.⁴⁵ In virally suppressed patients, a significant reduction in both IL-6 and LPS was shown after 12 weeks of supplementation with *Saccharomyces boulardii* compared with a placebo group, although changes within the treatment groups were not reported.⁴⁶ Hence, several studies indicate that targeted alteration of the gut microbiota could reduce inflammation.

We hypothesized that probiotic supplementation would attenuate inflammation and immune activation because of decreased microbial translocation. However, we could not demonstrate any significant effect on microbial translocation assessed by plasma LPS or sCD14 analyses. This is in line with most recent studies targeting the intestinal microbiota, including intervention with the probiotic/prebiotic combination given to SIV-infected macaques,²⁶ the LPS-binding agent Sevelamer,⁴⁷ the nonabsorbable antibiotic Rifaximin,⁴⁸ and the fungus *S. boulardii*,⁴⁶ showing no reduction in LPS or sCD14 levels.

Nevertheless, the results from previous studies are not fully consistent. Gori et al²⁴ observed reduced levels of sCD14 after 12 weeks of prebiotic therapy compared with a placebo group and a significant reduction of LPS at week 16. Interestingly, the *Bifidobacterium* fraction also increased. In our study, we observed a strong negative correlation between the expansion of *Bifidobacteria* and changes in LPS levels during the intervention period, indicating a possible effect of this probiotic strain on LPS levels. This finding is supported by both murine models and prebiotic intervention in humans, where an increase in fecal *Bifidobacteria* has been shown to be associated with reduced LPS levels.^{49,50}

We observed an increase in the relative abundance of both Firmicutes and Actinobacteria at phylum level and the latter was mainly induced by the expansion of *Bifidobacteria*. We also

noticed a tendency of increased levels of *Lactobacilli*. These shifts in the gut microbiota are consistent with the probiotic strains administered and thereby a robust measure of compliance. The expansion seen in the family Ruminococcaceae, which comprises several butyrate-producing bacteria, could perhaps also contribute to a favorable intestinal homeostasis.⁵¹ However, simultaneously there was a reduction of *Bacteroides*, which is the most common bacterial genus in the core gut microbiota.⁵² It has been reported that *Bifidobacteria* increase at the expense of *Bacteroides* after prebiotic supplementation, and competition for the same biological niche within the gut microbiota could be an aspect.^{50,53} Hence, there is a need to evaluate the potential long-term effect of these probiotic strains on the gut microbiota and whether such intervention is beneficial overall.

Our study has its limitations, the major one being the small sample size, increasing the risk of type II statistical errors and bias of the results. In this pilot trial, we experienced a relatively slow inclusion rate of patients and a demanding and costly production and distribution line of dairy-based probiotics and placebo products. In addition, the patients had to pick up 4 liters of dairy-based products every 2 weeks, which could have explained the relatively high drop-out rates. Hence, in future large-scale studies, probiotics in capsule or powder formulation will probably be a more feasible option. Our study also has its strengths and is one of the very first to target the gut microbiota in HIV infection with available deep sequencing data. Ideally, postintervention data could have determined whether changes in the gut microbiota persisted, and gut biopsies could also have provided more information of the possible effect on mucosa-adherent microbes as well as viral reservoirs and immune status of the gut mucosa.

In conclusion, 8 weeks of probiotic intervention was well-tolerated and seemed to reduce markers of coagulation and inflammation, without overt changes in microbial translocation. These findings warrant future studies in larger cohorts with long-term follow-up.

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