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Effect of 25 weeks probiotic supplementation on immune function of HIV patients

Ruben Hummelen,[1,5,*] John Changalucha,[2] Nicodemus L. Butamanya,[3] Tara E. Koyama,[1] Adrian Cook,[4] J. Dik F. Habbema[5] and Gregor Reid[1]

[1]Canadian Research & Development Centre for Probiotics; Lawson Health Research Institute; London, ON Canada; [2]National Institute for Medical Research; Mwanza Research Centre; [3]Sekou-Toure Regional Hospital; [4]Kivulini Womens' Rights Organisation; Mwanza, Tanzania; [5]Department of Public Health; Erasmus University Medical Centre Rotterdam; The Netherlands

Key words: HIV, probiotics, lactobacillus, diarrhea, immune function

Abbreviations: ART, anti-retroviral treatment; ALT, alanine transaminase; SD, standard deviation

Introduction: Studies with a follow-up of <8 weeks have indicated immune-preserving effects of yogurt probiotic supplementation among HIV patients. To evaluate the impact of 25 weeks use of probiotics, a randomized, double blind, controlled study was undertaken on 65 women who were naïve to anti-retroviral treatment.

Results: Ten participants were excluded post-randomization due to non-eligibility. Thirty participants were assigned placebo, of whom 25 completed the study versus 19 of 25 completing the study in the probiotics group ($p = 0.5$). From baseline to 10 weeks follow-up, the CD4 count declined on average 3 CD4 cells/μl (95% Confidence Interval: -97; 91) with placebo versus an increase of 50 cells/μl (95% CI: -61; 162) with probiotics ($p = 0.5$). From baseline to 25 weeks, the CD4 count increased with 19 cells/μl (95% CI: -90; 129) in the placebo group versus 46 cells/μl (95% CI: -100; 192) with probiotics ($p = 0.8$). No differences in immune markers, diarrhea incidence or adverse events were observed.

Discussion: Lactobacillus GR-1 and RC-14 may be safely consumed at 2×10^9 CFU/day by moderately immune compromised HIV patients but this did not universally preserve immune-function.

Patients and Methods: Women were randomized to receive oral capsules containing *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14 (2×10^9 colony forming units) or placebo twice daily for 25 weeks. The CD4 count and immune markers (IgG, IgE, IFNγ and IL-10) were measured at baseline and during follow-up, the occurrence of diarrhea was reported daily.

Introduction

The majority of the body's immune cells resides in the gut,[1] a compartment that is disproportionally affected by HIV infection. During the early stages of infection, the virus depletes gut associated lymphoid tissue (GALT) including $CD4^+$ lymphocytes[2,3] and dendritic cells.[4] These detrimental changes appear to lead to a reduced gut barrier function,[5] aberrant intestinal microbiota[6,7] and translocation of microbial products to the peripheral bloodstream.[8,9] The latter is associated with increased levels of inflammatory cytokines and immune cell activation,[8-10] which in turn can drive HIV progression.[8,11]

Though anti-retroviral treatment (ART) can reverse the detrimental changes to the GALT in most HIV patients,[8] in a subgroup of patients the damage cannot be undone.[9,12] The use of ART has significantly increased in the developing world, yet still approximately one in three patients do not have access to this therapy.[13] Moreover, ART is often only initiated when a moderate immune compromised state is reached (<350 CD4 cells/μl).

Studies in Nigeria, Brazil and Tanzania have suggested that probiotic organisms, defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host",[14] may be able to delay damage to, or help preserve, the immune function of HIV patients.[15-17] Potential mechanisms include stimulating Natural Killer cell activity,[18] improving intestinal barrier function[19,20] and lowering systemic inflammation.[21,22] However, no studies have been conducted to assess whether a preserved immune-function can be maintained for longer than two months. Furthermore, although the safety of probiotic use among HIV patients has been investigated,[7,23] the longer term safety has not been established. Therefore, we initiated a randomized, placebo controlled trial to assess the impact of 25 weeks probiotic *L. rhamnosus* GR-1 and *L. reuteri* RC-14 on immune function in moderately immune compromised HIV patients (>200 cells/μl) and confirm the safety of probiotic use among this population.

Results

A total of 229 women were screened of whom 65 were randomized to receive probiotics ($n = 32$) or placebo ($n = 33$) for 25

*Correspondence to: Ruben Hummelen; Email: rubenhummelen@gmail.com
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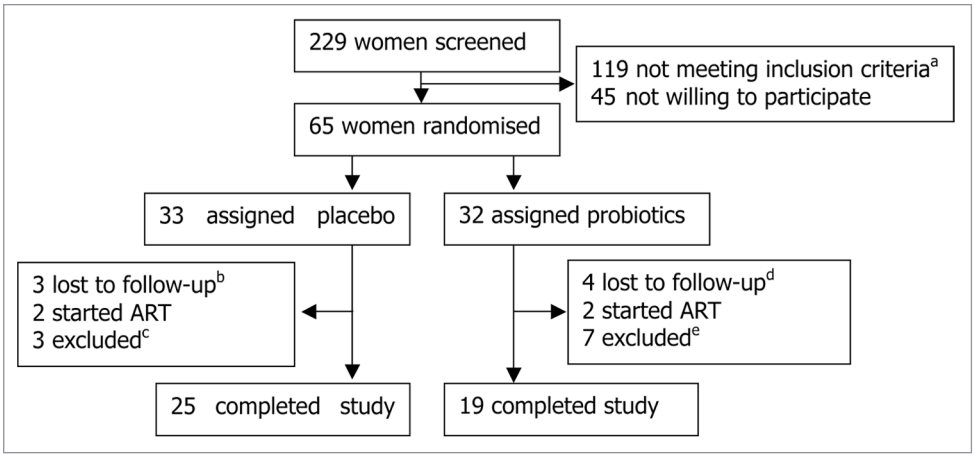

Figure 1. ART: Anti-retroviral treatment. [a]86 normal vaginal microbiota (Nugent score 1–3), 15 sexual transmitted infection and 18 eligible for ART (CD4 count <200 cells/μl). [b]2 losses due to travelling, and 1 for unknown reasons. [c]2 baseline CD4 <200 cells/μl, 1 baseline CD4 invalid. [d]2 losses due to travelling, and 2 for unknown reasons. [e]4 baseline CD4 <200 cells/μl, 1 baseline CD4 invalid, 2 HIV negative.

Table 1. Baseline characteristics

Characteristic		Placebo (*n* = 29)	Probiotic (*n* = 24)	*p*
		% (*n*)	% (*n*)	
Age (years)	<30	31 (9)	38 (9)	0.8
	≥30	69 (20)	63 (15)	
Education (years)	0	3 (1)	4 (1)	1.0
	≥1	97 (28)	96 (23)	
Marital status	Single	79 (23)	63 (15)	0.2
	Married	21 (6)	38 (9)	
HIV diagnosis (years)	<1	69 (20)	79 (19)	0.5
	≥1	31 (9)	21 (5)	
CD4 count (cells/μl)	<350	59 (17)	67 (16)	0.6
	≥350	41 (12)	33 (8)	
Any HIV symptom[a]	None	76 (22)	75 (18)	1.0
	Present	24 (7)	25 (6)	
Co-trimoxazole[b]	No	66 (19)	46 (11)	0.2
	Yes	35 (10)	54 (13)	
BMI (kg/m2)[c]	<18.5	12 (3)	23 (5)	0.4
	≥18.5	89 (23)	77 (17)	
Albumin (gr/ml)[c]	<36	67 (16)	59 (10)	0.7
	≥36	33 (8)	41 (7)	

BMI = Body Mass Index. [a]Any HIV related symptoms: Defined as having any of the following symptoms; diarrhea, coughing, fever for >1 month, weight loss >4.5 kg or skin rash during past year. [b]Co-trimoxazole use as prophylaxis for ≥30 days during follow-up. [c]Numbers do not add up due to missing values.

weeks (**Fig. 1**). A total of 10 women were excluded from analyses due to a missing CD4 count at baseline (*n* = 2), a CD4 count lower than 200 at baseline (*n* = 6), or a HIV-negative test during follow-up (*n* = 2). Two other participants, one in each group, were not included in the analyses after failing to return for any follow-up visit. At baseline, no clinically relevant differences could be detected between the placebo and probiotics group (**Table 1**). Overall, half of the women had low albumin levels (<36 gram/ml), indicating malnutrition or systemic inflammation. Furthermore, 43 of 48 women had levels of IgG higher than normal (≥18 gr/l), and 35 of 48 women had abnormally high IgE levels (≥400 ng/ml, **Fig. 2**).[27]

From baseline to 10 weeks follow-up the CD4 count dropped on average with 3 CD4 cells/μl (95% CI: -97; 91) in the placebo group versus an increase of 50 cells/μl (95% CI: -61; 162) in the probiotics group (p = 0.5). At 25 weeks, the CD4 count increased from baseline in the placebo group with 19 cells/μl (95% CI: -90; 129) versus an average increase of 46 cells/μl (95% CI: -100; 192) in the probiotics group (p = 0.8). Changes in the immune parameters IFNγ, IL-10, IgG and IgE did not differ between the groups (**Fig. 2**).

The median number of days reported with diarrhea was low in both groups with two days in the placebo group (median 151 days recorded) versus five days in the probiotic group (median 148 days recorded) (p = 0.4, Wilcoxon rank-sum test). The average length of each episode was similar with in both groups a median of 1 day diarrhea per episode (p = 0.2, Wilcoxon rank-sum test). The odds of having stomach pain at any time during follow-up (Odds Ratio for probiotics group (OR) = 0.7; 95% CI: 0.4–1.3), flatulence (OR = 0.9; 95% CI: 0.5–1.7), nausea (OR = 1.3, 95% CI: 0.7–2.5) and the impact of gastro-intestinal symptoms on daily life (OR = 0.7, 95% CI: 0.4–1.2) did not differ between groups.

Adherence estimates based on pill counts indicated that the product was well-tolerated and that compliance was good with a median of 92% (range 47–100%) of days compliant in the placebo group versus 94% (range 84–100%) of days in the probiotics group (p = 0.5, Wilcoxon rank-sum test). Furthermore, loss to follow-up was similar between groups with 4 of 29 in the placebo group not returning for a follow-up visit versus 5 of 24 in the probiotics group (p = 0.5).

A total of 15 participants reported at least one adverse event, of which 10 of 29 among the placebo group and 5 of 24 among the probiotics group (p = 0.3). One participant in the placebo group reported constipation, two diarrhea, three nausea, two itching or peeling skin, one dizziness and one vaginal odor as main adverse event. In the probiotic group one participant reported abdominal discomfort, three nausea and one reported vomiting as adverse event. A physician rated all events mild or moderate and none was rated severe. Over the course of 25 weeks probiotics supplementation, no differences in ALT, albumin and creatinine level were detected between the groups, indicating no adverse effect on liver or kidney function (**Table 2**).

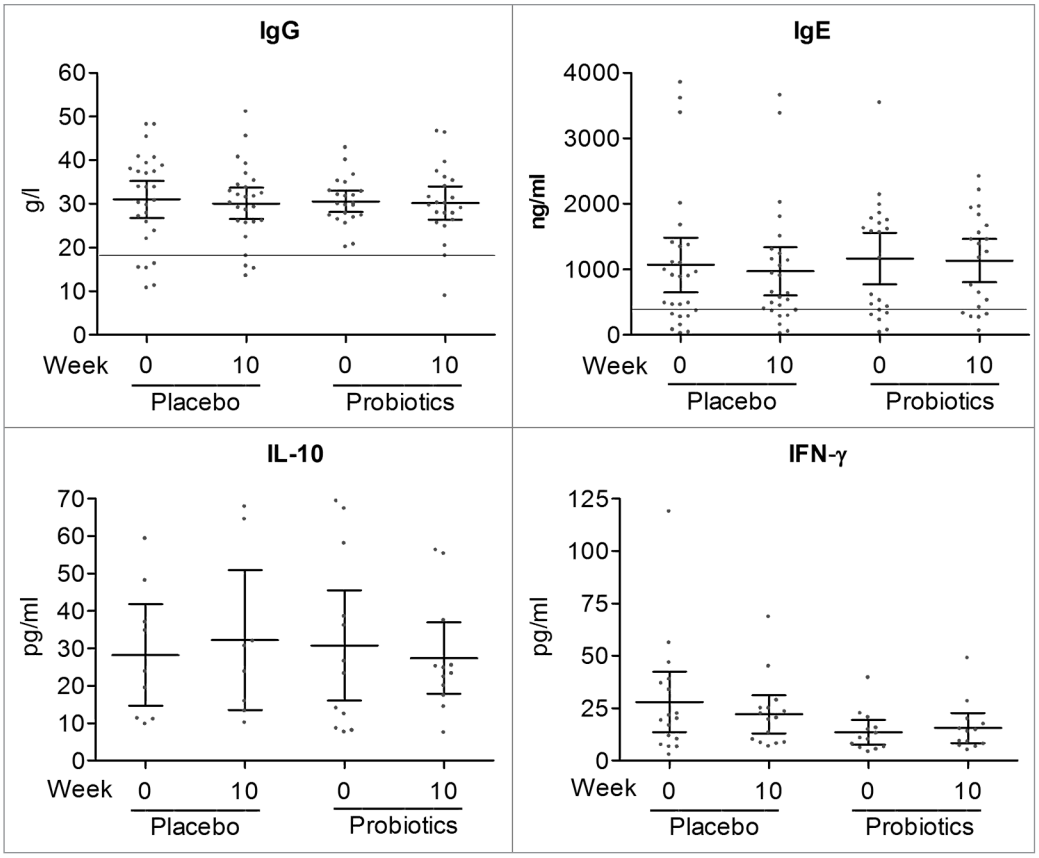

Figure 2. The horizontal lines in the IgG and IgE graphs indicate the upper reference values.[27] Individual values are shown as dots with the short horizontal line representing the mean and the 95% confidence intervals displayed as the error-bars.

Table 2. Laboratory parameters at baseline and 25 weeks follow-up

		Placebo			Probiotics			
Measure	**Reference** Value/unit	**Week 0** n/total	**Week 25** n/total	**Change**[a] mean ± SD	**Week 0** n/total	**Week 25** n/total	**Change** mean ± SD	**P**[b]
Creatinine	≥139 μmol/l	1/24	1/25	-5.0 ± 46	0/18	1/18	-1.3 ± 18	0.7
Albumin	<36 g/l	16/24	16/25	-0.8 ± 4	10/17	8/15	-0.8 ± 5	1.0
ALT	≥50 IU/l	0/24	1/25	-3.8 ± 11	0/18	0/18	-2.7 ± 8	0.7

SD = standard deviation. [a]Mean within-subject change from baseline to follow-up. [b]Tested using within-subject change from week 0 to week 25.

Discussion

To our knowledge, this is the first randomized, placebo controlled study to assess the impact of long-term probiotic use on immune function among people living with HIV. Previous trials have shown a preservation of the immune function with probiotic use among children treated with, or non-responsive to ART, *Bifidobacterium bifidum* and *Streptococcus thermophilus* in Brazil,[16] and among women naïve to ART with *L. rhamnosus* GR-1 in Nigeria.[15] The present study did not show a significant impact of *L. rhamnosus* GR-1 and *L. reuteri* RC-14 on the immune function. The disconcordance of this finding has three potential explanations; the variability in CD4 count fluctuations was higher among this study population than the one from which the sample size was calculated,[15] resulting in a potential too small sample size; the populations previously studied, children in Brazil, and women with diarrhea in Nigeria, differed in their immune response to the probiotic formulation due to a different and less acute disease state of the host; the daily ingestion of encapsulated probiotic *L. rhamnosus* GR-1 and *L. reuteri* RC-14 did not have a long-term impact on the immune function of people living with HIV. The inefficacy of the probiotic strains in preserving the immune function may be the result of using encapsulated probiotics versus the use of probiotic yogurt with the same probiotic strains in previous studies in reference 15 and 17. However, as yogurt was not regarded as a sustainable long-term intervention for those patients living far from a distribution center, the probiotics were encapsulated. Though in vitro analyses of the viability of the probiotics from yogurt or capsules did not differ, analyses of the survival and colonization of the probiotic

strain in the intestinal tract could have aided further explanation of discrepancies. This analyses was not included to minimize the attrition rate by reducing the number of samples taken.

Of note, within our population, those who were moderately immune deficient at baseline (CD4 count 200–350 cells/μl) experienced an increase of 34 cells/μl (95% CI: -37; 105) in the placebo group versus a mean increase of 158 cells/μl (95% CI: 35; 281) in the probiotics group at ten weeks (p = 0.1). In the six participants who were not included in the main analyses because of a baseline CD4 count <200 cells, the four participants receiving probiotics experienced a mean increase of 93 cells/μl (95% CI: 26; 159) while the two placebo participants experienced a mean decrease of 69 cells/μl (95% CI: -95; -42) (p = 0.04). In both subgroups, these effects were not sustained at 25 weeks. These findings suggest that probiotics may have a different impact among sub-populations, with those who have a more compromised immune state responding better to the probiotic interventions. This emphasizes the importance of trying to increase the chance of patients responding to therapy, by using biological or genetic markers when available to stratify the patient population before enrollment and decide at what point intervention will provide the best outcome (for example, in subjects with a starting CD4 count less than 350 cells/μl).[28]

The immune markers IgE, IgG, IFNγ and IL-10 did not show a significant impact of probiotic supplementation. Markers that are more specific for the intestinal barrier including plasma lipopolysaccharide, plasma total bacterial DNA and urine lactoferrin: mannitol ratio, may be more useful in future studies.[8,29] The finding that IgE is elevated compared to reference values[27] confirms previous findings of a Th-2 dominated immune-state among this population.[30,31] The increased levels may be partly due to helmintic colonization (IgE), though this effect is likely to be limited as previous studies show levels that do not significantly increase among HIV patients harboring helminths.[30] Elevated levels of IgG among HIV patients have been previously reported, although these may have been due to sub-clinical opportunistic infections.[32]

This is one of few studies to examine the safety of long-term use of probiotics, and the first to do so in a population with HIV.[33] No adverse events were associated with the long term use of probiotics. Minor adverse events were reported by twice as many participants in the placebo group than in the probiotic group. Given the challenges of living in this impoverished community, participants' compliance was remarkable with 80% (n = 44) completing the 25-weeks trial. This might partly be explained by the selection of participants who were keen to take prolonged action that they believed might improve the course of their HIV infection.

This study shows that long term probiotic supplementation is feasible in HIV patients in sub-Saharan Africa. Unfortunately, so-called 'probiotic' products that have never been tested in humans, never shown to provide measurable benefits, and with strain combinations that have not been assessed in malnourished or HIV-positive subjects, are now becoming available in countries including Zambia and South Africa. This is not unexpected given that similar or identical products have not yet been banned in the developed world, or instructed to not use the term probiotic until their health benefits have been substantiated.[14] For people in developing countries who face the day-to-day challenges from malnutrition, infectious diseases and poverty, the alleviation of gastro-intestinal symptoms, improvement in immunity and increased ability to work could contribute significantly to their life and that of their family. The potential to provide benefits through probiotic food or supplements, is worthy of further study, but selling untested products is tantamount to an unethical practice. As shown herein and as mechanistic studies would support the rationale for probiotics for HIV management,[34] there are some encouraging signs that well-documented probiotics may have potential.[15-17,35] This has to be proven in targeted large randomized trial. Just as important, if certain probiotics do not have a tangible effect on the health (as assessed by immune parameters, diarrhea incidence, other markers) of subgroups of HIV patients, there is no reason for them to be recommended.

Methods

Patients and methods. The primary outcome of the trial was the change in CD4 count from baseline to 25 weeks. Secondary outcomes included the effect of probiotic supplementation on the vaginal microbiota of women living with HIV, results which were reported elsewhere in reference 24. Between October 2007 and February 2008, HIV seropositive women attending the HIV care and treatment clinic at Sekou-Toure regional hospital, Mwanza, Tanzania, were enrolled. Inclusion criteria were confirmed HIV infection, not eligible to be treated with antiretroviral medication (i.e., CD4 count >200 cells/μl), not-normal vaginal microbiota according to the Nugent score,[25] and being 18–45 years old. Exclusion criteria were pregnancy, lactation, menstruation at time of screening, hypersensitive to metronidazole and presence of sexually transmitted diseases.[24]

Participants visited the clinic for a screening, which included CD4 cell count, serum storage, and a physical and gynecologic examination performed by a physician. Participants diagnosed with bacterial vaginosis[25] were treated with metronidazole for 10 days (twice daily 400 mg orally). Within one week of screening, eligible participants were interviewed using a structured questionnaire to collect information on demographics, medical history and drug use. According to a computer generated block randomization list, participants were supplied with a tube of capsules containing freeze-dried *L. rhamnosus* GR-1 and *L. reuteri* RC-14 (2 x 10^9 viable organisms/capsule) or identical looking placebo capsules. The tube sets, only bearing the identification number of each participant, had been prepared by a statistician not involved in the data collection, to ensure blinding of participants and study staff. Tube-sets were supplied at each follow-up visit and capsules had to be taken twice daily for 25 consecutive weeks. Viability testing after transport on dry ice from Denmark and storage on site for eight months confirmed a minimum of 10^9 colony forming units per probiotic capsule.

During follow-up, participants were interviewed at 2, 5, 10, 15, 20 and 25 weeks to evaluate adverse events, assess gastrointestinal symptoms, and to count remaining capsules. The

participants recorded the occurrence of diarrheal episodes using a diary, which was collected at each visit. Diarrhea was defined as three or more loose or watery stools in a 24-hour period.[26] At baseline and after 10 and 25 weeks the CD4 was measured using the Partec FACS (Partec GmbH, Münster, Germany). At baseline and 10 weeks, ELISA assays were used to measure serum levels of IgE, IgG, (E80-108 & E80-104, Bethyl Laboratories, Montgomery, USA) and IFNγ, IL-10 (99-7799, eBioscience, San Diego, USA). Alanine transaminase (ALT), creatinine and albumin were measured at baseline and twenty-five weeks using a Beckman CX5 biochemistry machine (Beckman coulter, Brea, USA). All laboratory tests were performed on site at National Institute for Medical Research (NIMR), Mwanza Research Centre, according to good laboratory practices.

Ethics. The ethics boards of Erasmus University Medical Centre, Rotterdam, The Netherlands, and NIMR, Tanzania, approved the study design and protocol. Participants were informed of the purpose of the trial and had to give their signed or thumb printed informed consent before participation. This trial was registered at clinicaltrials.gov NCT00536848.

Sample size and statistical analyses. We calculated that a sample size of 30 participants per treatment arm would be required to detect a 15 cells/μl difference in CD4 count (from baseline to 25 weeks) with 90% power and a two-sided alpha of 0.05, a 25% loss to follow up and a standard deviation of 10 CD4 cells/μl of within subject differences.[15] Laboratory parameters were analyzed as continuous data using within-subject differences from baseline to 10 or 25 weeks, and tested using an unpaired t-test. When non-parametric data was analyzed using a Wilcoxon-rank-sum, this is indicated in the text. Gastro-intestinal symptoms' data were analyzed as dichotomous data by taking into account the correlation between measurements using generalized estimating equations, with an exchangeable correlation structure and a logit link. Baseline variables were analyzed using a χ^2 test; Fishers exact test was used if one or more sub-groups had fewer than five participants. All tests were performed two-sided at the $\alpha = 0.05$ level with no adjustments made for multiple comparisons. Data was stored in an Access database and analyzed using SPSS 15.0 software.

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Potential Conflicts of Interests

We declare that no conflict of interest exists. Gregor Reid no longer owns patents for Lactobacillus GR-1 and RC-14 and none of these patents cover HIV/AIDS treatment.

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