Probiotics to Target the Intestinal and Vaginal Microbiota in HIV

Ruben Hummelen

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Probiotica als interventie voor de darm- en vaginale flora bij HIV

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Promotoren: Prof.dr. J.D.F. Habbema
                         Prof.dr. G. Reid

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"To the women living with AIDS in Africa. Indomitable, resilient, courageous."

Stephen Lewis

Aan mijn ouders en Jaimie

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Part III. Probiotics and bacterial vaginosis in HIV

1.

General introduction

HIV

The human immunodeficiency virus (HIV) preferentially targets, infects and kills CD4+ lymphocytes, which are essential for initiating an immune response against invading micro-organisms. Ultimately the immune response becomes severely compromised and opportunistic infections and virus-related cancers develop, at which stage the acquired immune deficiency syndrome (AIDS) is apparent. The time between HIV infection and the development of AIDS shows enormous variety, with less than two years in some individuals to more than ten years without treatment [1]. This is influenced by multiple factors, including the hosts immune response, nutritional status and pharmacologic prophylaxis [2,3,4].

Early in the twentieth century the virus likely transferred from an animal source to humans [5]. Observations were made in the 1970s when African doctors noted an increase in wasting and infections associated with a failing immune system. The disease took its toll in many villages and became known to the African public as "slim disease". Western physicians and scientists remained unaware of the epidemic until the first reports of an unusual cluster of *Pneumocystis carinii* pneumonia were made in New York in 1981 [6]. Although HIV was already identified as the cause for AIDS in 1984 [7], only in 1996 a major breakthrough was made in treatment, when a combination of anti-retroviral drugs was shown to dramatically increase life-expectancy among HIV patients. Currently, these anti-retroviral treatments (ART) are almost universally available in developed countries. However, despite major efforts since the G8 summit in 2005, globally only one out of three patients in need of ART have access to the intervention [8]. For those who are not eligible, and those who do not have access to the treatment, adjuvant interventions to reduce the progression of HIV would fill a significant void in the range of treatment options.

HIV and the intestinal microbiota

In a healthy state, the interplay between the intestinal microbiota and the host is symbiotic in nature. Microbes are provided with a range of habitats and in turn supply energy, provide nutrients, and prevent colonization of the host by pathogens [9]. In this setting, the

immune system has to balance permissive, tolerogenic responses to food antigens and commensal microbes with inducing potentially damaging inflammatory responses to ward off pathogens.

Infection with HIV has a disruptive impact on this physiological interplay. In the early stages of disease, the virus preferentially infects and depletes immune cells associated with the intestinal immune system, including CD4+ lymphocytes and dendritic cells [10,11,12]. Their infection is likely due to the relatively high expression of HIV co-receptors as a result of their activated state [13,14]. The intestinal epithelial cells are also affected by the infection leading to a reduced barrier function and compromised mucosal regeneration mechanisms [15,16]. The net result is an increased risk of gastro-intestinal infections at all stages of HIV infection and a high prevalence of gastro-intestinal disorders with unknown etiology [17,18].

Furthermore, the breakdown of the intestinal immune system results in an aberrant microbiota with higher levels of pathogens such as *Pseudomonas aeruginosa* and *Candida albicans* and reduced or undetectable levels of *Bifidobacterium* and *Lactobacillus* species [19,20]. Collectively these changes can lead to leakage of microbial products, including lipopolysaccharide (LPS) and bacterial DNA, to the peripheral circulation of HIV patients [15,16]. Increased serum levels of microbial products have been associated with enhanced levels of inflammatory cytokines and immune cell activation [21,22,23]. This, in turn, can be a factor for enhanced HIV progression [21,24].

The composition of the intestinal microbiota and its interplay with the immune system could have an impact on leakage of microbial products from the intestinal tract and the associated inflammatory response. If modulating it by using beneficial microbes (probiotics) can influence these factors, this could have an impact on HIV progression.

The intestinal microbiota and probiotics

Probiotics, defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" [25], have been studied in several conditions related to a compromised intestinal epithelial barrier and an aberrant intestinal microbiota. Although most of these studies have been conducted

among persons without HIV, some of these findings can be applied to HIV subjects. For example, various trials have been conducted among critically ill patients to assess whether a probiotic formulation could impact a compromised intestinal epithelial barrier and reduce leakage of bacterial products to the peripheral circulation. A product, combining probiotic strains with a prebiotic [Synbiotic 2000 Forte], was shown to reduce the rates of bacteria detected in the bloodstream and mortality [26], findings which were confirmed in consecutive trials [27,28,29]. In addition, these studies also showed an enhanced intestinal immune defense with an increased secretion of fecal IgA. Among non-critically ill patients, similar observations were made [30,31]. However, the evidence is not conclusive, as three other randomized clinical trials using *L. acidophilus* La-5 and *Bifidobacterium lactis* Bb-12 [32,33] or *L. plantarum* 299v [34] reported no reduction in leakage of microbial products to the peripheral circulation. This suggests strain specific effects or the need of prebiotics for efficacy.

Specific probiotic strains have been shown to modify the intestinal microbiota at least temporarily in a range of populations [35,36,37]. Furthermore, they can interfere with the colonization and proliferation of pathogens in the intestinal tract in various ways; by enhancing the secretion of pathogen-specific IgA [38], inducing β-defensin production by the host [39], or secreting bactericidal compounds themselves [40,41]. The net effect is reduced adhesion and invasive properties of intestinal pathogens [42,43]. As a result, certain probiotic strains have been firmly established as having the ability to prevent or treat gastro-intestinal infections (reviewed in [44]).

Probiotics to modify the intestinal microbiota in HIV

Although the application of probiotics to prevent gastro-intestinal infections and potentially reduce a compromised state of the intestinal barrier bears promise, only a few studies have assessed the potential of probiotics for HIV. The first small trials concluded that supplementation with *L. rhamnosus* GG or *L. reuteri* to a level of 10^{10} cfu/day was safe and not associated with adverse events [20,45]. These were followed by randomized controlled trials assessing the potential for specific strains to reduce the progression of HIV. One

study of 77 children in Brazil showed an increase of 118 CD4^{+}cells/µl among those receiving *B. bifidum* and *Streptococcus thermophilus* for two months compared to a decrease of 42 CD4^{+}cells/µl among the placebo group (p = 0.05) [46]. Another study of 24 HIV patients in Nigeria showed after four weeks of *L. rhamnosus* GR-1 and *L. reuteri* RC-14 an increase of 6.7 CD4^{+}cells/µl compared to a decrease of 2.2 CD4^{+}cells/µl among the placebo group (p < 0.05) [47].

However, the impact of probiotics on the immune function has not been evaluated during follow-up periods of more than two months. Therefore, this thesis aims to answer the following question: **What is the long-term impact of probiotic use on the immune function of HIV patients?**

HIV and the vaginal microbiota

The quantitative composition of microorganisms living on the vaginal epithelium has a major impact on the health of a woman. The composition is determined by multiple factors, including the nutrients available in the vaginal tract, the ability of organisms to ascend from the rectum and the host immune response. High levels of estrogen in reproductive age women result in glycogen being deposited on the vaginal epithelium. This complex sugar molecule is mainly metabolized by *Lactobacillus* species with concurrent production of organic acids [48]. The resulting low pH creates an environment inhospitable to many pathogenic organisms [49].

For reasons poorly understood, a shift can occur from a vaginal microbiota dominated by lactobacilli and characterized by a low pH, to a complex mix of anaerobic bacteria and a higher pH [50]. This condition is termed bacterial vaginosis (BV) and is diagnosed by the clinical Amsel criteria or the microbiological Nugent score [51,52]. Among women with HIV, the condition is highly prevalent and its resulting higher pH disrupts physiological mechanisms that might inactivate or contain the virus [49,53,54,55]. The physiologically aberrant environment associated with BV results in an increased risk of viral transmission from the host to the vaginally born child or to a sexual partner [56]. In addition, BV appears to be more recalcitrant to antibiotic treatment in HIV patients and treatment is ineffective in reducing shedding of viral HIV particles from the vaginal tract [57,58].

An understanding of the vaginal microbiota among women living with HIV is important as different micro-organisms may represent distinct risk-profiles in the transmission of HIV or severity of BV. Therefore, a study was undertaken to answer the following question: **Which micro-organisms constitute the vaginal microbiota among women with HIV?**

Probiotics to modify the vaginal microbiota in HIV

Conventional antibiotic treatment for BV has a cure rate of 50 – 80% among women not infected with HIV. However, more than half of women have recurrent BV within six months after antibiotic treatment [59,60]. The rate of recurrence is higher among women living with HIV, with antibiotic cure rates being negligible [57]. In an attempt to provide an adjunct treatment for BV, some effort has been made to select lactobacilli from a healthy vaginal microbiota with the ability to modify the vaginal microbiota of women with BV. Of those, *L. rhamnosus* GR-1*, L. reuteri* RC-14 and to a lesser extent *L. crispatus* CTV-5 are the most thoroughly studied [61,62]. *L. rhamnosus* GR-1 and *L. reuteri* RC-14 have been shown to persist in the vagina for up to 21 days after vaginal application [63,64,65]. Using molecular techniques, Morrelli and co-workers confirmed that the probiotic strains could reach the vagina after oral ingestion [66]. Consecutive studies then showed that oral application of *L. rhamnosus* GR-1 and *L. reuteri* RC-14 could improve the vaginal microbiota of healthy women in terms of reduced Nugent score's [67,68,69,70]. Also, application of the probiotic strains in addition to metronidazole treatment was shown to improve the cure-rate of BV. For example, one randomized controlled trial conducted among 125 African women with BV showed that combining metronidazole with *L. rhamnosus* GR-1 and *L. reuteri* RC-14 had a cure rate of 88% versus 40% among those not additionally supplemented (p < 0.001) [71]. The improved cure rate of antibiotics in combination with the probiotic strains was confirmed in a Brazilian trial [72].

This concept has great potential for women living with HIV, who bear many of the health consequences of BV. However, no studies have been conducted evaluating this concept in such populations, nor has the long term impact of this strategy been assessed. A study was

designed to answer the following question: **Can probiotics prevent or improve the cure rate of BV among women with HIV?**

Overview of the thesis

Several studies have been undertaken to answer the research questions. In part I (chapter 2 – 3) the question "What is the long-term impact of probiotic use on the immune function of HIV patients?" is addressed. **Chapter 2** provides a systematic assessment of the effectiveness of nutritional interventions in reducing the progression of HIV. **Chapter 3** describes a conceptual framework for the use of probiotics to impact HIV progression. **Chapter 4** evaluates the effectiveness of probiotics in preserving the immune function among a cohort of Tanzanian users with HIV. A randomized controlled trial is described in **chapter 5** to evaluate the impact of 25 weeks probiotic use on the immune function of HIV patients.

The second research question on "Which microorganisms constitute the vaginal microbiota among women with HIV?" is addressed in part II (chapter 6 – 7). In **chapter 6** an in depth assessment of the vaginal microbiota among HIV infected women is performed and **chapter 7** describes the characteristics of the methodology developed to analyze the vaginal microbiota.

The third research question on "Can probiotics prevent or improve the cure rate of BV among women with HIV?" is addressed in part III (chapter 8 – 9). A critical assessment of the studies that have been conducted to assess the impact of probiotic supplementation on the vaginal microbiota is provided in **chapter 8**. This is followed, in **chapter 9,** by the description of a randomized controlled trial on the impact of probiotics on the cure rate of BV among women with HIV.

The thesis concludes with a general discussion, along with conclusions and recommendations in **chapter 10**.

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2.

Micronutrients, *N*-acetyl cysteine, probiotics and prebiotics, a review of effectiveness in reducing HIV progression

Ruben Hummelen
Jaimie Hemsworth
Gregor Reid

Abstract

Low serum concentrations of micronutrients, intestinal abnormalities, and an inflammatory state have been associated with HIV progression. These may be ameliorated by micronutrients, *N*-acetyl cysteine, probiotics, and prebiotics. This review aims to integrate the evidence from clinical trials of these interventions on the progression of HIV. Vitamin B, C, E, and folic acid have been shown to delay the progression of HIV. Supplementation with selenium, *N*-acetyl cysteine, probiotics, and prebiotics has considerable potential, but the evidence needs to be further substantiated. Vitamin A, iron, and zinc have been associated with adverse effects and caution is warranted for their use.

Introduction

Infections caused by the Human Immunodeficiency Virus (HIV) are one of the leading public health concerns around the world. Over 33 million people are living with HIV and nearly three million became infected during 2008 [1]. While antiretroviral therapies (ART) are almost universally available in developed countries, only since the G8 summit in 2005 have they been made more readily accessible to patients in developing countries [2]. As there is considerable risk for developing ART-induced toxic effects and metabolic dysfunction, the therapy is initiated only when the immune function is compromised (<350 CD4 cells/µL). For patients who have not developed severe immune deficiency, there is a significant void in methods to provide improved health. HIV causes certain dietary complications, including increased resting energy expenditure [3,4], enhanced oxidative stress [5], and a deleterious impact on the gastrointestinal system [6] that may require interventions specifically targeted to HIV. Therefore, the provision of a balanced and adequate diet is of prime importance for people living with HIV [7].

Vitamins and minerals (referred to as micronutrients) have received widespread and recent attention as potential interventions to delay HIV progression [8]. Although micronutrient interventions for people living with HIV have been thoroughly reviewed [9], to our knowledge, no review articles have integrated the evidence of the potential of various bioactive components, such as *N*-acetyl cysteine, probiotics, and prebiotics with micronutrients for this population. These interventions could potentially delay the progression of HIV and therefore postpone the moment that a patient becomes immune-compromised, and thus eligible for ART. Moreover, they could act in a complementary fashion with the ART, once the latter is initiated. Therefore, we present an assessment of randomized controlled trials (RCT) that evaluates the impact of micronutrients, *N*-acetyl cysteine, probiotics, and prebiotics on mortality, CD4 count, or HIV viral load among people living with HIV. Although this review emphasizes results from trials, it also briefly explores results from observational studies.

Micronutrients

The use of micronutrients among people living with HIV is widespread and comes from the concept that the virus causes a diverse range of nutrient abnormalities (reviewed in [10]). The depletion of micronutrients may occur through increased metabolic requirements, enhanced excretion, and intestinal mal-absorption (reviewed in [11]).

Early studies of micronutrients tested the effect of single vitamins or minerals on delaying the progression of HIV. While some successes were reported, the focus has now turned to multivitamin/mineral supplementation as a more comprehensive approach to delaying HIV progression. The levels of micronutrients consumed by people living with HIV are often much higher than recommended by the Dietary Reference Intakes, potentially leading to adverse events [12]. Therefore, health care professionals should be aware of the beneficial and potential adverse effects of micronutrients in order to make informed, safe, and practical decisions for people living with HIV.

Vitamin A

Vitamin A is essential for normal immune function, the maintenance of mucosal surfaces, and haematopoiesis [13]. Also, vitamin A supplementation is recognized for its ability to decrease morbidity and mortality rates for some infectious diseases [14]. Vitamin A deficiency has consistently been associated with an increase in mother-to-child transmission of HIV (MTCT) [15]. However, there is conflicting evidence coming from observational studies on HIV progression and vitamin A where in one study, a cohort (n = 179) of HIV infected drug users, reported increases in mortality and lower CD4 counts associated with lower serum vitamin A levels [16]. In contrast, another observational study of HIV-infected men (n = 311) did not find a relationship between lower serum retinol (vitamin A) status and HIV disease progression or CD4 count [17].

There seems to be a potential role for vitamin A in children living with HIV. During an RCT in Tanzania, 687 children without signs of vitamin A deficiency who were admitted to the hospital with pneumonia were randomized. Some were given a large dose of vitamin A (400,000 IU) at baseline and again at four and eight

months post-discharge. Among those, 58 were diagnosed with HIV and naïve to ART. Overall, a 49% reduction in mortality (p = 0.05) and a 92% reduction in diarrhea-related death (p = 0.01) was observed. These effects were more profound among the sub-group of children with HIV with a reduction in mortality of 53% (p = 0.04) and a reduction of 68% in AIDS related deaths (p = 0.05) [18]. Another RCT among 181 ART naïve HIV-infected children (15–36 months) in Uganda found similar results. Vitamin A supplementation (60 mg retinol) at three month intervals for a median of 17.8 months reduced mortality rates by 46% (p = 0.04), as well as persistent cough and chronic diarrhea. No effects were noted on CD4 count or HIV viral load [19]. Thus, there are potential benefits conferred by vitamin A supplementation for reducing the morbidity and mortality of children living with HIV in developing countries.

Women living with HIV are at increased risk for experiencing adverse pregnancy outcomes, such as preterm birth and the delivery of low-birth-weight infants. Moreover, women can transmit the virus to their newborns through intrauterine, delivery, or breastfeeding routes. Research on the role of vitamin A in birth outcomes is critical as low levels of the vitamin have been associated with adverse pregnancy outcomes and vertical transmission of HIV [15]. To evaluate the effect of vitamin A supplementation on MTCT, RCTs were conducted among ART naïve pregnant women in South Africa, Malawi, and Tanzania. In South Africa, an RCT among 728 pregnant women showed that daily Vitamin A supplementation (5000 IU and 30 mg β-carotene) and a large dose at delivery (200,000 IU) did not reduce MTCT or mortality among women and infants, although among those infants born pre-term, vitamin A was associated with a reduction in MTCT [20]. In the Malawian RCT, pregnant women with HIV (n = 697) were randomized to receive daily vitamin A (3 mg retinol) or placebo. Infants whose mothers were in the vitamin A group had a higher birth weight (p = 0.05) and fewer incidences of anemia at six weeks, from 41% to 23% (p = < 0.001). This trial, however, did not show vitamin A supplementation to have an effect on MTCT [21]. Another trial among HIV-infected pregnant women in Tanzania (n = 1078) did not show any effects of vitamin A supplementation (30 mg β-carotene plus 5000 IU vitamin A) on improved pregnancy outcomes. In contrast, vitamin A supplementation appeared to increase the MTCT of HIV by an

additional 38% compared to the placebo (p = 0.009) [22]. Furthermore, vitamin A did not have any effect on HIV-associated mortality or CD4 count during a median 71 months of follow-up among the women [23,24].

In conclusion, two studies have indicated a potential benefit of vitamin A supplementation for children with HIV in developing countries who are naïve to ART. However, these benefits need to be confirmed in larger RCTs with HIV progression, mortality, and morbidity as primary outcomes. With respect to maternal supplementation, little evidence exists to support the use of vitamin A supplements in HIV-positive pregnant women. Rather, the possibility that maternal vitamin A supplementation could increase the risk of vertical transmission warrants caution for its use among this population. The notion that vitamin A supplementation reduced the benefits of multivitamin supplementation indicates that no profound benefits may exist for supplementing vitamin A among adults.

Table 1. Randomized controlled trials on vitamin A and HIV progression.

Reference	Population, in- and exclusion criteria.	Intervention and follow-up	Primary outcomes	Major findings	Conclusions
Fawzi 1998 [22-24]	Tanzania, 1078 ART naïve pregnant women.	Multifactorial design with Vit. A (5000 IU) and β-carotene (30 mg) daily during pregnancy and lactation.	Mortality, CD4 count and viral load.	No differences in mortality, CD4 count or viral load among women or children. Vit. A increased MCTC.	Vit. A does not reduce mortality among women but increased MTCT.
Fawzi 1999 [18]	Tanzania, 58 ART naïve children admitted with pneumonia.	Dose vit. A (400,000 IU) at baseline, 4 and 8 months.	Mortality	Reduced overall and AIDS related mortality. Reduction on diarrhea related death.	Vit. A reduces mortality among children admitted with acute infections.
Coutsoudis 1999 [20]	South-Africa, 728 ART naïve pregnant women.	Vit. A (5000 IU), β-carotene (30 mg) third trimester and vit. A (200,000 IU) at delivery.	MCTC, fetal and infant mortality.	No reduction in MCTC, fetal or infant mortality. Reduction in preterm delivery.	Vit. A administered to the mother does not affect fetal or infant mortality or MCTC.
Kumwenda 2002 [21]	Malawi, 697 ART naïve pregnant women.	Vit. A (3 mg) daily from 18-28 weeks of gestation until delivery.	MTCT, birth weight.	Increase in birth weight and a reduction of the number of anemic children. No effect on MTCT.	Vit. A administered to the mother increases birth weight and prevents anemia. No effect on MTCT
Semba 2005 [19]	Uganda, 181 ART naïve children.	Vit. A (60 mg) every three months for 18 months.	Mortality, CD4 count, HIV viral load.	Reduction in mortality. No effect on CD4 count or viral load.	Vit. A reduces mortality among children.

MTCT = mother to child transmission

Vitamin B, C, E and Folic Acid

B-vitamins are essential components of our immune system. Riboflavin deficiency hampers the generation of a humoral antibody response, vitamin B-6 deficiency reduces the maturation of lymphocytes, and vitamin B-12 deficiency impairs the function of neutrophils [25]. It is therefore not surprising that among people living with HIV, higher serum levels and intake of B vitamins have been associated with improved survival. In a large cohort study of people living with HIV in the US (n = 241), the highest quartile of intake for each B-group vitamin was independently associated with improved survival over the course of an eight-year follow-up: B1, (relative hazard (RH) = 0.60), B2 (RH = 0.59), B6, (RH = 0.45), and niacin (RH = 0.57) [26]. Higher serum levels of vitamin B12 were also associated with delayed HIV progression [27]. These results were confirmed in a matched case-control study within a cohort of South African HIV patients. The median survival time of patients using vitamin B supplements (264 weeks) was significantly longer than of those not taking B vitamins (144 weeks) (p = 0.0014) [28].

In several observational studies, vitamins C and E were found to be lower among people living with HIV [5,10]. These findings were confirmed by the group of Baum *et al.*, who compared the nutrient levels of 108 HIV-infected gay men, almost all with high CD4 counts, to a similar group of HIV-negative controls. In this seminal study, they found that deficiencies of vitamins B6, B12, A, E, and zinc were highly prevalent in the HIV-infected cohort. Thus, the conclusion was that HIV-infected individuals most likely require micronutrient intake in multiples of the DRI in order to maintain normal serum levels [29]. Lower levels of vitamin C and E were also related to significantly higher levels of oxidative stress [5], which presumably leads to increased viral replication [30]. In longitudinal studies, the dietary intake of vitamin E [31] and higher vitamin E serum levels [17] were associated with higher CD4 counts and a reduced risk of HIV progression [32].

These findings were the impetus for studies assessing whether exogenous supplementation of vitamins could reduce oxidative stress and therefore potentially reduce HIV viral load. A pilot RCT (n = 49) among HIV patients receiving ART showed that the group receiving vitamin E (800 IU) and C (1000 mg) for three months had

significantly less oxidative stress (reduction of plasma peroxides and malondialdehyde) and a trend was seen (p = 0.1) towards a reduction of the viral load (-0.45 log^{10} copies/ml) compared to the placebo group (+0.50 log^{10} copies/mL) [33]. A study in Uganda with a short follow-up period of six weeks did not find a benefit of supplementation. The RCT (n = 141) among ART naïve HIV patients admitted with diarrhea showed that supplementation with a combination of vitamin A (10,500 IU), E (300 mg), Selenium (150 µg), and Zinc (200 mg) for two weeks did not reduce the length of time with diarrhea. Moreover, no effects were seen on mortality or CD4 count after the six weeks of follow-up. Low serum levels of vitamin A and E were confirmed to be predictors of mortality in this study, but exogenous supplementation did not increase these levels [32]. The expectation to see an effect of supplementation within such a short time-frame might have been unrealistic, as an impact on HIV progression is typically assessed over longer periods of time.

The first long term study with HIV progression and mortality as primary outcome was conducted in Tanzania. A specific multivitamin combination of vitamins B, C, E, and folic acid (20 mg B_1, 20 mg B_2, 25 mg B_6, 100 mg B3, 50 µg B12, 500mg C, 0.8mg folic acid) was repeatedly shown to increase CD4 and CD8 counts, lower viral loads, and reduce mortality among ART naïve HIV infected women [34]. The large RCT (n = 1075) among pregnant women showed an increase in CD4 count from baseline (between 12 and 27 weeks gestation) to six weeks postpartum of 176 cells/µL *vs.* 112 cells/µL (p = < 0.001) in the placebo group. At 30 weeks postpartum, there was still a significant difference with an increase of 99 CD4 cells/µL in the multivitamin group *vs.* an increase of 59 CD4 cells/µL in the placebo group (p = 0.05), in addition to an increase in CD8 and CD3 cells. Furthermore, there were significant reductions in fetal death (Relative Risk (RR) = 0.61), low birth weight (RR = 0.56), and severe preterm birth (RR = 0.61) with multivitamin supplementation [34]. Long term (71 months) follow-up of this cohort showed that only 67 of 271 (25%) women receiving multivitamins died or developed stage 4 disease compared to 83 of 276 (30%) receiving placebo (RR = 0.71, P = 0.04). Moreover, women receiving multivitamin had on average a CD4 count of 48 cells/µL higher than the placebo group (p = 0.01), and a significantly lower viral load (p = 0.02) [24]. Multivitamins also reduced the risk of wasting (RR = 0.66, p = 0.02) and the incidence

of weight loss episodes amongst HIV infected women [35]. Interestingly, women with impaired immunological (low lymphocyte count) and nutritional status (low haemoglobin levels) at baseline had reduced transmission of the virus from mother to child (RR = 0.37, p = 0.02) and decreased child mortality after 24 months (RR = 0.82, p = 0.08) when given multivitamins [22]. These results suggest that this particular multivitamin supplement (20 mg B_1, 20 mg B_2, 25 mg B_6, 100 mg B3, 50 µg B12, 500mg C, 0.8 mg folic acid) can be safely administered to pregnant women and has considerable immunological benefits.

To assess the impact of the same multivitamin formula and selenium (200 µg daily for six weeks) on the infectivity (vaginal HIV shedding) of ART naïve women with HIV, an RCT (n = 400) was launched in Kenya. Although a relatively short intervention period of six weeks was used and the primary outcome was infectivity, the study confirmed the beneficial effects of the multivitamin supplement on the CD4 count (a mean of 23 cells/µL higher than placebo) and CD8 count (a mean of 74 cells/µL higher than placebo). However, the trial showed a potential increase of HIV infectivity amongst women receiving supplements as the odds of detecting HIV infected vaginal epithelial cells increased 2.5 fold (p = 0.001), as well as the quantity of vaginal HIV RNA (p = 0.004) [36].

In Bangkok, an RCT (n = 481) was conducted with an intervention using similar concentrations of vitamin B, C, E, and folic acid (24 mg B_1, 15 mg B_2, 40 mg B_6, 30 µg B12, 400mg C, 3 mg A, 0.1 mg folic acid), but with the addition of vitamin A, zinc, and various other micronutrients (table 2.). Overall, a trend was seen towards a reduced mortality in the multivitamin group (RR = 0.53, p = 0.1) and a significant reduction among subgroups of those with a CD4 count <200 (RR = 0.37, p = 0.05) and <100 (RR = 0.26, p = 0.03). However, no effect was seen on viral load and CD4 count [37].

A multivitamin combining vitamins A, B, C, D, E, zinc, copper, selenium, iodine, and folic acid with concentrations approximately 10 times lower than the Tanzanian combination was tested within a cluster randomized trial in Zambia. Households (n = 500 individuals) were randomized to receive the multivitamin combination for an average of 3.3 years [45]. No differences were seen in terms of CD4 count or diarrhea incidence amongst those living with HIV who are ART naïve (n = 135). However, a significant difference in mortality

was reported, as 20% (12/61) died during follow-up with placebo as compared to 5% (4/74) amongst the multivitamin supplemented group (p = 0.03 by log-rank test).

In the United States, an RCT (n = 40) among HIV patients treated with ART who displayed related neurotoxicity, tested the impact of an intervention combining vitamins B, C, E, and folic acid in a daily dose approximately 6 times higher than the Tanzanian multivitamin combination and with the addition of vitamins A, D, zinc, *N*-acetyl cysteine (1.2g), and various other minerals (Table 2). The intervention group experienced an increase in CD4 count from a baseline of 65 cells/μL after 12 weeks of supplementation, as compared to -6 cells/μL in the placebo group. However, no differences were seen in viral load or neuropathy scores [38].

In summary, several well designed RCTs have shown a considerable benefit of supplementation of vitamins B, C, E, and folic acid (20 mg B_1, 20 mg B_2, 25 mg B_6, 100 mg B3, 50 μg B12, 500 mg C, 0.8mg folic acid) to ART naïve HIV patients. Although in subpopulations a decrease in MTCT has been reported, further studies are needed to assess if the potential increase in infectivity is present and may enhance HIV transmission. Furthermore, as one trial has shown potential for this approach among an ART-treated population, these results need to be confirmed by larger RCTs with long term follow-up.

Vitamin D

The interest in vitamin D and the immune system has been catalyzed by the discovery of vitamin D receptors in peripheral blood mononuclear cells involved in immune system regulation [39]. Vitamin D is involved in immune-related diseases, such as Ulcerative Colitis and Crohn's Disease, and has recently gained attention for its mediating role in innate immunity and the body's response to intracellular micro-organisms, such as *Mycobacterium tuberculosis* [40]. The role of vitamin D in HIV progression is less well understood. In Tanzania, an observational study (n = 1078) was conducted within a trial to assess the effects of the vitamin D status of pregnant women on adverse pregnancy outcomes, MTCT, and child mortality [41]. Children born to mothers with low vitamin D levels had a 64% greater risk of dying during follow-up and an overall 46% higher risk of contracting HIV. In this observational study of vitamin D status'

effects on child morbidity and mortality, a non-linear relationship was found between vitamin D levels and MTCT (p = 0.01), where the risk of MTCT decreased as the levels of vitamin D increased [41]. The only RCT with findings on vitamin D and HIV progression to date was conducted in Guinea-Bissau. The trial (n = 367) among Tuberculosis (TB) patients assessed the impact of supplements of vitamin D every four months (100,000 IU of oral cholecalciferol) over a 12-month period [42] on mortality and the clinical severity of TB (Table 2). Vitamin D did not appear to improve the severity of tuberculosis, nor was there an impact on mortality in both the complete group and the sub-group infected with HIV (n = 135) [42]. Therefore, there is insufficient evidence to recommend vitamin D use among people living with HIV. Larger RCTs are warranted, as there might be considerable potential.

Table 2. Randomized controlled trials on vitamin B, C, D, E and folic acid, and HIV progression.

Reference	Population, in- and exclusion criteria.	Intervention and follow-up	Primary outcomes	Major findings	Conclusions
Allard 1998 [35]	Canada, 49 individuals receiving ART.	Daily vit. E (800 IU) and C (1000mg) for 3 months.	HIV viral load, oxidative stress	Trend towards reduction in viral load. Significant reduction of oxidative stress.	Vit. E and C may reduce viral load and reduces oxidative stress.
Kelly 1999 [34]	Zambia, 141 ART naïve patients admitted with diarrhea.	Daily vit. A (10.500 IU), C (300 mg), E (300 mg) Selenium (300 µg) and Zinc (200mg) for two weeks.	Diarrhea, mortality, serum A and E, CD4 count.	No difference in length of diarrheal episodes, mortality or CD4 count after 6 weeks.	No additional value of multivitamin to treatment of diarrhea among HIV patients.
Fawzi 1998. [22,24,,43]	Tanzania, 1078 ART naïve pregnant women.	Multifactorial design with Vit. B, C, E and folic acid[1].	MTCT, Mortality, CD4 count and viral load.	No effect on MTCT, Reduced mortality, delayed HIV progression, increased CD4, CD8 count and decreased viral load for the multivitamin.	Multivitamin does not reduce MTCT but reduces HIV progression and mortality among children and pregnant women.
Jiamton 2003 [44]	Bangkok, 481 ART naïve individuals with CD4 count 50 - 500 cells/µL.	Daily vit. A, B, C, D, E, K, zinc, selenium and various minerals[2] for 48 weeks.	Mortality, CD4 count	Reduction of mortality with no differences in viral load and CD4 count.	Micronutrients reduce mortality among people living with HIV.
McClelland 2004 [36]	Kenya, 400 ART naïve women.	Daily vit. B, C, E, folic acid[1] and selenium (200 mg) for 6 weeks.	HIV infectivity	Increase in HIV infectivity. Increase in CD4 and CD8 count and no difference in viral load.	Multivitamin may increase HIV infectivity.

Table 2. Cont. Randomized controlled trials on vitamin B, C, D, E and folic acid, and HIV progression.

Reference	Population, in- and exclusion criteria.	Intervention and follow-up	Primary outcomes	Major findings	Conclusions
Kaiser 2006 [38]	United states, 40 individuals receiving ART and signs of ART related neuropathy.	Twice daily vit. A, B, C, D, E, zinc, selenium, NAC and various minerals[3] for 12 weeks.	CD4 count, viral load, neuropathy, safety parameters.	Intervention increased CD4 count with no effect on viral load or neuropathy.	Micronutrients and NAC increases CD4 count and was found to be safe.
Kelly 2008 [45]	Uganda, cluster randomized trial among 500 individuals of which 135 with HIV and ART naïve.	Daily vit.A, B, C, D, E, folic acid, selenium, iron, zinc, copper, iodine[4].	Diarrhea incidence, CD4 count, mortality.	Intervention did not reduce the incidence of diarrhea. No effect on CD4 count but decrease in mortality.	Low dose multivitamin may reduce mortality.
Wejse 2009 [42]	Guineau-Bassau, West Africa, 367 TB-infected, 131 co-infected with HIV and ART naïve.	Vitamin D 100,000 IU every 3 months for 12 months	Clinical severity score of TB and overall mortality at 12 months	No difference in clinical severity or mortality between vitamin D and control group.	Vit. D not effective at improving clinical outcomes of TB.

MTCT = Mother to child transmission, TB = Tuberculosis
1. Micronutrient included vit. B1 20 mg , B2 20 mg, B6 25 mg, B3 100 mg, B12 50 µg, C 500 mg and folic acid 0.8 mg.
2. Micronutrient supplement included vitamin A 3000 µg, beta-carotene 6 mg, vitamin D3 20 µ g, E 80 mg, K 180 µg, C 400 mg, B1 24 mg, vitamin B2 15 mg, vitamin B6 40 mg, vitamin B12 30 µg, folacin 100 µ g, panthothenic acid 40 mg, iron 10 mg, magnesium 200 mg, manganese 8 mg, zinc 30 mg, iodine 300 µg, copper 3 mg, selenium 400 µg, chromium 150 µg and cystine 66 mg.
3. Micronutrient supplement included N-acetyl cysteine 1200 mg , acetyl L-carnitine 1000 mg, α-lipoic acid 400 mg, β-carotene 20 000 IU, vitamin A 8000 IU, vitamin C 1800 mg, thiamine 60 mg , riboflavin 60 mg, pantothenic acid 60 mg, niacinamide 60 mg, inositol 60 mg, vitamin B6 260 mg, vitamin B12 2.5 mg, vitamin D 400 IU, vitamin E 800 IU, folic acid 800 µg, Ca 800 mg, Mg 400 mg, Se 200 µg, Iodine 150 µg, Zn 30 mg, Cu 2 mg, B 2 mg, K 99 mg, Fe 18 mg, Mn 10 mg, biotin 50 µg, Cr 100 µg, Mo 300 µg, choline 60 mg, bioflavonoid complex 300 mg, L-glutamine 100 mg, and betaine HCL 150 mg.
4. Micronutrient supplement included Vitamin A as β-carotene 4,8 mg, vitamin B1 1,4 mg , vitamin B2 1,4 mg , vitamin B6 1,6 mg, vitamin B12 2,6 µg, vitamin C 18 mg, vitamin D35 mg, vitamin E 10 mg, iron 30 mg, zinc 15 mg, copper 12 mg, selenium 65 µg iodine 150 µg and folic acid 400 µg.

Table 3. Randomized controlled trials on iron and HIV progression

Reference	Population, in- and exclusion criteria	Intervention and follow-up	Primary outcomes	Major findings	Conclusions
Olsen 2004 [55]	Kenya, 45 ART naïve HIV patients.	60 mg iron twice weekly for 4 months.	HIV viral load.	No increase in HIV viral load.	Low dose iron can be safely administered.
Semba 2007 [56]	United States, 485 HCV infected of which 138 co-infected with HIV of whom 50 receiving ART.	18 mg iron daily for 12 months.	HCV and HIV viral load, anemia.	No increase in HCV or HIV viral load. Reduced occurrence of anemia.	Iron is safe administered and is effective in treating anemia.

Iron

Iron deficiency is the most common nutritional deficiency in the world, and is a condition mainly affecting women. Among people living with HIV, anemia (defined as low haemoglobin) is highly prevalent and is associated with increased mortality [46,47] and enhanced HIV progression [47]. Although anemia can generally be treated by iron supplementation, various clinical observations have raised the concern that iron supplementation may adversely affect HIV progression and increase mortality [48]. Firstly, in a retrospective study among thalassemia major patients (n = 64), the rate of progression of HIV was significantly faster amongst those receiving lower doses of the iron chelating agent, desferrioxamine, and who have higher serum ferritin concentrations [49,50]. Second, the concurrent administration of low doses of iron with dapsone for the prophylaxis of *Pneumocystis carinii* pneumonia in HIV-positive patients was associated with an increased risk of mortality in a randomized study among 196 HIV patients [51]. In contrast, the administration of Dapsone for the prophylaxis of *Pneumocystis carinii* without a low dose of iron did not lead to excess mortality in another trial [52]. Thirdly, a study on polymorphisms of haemoglobin-binding haptoglobin indicated that the haptoglobin 2-2 polymorphism is associated with higher iron stores and shortened survival among HIV patients [53]. Finally, a retrospective study of iron load in bone marrow macrophages among HIV patients suggested that high iron stores were associated with a shorter survival in HIV-positive patients [54]. As none of these observations could provide conclusive evidence for an adverse effect of iron on HIV progression, two prospective studies were conducted. Within a larger RCT of non-anemic participants (n = 181), a subpopulation of ART naïve HIV patients (n = 45) was studied to assess the effect of daily 60 mg iron supplementation (twice weekly for 4 months) on HIV viral load (table 3). In this small study, no increase in HIV viral load could be detected [55]. In the United States, an RCT was conducted among female drug users infected with Hepatitis C Virus (n = 485) and among them, a subgroup co-infected with HIV (n = 138), of whom 50 were treated with ART. An assessment was made on whether iron supplementation

(18 mg daily) would increase HIV viral load and reduce anemia over a 12-month period. At follow-ups, the proportion with anemia in the iron group was only 21% *vs.* 31% in the placebo group (P = 0.03) at 6 months and, respectively, 26% *versus* 30% (P = 0.5) at 12 months. Furthermore, no increase in HIV viral load could be detected [56]. This study could exclude a large increase in HIV viral load due to iron supplementation, but was not powered to detect small increases over a larger period of time. Furthermore, the conclusions from this study cannot readily be extrapolated to other HIV populations. It remains to be determined what the influence is of iron supplementation among patients who are receiving ART. Given the potential adverse role of iron in HIV progression, caution is warranted for iron supplementation among HIV infected populations. Routine supplementation without a specific indication is contra-indicated.

Zinc

Various studies have investigated the role of zinc in HIV disease progression. The immunologic consequences of zinc deficiency are manifold, given the involvement of the mineral in basic cellular processes, which are essential in immunological mechanisms [57]. Consequently, zinc deficiency has been associated with a higher susceptibility to infectious diseases, such as pneumonia, malaria, and diarrhea [58]. There is evidence that zinc deficiency amongst people living with HIV may account for an improper maturation of CD4 count cells mediated through low levels of the zinc-dependent hormone, thymuline. This may lead to a less effective immune response and a higher susceptibility to opportunistic infections [59]. However, evidence for the relationship between zinc status and HIV is inconclusive. In an observational study of asymptomatic HIV-infected men in the United States, high zinc intake has been shown to be significantly associated with faster HIV progression and an increased mortality amongst men [17,60]. In contrast, another observational US cohort study of HIV positive men suggested that higher serum zinc levels were inversely associated with mortality [61].

A first interventional study was conducted in Italy among 57 Zidovudine treated HIV patients that had developed or were at risk of developing AIDS (Table 4.). Those supplemented daily with zinc (45 mg) for 30 days experienced, at follow-up, a lower frequency of opportunistic infections after 24 months and an increase in CD4 count

after four months (compared to the controls) [59]. To assess the safety of daily zinc supplementation (10 mg) to ART naïve children with HIV, an RCT (n = 96) was conducted in South Africa. This trial did not show any apparent benefit of zinc supplementation. No differences were found in CD4 count, viral load, or haemoglobin concentration, while supplementation was found to be safe [62]. This trial was followed by the largest study to date on zinc supplementation for people living with HIV and was aimed at assessing the potential benefits of zinc supplementation on pregnancy outcomes. The study population of the RCT (n = 400) consisted of pregnant ART naïve women with HIV in Tanzania [63]. All women received the multivitamin supplement (discussed in the section on vitamins B, C, E, and folic acid) and were randomized to receive, in addition, either a placebo or a 25 mg daily dosage of zinc between recruitment and six weeks after delivery. The results showed no differences in pregnancy outcomes, HIV transmission, CD4 or CD8 count, and viral load. However, zinc supplementation was inversely associated with haemoglobin levels, and was related to a threefold increase in the probability of wasting (RR = 2.7, p = 0.03) [64].

In conclusion, although an initial trial has shown potential benefits of zinc supplementation among ART treated patients, these results have not been replicated in a trial among ART naïve pregnant women. Further research is warranted to assess whether there is a potential role for zinc among HIV patients treated with ART.

Selenium

The trace selement selenium has been suggested to be a key nutrient for people living with HIV. Lower serum concentrations of selenium in both adults and children infected with HIV have been linked to increased mortality [65] and an increased viral load [66,67,68]. The role of selenium in immunity and antioxidant defence may be the underlying mechanism of an enhanced HIV progression with lower levels of the micronutrient [69]. These observational findings instigated clinical trials to assess whether selenium supplementation could have an impact on HIV viral load, CD4 counts, or HIV progression (Table 5). An earlier pilot RCT among ART treated HIV patients (n = 52) showed that selenium supplementation (100 µg) could enhance the antioxidant status of HIV patients [70]. A larger RCT (n = 186, of which 124 were receiving ART) showed that

daily supplementation with selenium (200 µg) did not affect CD4 count levels or viral load after two years of follow-up. However, a smaller proportion (25%) among the selenium group experienced a substantial decline in CD4 count (>50 cells/µL) when compared to the placebo group (46%, p = 0.01). In addition, the relative risk of being admitted at a hospital was 2.4-times lower among the selenium supplemented group in comparison to the placebo group (p = 0.008) [71]. In a more recent RCT among 262 HIV-infected patients (192 receiving ART), nine-month supplementation with selenium (200 µg daily) resulted in an increase in CD4 count (p = 0.04) and a decrease in viral load (p = 0.02) [72]. To assess the impact of selenium supplementation on mother and child mortality, a large RCT (n = 913) was conducted amongst pregnant women in Tanzania. Daily selenium (200 µg) supplementation until six months post partum did not affect CD4 count, viral load, or overall mother and child mortality. However, at six weeks after delivery, a reduction of 57% in child mortality was found (p = 0.05) amongst the selenium supplemented group [73]. The authors concluded that the lack of a beneficial effect of selenium supplementation on HIV progression may have been due to the low prevalence of ART use and selenium deficiency amongst their study population or because of a reduced effect of selenium amongst patients already supplemented with micronutrients [73].

At present, there is no support for providing selenium supplements to HIV patients who are not selenium deficient and who are already receiving a high-dose multivitamin. Although two RCTs have shown that selenium supplementation can potentially reduce the viral load and enhance the immunological status of patients treated with ARTs, well designed trials with a long term follow-up and an appropriate sample size among similar populations are needed to substantiate the evidence for the use of selenium.

Table 4. Randomized controlled trials on zinc and HIV progression.

Reference	Population, in- and exclusion criteria	Intervention and follow-up	Primary outcomes	Major findings	Conclusions
Mocchegiani 1995 [59]	Italy, 57 AZT treated patients. At risk or developed AIDS.	Zinc (45 mg) for 30 days.	Incidence opportunistic infections, body weight.	Reduction opportunistic infections, stabilization or increase body weight, CD4 count, thymulin.	Zinc increases CD4 count.
Bobat 2005 [62]	South Africa, 96 ART naïve children.	Daily zinc (10 mg) for 6 months.	CD4 count, viral load and diarrhea incidence.	No difference in CD4 count or viral load but a reduction in watery diarrhea.	Zinc safe among children with HIV.
Fawzi 2005 [63,64]	Tanzania, 400 ART naïve pregnant women.	Daily zinc (25 mg) additional to multivitamin[1] until 6 weeks after delivery.	CD4 count, viral load and MTCT.	No effects on CD4 count, viral load and MCTC but adverse effects on haemoglobin level and an increase risk of wasting.	Zinc has no impact on HIV progression and may cause adverse effects.

1. Micronutrient included vitamin B1 20 mg , vitamin B2 20 mg, vitamin B6 25 mg, vitamin B3 100 mg, vitamin B12 50 µg, vitamin C 500 mg and folic acid 0.8 mg.

Table 5. Randomized controlled trials on selenium and HIV progression

Reference	Population, in- and exclusion criteria	Intervention and follow-up	Primary outcomes	Major findings	Conclusions
Constans 1996 [70]	France, 52 individuals receiving ART.	Selenium (100 µg) vs. β-carotene (30 mg) vs. placebo	CD4 count and serum anti-oxidant level.	Both selenium and β-carotene did not have an impact on the CD4 count but enhanced serum antioxidant levels.	Selenium and β-carotene improve anti-oxidant status.
Burbano 2002 [71]	United states, 186 injection drug users of whom 124 receiving ART.	Daily Selenium (200 µg) for 2 years.	CD4 count and hospital admissions	No differences in CD4 count and viral load. Reduction in number with drop in CD4 count of >50 cells/µL and risk of admission was lower.	Selenium may reduce decline in CD4 count and risk of hospital admission.
Hurwitz 2007 [72]	United states, 262 individuals of whom 192 receiving ART	Daily selenium (200 µg) for 9 months.	CD4 count, viral load.	Increase in CD4 count, and decrease in viral load.	Selenium reduces HIV progression.
Kupka 2008 [73]	Tanzania, 913 ART naïve pregnant women.	Daily selenium (200 µg) until 6 months after delivery.	Mortality, CD4 count, viral load.	No differences in mortality, CD4 count or viral load.	Selenium does not affect HIV progression.

N-acetyl Cysteine

Glutathione (GSH) is a principal regulator of oxidative stress, which occurs in the intracellular space and on cellular membranes. HIV-infected people tend to have abnormal GSH levels in various components of the immune-system, including CD4 cells. *In vitro* studies have shown that lower intracellular GSH levels decrease cell survival and increase NF-κB activation and consequently, HIV replication. Observational studies have linked GSH deficiency directly to patient survival [74]. Various interventions have been tested to increase GSH levels to normal among HIV patients. N-acetyl cysteine (NAC), a pro-drug used to replenish GSH after paracetamol intoxication, was shown to restore normal GSH levels among HIV patients [74-76].

One of the first RCTs was conducted by Herzenberg *et al*., who randomized HIV patients (n = 246, mixed ART status) with low GSH levels to NAC supplementation (3.2–8 g daily) or to a placebo for eight weeks, followed by an open label treatment of two years (Table 6). The results suggested that those using NAC were more likely to survive during follow-up (RR = 1.8, p = 0.02), but no CD4 count or viral load data were collected [74]. A smaller RCT (n = 45) among ART naïve HIV patients taking NAC supplementation (400 mg) for four months showed significantly reduced TNF-α levels, but no effect upon CD4 count during the study. However, it was noted that the NAC supplemented group had a less profound decline in CD4 count when compared to the 36 months before randomization (p = <0.05) [75]. Another RCT (n = 29) assessing the effect of daily NAC supplementation (0.6–3.6 g) during 7 months did not show differences in CD4 and CD8 counts, although an increase in natural killer (NK) cell activity (p = <0.05) was noted [76]. A controlled cross-over study (n = 24) showed a trend towards an increased CD4 count and CD4/CD8 ratio during daily supplementation of NAC (600 mg) when combined with selenium (500 µg) [77]. Serum GSH concentrations were not affected by supplementation.

Another approach to replenishing GSH levels is through the supplementation of whey protein, which is a source of the amino acid, cysteine. This intervention was shown to be effective in enhancing GSH levels in a small RCT amongst patients receiving ART (n = 30) [78]. The effectiveness of whey protein on CD4 count levels was

assessed in a larger RCT, which was also amongst ART treated patients (n = 59). In this study, the whey supplemented group (40 g daily) had an increase of CD4 count of 31 cells/μL, compared to 15 cells/μL amongst the placebo group during the 12-week follow-up (p = 0.03) [79].

Replenishing GSH stores with either NAC or whey protein appears to have a beneficial impact upon the immune function of people living with HIV. These results are promising and warrant the conduct of a large RCT to assess either of the effects of these supplements upon the progression of HIV.

Table 6. Randomized, placebo controlled trials on cysteine and HIV progression.

Reference	Population, in- and exclusion criteria	Intervention and follow-up	Primary outcomes	Major findings	Conclusions
Akerlund 1991 [75]	Sweden, 45 ART naïve individuals with CD4 > 200 cells/uL	Daily NAC (400 mg) for 4 months.	CD4 count and clinical signs and symptoms.	No effect on CD4 count. But increased level of GSH, reduced TNFα levels and reduced decline of CD4 count than before baseline.	NAC may reduce decline in CD4 count and restores GSH levels.
Herzenberg 1997 [74]	United states, 246 individuals with low glutathione levels (ART unknown).	Daily NAC (3.2– 8.0 g) for 8 weeks, followed by open label treatment for 2 years.	Mortality, GSH levels.	Supplementation restored GSH levels and reduced mortality.	NAC restores GSH levels and may reduce mortality.
Look 1998 [77]	Germany, Cross-over RCT, 24 ART naïve individuals.	Daily NAC (0.6 g) and selenium (500 µg) for 24 weeks.	CD4 count, GSH levels	Trend towards increase in CD4 count and CD4/CD8 ratio at particular time points, no increase in GSH level.	NAC and selenium may improve immune status.
Breitkreutz 2000 [76]	Germany, 29 ART naïve individuals.	Daily NAC (0.6– 3.6 g) for 7 months.	CD4 count, GSH levels	Supplementation restored GSH levels, no difference in CD4 or CD8 cells but increase in NK cell activity.	NAC restores GSH levels and may enhance antiviral response.
Micke 2002 [78]	Germany, 30 individuals receiving ART.	Daily whey protein (45 g) for 6 months.	GSH levels, CD4 count and body weight.	Increase in GSH levels, no differences in CD4 count or body weight.	Whey protein increases GSH levels.
Sattler 2008 [79]	United states, 59 individuals receiving ART.	Daily whey protein (40 g) for 12 weeks.	Weight, lean body mass.	No difference in weight or lean body mass, but significant increase in CD4 count.	Whey protein increases CD4 count.

NAC = N-acetyl cysteine, GSH = gluthathione

Probiotics

Recent findings suggest that the mucosal immune system is rapidly and preferentially [80] depleted of CD4 cells during initial HIV infection. This is associated with an enhanced intestinal epithelial permeability, as evidenced by increased levels of lipopolysaccharides (LPS) [81]. Plasma LPS levels correlate with systemic inflammation, which may drive HIV replication and consequently, progression of the disease [82]. Also, HIV is associated with increased markers of intestinal inflammation [83] and has been shown to elicit a Th-2 skewed immune response, which may inhibit an effective antiviral response [84,85]. The gut microbiota of people living with HIV is dramatically different than that of healthy populations. The former group have a notable reduction of both lactobacilli and bifidobacteria, while pathogenic species, such as *Candida albicans* and *Pseudomonas aeruginosa*, are more prominent [83,86].

Probiotics are defined as "live micro-organisms, which, when administered in adequate amounts, confer a health benefit on the host" [87]. Studies indicate that specific probiotic strains may reduce epithelial permeability [88-91], down-regulate systemic and mucosal inflammation [92,93,94], and regulate "allergy-like" Th-2 responses [95]. Therefore, specific probiotic strains may potentially ameliorate HIV-induced changes to the mucosal and systemic immune systems.

To test this hypothesis, a small RCT (n = 77, of which 42 were receiving ART) was conducted in Brazil amongst children living with HIV. After daily supplementation of candidate probiotic containing strains of *Bifidobacterium bifidum* and *Streptococcus* thermophilus (2.5 × 1010 colony forming units) for two months, the CD4 count among the treatment group increased +118 cells/μL versus a decrease of −42 cells cells/μL among the placebo group (p = 0.05). However, the viral load was not measured, and no reduction in diarrhea incidence was shown [96]. Encouraging results were also reported in a small RCT (n = 24) among HIV infected women in Nigeria (table 7). After four weeks of *Lactobacillus* rhamnosus GR-1 and *Lactobacillus* reuteri RC-14 supplementation, the probiotic group experienced an increase of +6.7 CD4 cells/μL, compared to a decrease of −2.2 CD4 cells/μL among the placebo group (p = 0.05) [97]. Although preliminary results are encouraging for probiotic supplementation for people living with HIV, the trials conducted to

date have not had sufficiently large sample sizes or length of follow-up to substantiate the evidence for increases in CD4 count or clinical parameters of HIV progression.

Prebiotics

A prebiotic is defined as "a selectively fermented ingredient that allows specific changes, both in the composition or activity, in the gastrointestinal microflora that confers benefits upon host well-being and health" [98]. Specific prebiotic formulations have been shown to reduce intestinal permeability [99], down-regulate mucosal inflammation [100], and enhance a systemic Th1 immune profile [101,102]. A recently conducted RCT (n = 57) was the first to show the potential of prebiotic supplementation amongst a population living with HIV. Participants were randomized to receive a placebo, a single dose (15g), or a double dose (30g), of a specific mixture of galacto-oligosacharides (GOS), which are long-chain fructans and pectin-derived oligosaccharides. The prebiotic intervention resulted in increased bifidobacterial levels and reduced numbers of the pathogenic *Clostridium histolyticum* cluster [103]. Furthermore, the prebiotic intervention was associated with reduced CD4+/CD25+ cell activation and an improved Natural Killer (NK) cell activity. Effects on CD4 count or viral load were not reported [104]. Prebiotics can be supplemented with probiotics (once combined, they are referred to as synbiotics). A large RCT in Malawi (n = 795) testing the effect of SynbioticForte 2000 (Table 5) on malnutrition also included a proportion of HIV infected children (n = 361). Although there was no improvement in nutritional cure, there was an overall reduction in outpatient mortality, including a trend towards a reduced mortality among the subgroup of HIV infected children [105]. In conclusion, the application of prebiotics to reverse HIV induced intestinal changes has considerable potential, and further design and testing of products specifically for HIV is warranted.

Table 7. Randomized, placebo controlled trials on probiotics and prebiotics and HIV progression.

Reference	Population, in- and exclusion criteria	Intervention and follow-up	Primary outcomes	Major findings	Conclusions
Trois 2007 [96]	Brazil, 77 children (42 receiving ART).	Daily *B. bifidum* and *S. thermophilus* (2.5 x 10^{10} CFU/ml) for two months.	CD4 count and diarrhea prevalence.	Increase in CD4 count, no reduction in diarrhea prevalence.	The candidate probiotic strains may increase CD4 count among children.
Van 't Land 2008 [104]	Italy, 57 ART naïve individuals.	Daily prebiotic 15 g or 30 g for 12 weeks[1].	NK cell activity and CD4 cell activation.	Increased NK cell activity, reduced CD4+/CD25+ cell activation and beneficial effect of intestinal flora.	Prebiotics may reduce HIV associated hyper-immune activation and enhance anti-viral response.
Anukam 2008 [97]	Nigeria, 24 ART naïve women	Daily *L. rhamnosus* GR-1 and *L. reuteri* RC-14 (>1.0 x 10^7 CFU/mL) for 4 weeks.	CD4 count and resolution of diarrhea	Increase in CD4 count and faster resolution of diarrhea.	The probiotics may increase the CD4 count and reduce the length of diarrheal episodes.
Kerac 2009 [105]	Malawi, 361 ART naïve children with malnutrition.	Daily Synbiotic Forte 2000[1] for 9 months.	Nutritional cure, mortality.	No difference in nutritional cure but trend towards reduced mortality.	Synbiotic may reduce mortality.
Lange 2009 [106]	Various countries, 340 ART naïve individuals.	Daily prebiotics, NAC, bovine colostrum, omega-3 PUFA's and micronutrients for one year[2].	CD4 count and viral load.	Increase in CD4 count, no decrease in viral load.	Intervention increases CD4 count and may delay the progression of HIV.

1. Synbiotic included, *Pediococcus pentosaceus*, *Leuconostoc mesenteroides*, 1. *Lactobacillus paracasei ssp paracasei*, and *Lactobacillus plantarum* (10^{11} colony-forming units of bacteria total) and four prebiotic fermentable bioactive fibers (2.5 g of each per 10^{11} bacteria) (oat bran [rich in β-glucans], inulin, pectin, and resistant starch).
2. Formula included short-chain Galactooligosaccharides (scGOS), long-chain Fructooligosaccharides (lcFOS) and Acidic Oligosaccharides from pectin hydrolysate (AOS) (ratio 9,1,10), NAC, bovine colostrum, omega-3 PUFA's and micronutrients.

Conclusions

The beneficial effects of supplementation with vitamins B, C, E, and folic acid to HIV patients have been firmly established, and enough evidence exists to recommend the Tanzanian supplement to pregnant women with HIV in developing countries. The potential beneficial effects of this multivitamin supplement among HIV patients who are being treated with ARTs and among male HIV patients remain to be determined.

Among children living with HIV, evidence indicates that vitamin A is beneficial in reducing morbidity and mortality in developing countries. Further large RCTs with HIV progression and mortality as primary outcomes are warranted to substantiate these findings. Among pregnant women with HIV, there is little evidence to recommend vitamin A supplementation. The potential increase in MTCT found in one study due to vitamin A supplementation warrants caution for its use among this population.

Only one RCT has been conducted to date to assess the effect of vitamin D on HIV progression. The vitamin did not show apparent benefits. This might have been due to an insufficient dose or a relatively small sample size of the sub-population co-infected with HIV. Therefore, a large RCT with HIV progression as the primary outcome and a potentially different dose would be of great value.

A small RCT on zinc has provided some evidence of beneficial effects among patients treated with ART. However, a study among ART-naïve pregnant women did not show similar results. In contrast, supplementation of zinc was associated with an increased risk of wasting and anemia. Until these concerns are addressed by further research, caution is warranted for the use of zinc among people living with HIV.

No apparent adverse effects on HIV progression have been detected with low-dose iron supplementation. However, the studies conducted do not exclude more subtle adverse effects of iron supplementation on HIV progression. Therefore only an RCT with a sufficient sample size to detect more subtle changes would be able to rule out potential adverse effects of iron.

Small studies have indicated beneficial effects of selenium, NAC, or whey protein on HIV progression. However, these findings need to be confirmed by larger, well designed RCTs. Furthermore, probiotics and

prebiotics have limited preliminary data for their use among people living with HIV and further research is warranted.

An integrated approach that combines micronutrients with the various functional food components may have the greatest potential to delay HIV progression. A large multicenter trial, recently conducted in Europe, provides a prime example for this approach. The RCT among ART naïve patients (n = 370) showed that a novel formulation (not fully disclosed, but including prebiotic oligosaccharides with NAC, bovine colostrum, omega-3 poly-unsaturated fatty acids and a combination of vitamins and minerals) slowed down the decline of the CD4 count with 28 cells/µL in the intervention group, compared to −68 cells/µL among the placebo group after one year of supplementation ($p < 0.05$) [106]. This is very encouraging, although the formulation would be expensive and perhaps not so readily available to people living in developing countries.

It should be emphasized that an adequate micronutrient intake among people living with HIV is best achieved through an adequate diet [7]. On the other hand, various interventions have been shown to have beneficial effects in dosages not feasible to be obtained from a diet or not naturally present in high concentrations in food. Therefore, in addition to a diet-based approach, the development of functional food interventions specifically for people living with HIV are much needed.

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3.

Altered host-microbe interaction in HIV:

target for intervention with

pro- and prebiotics

Ruben Hummelen

Arjan P Vos

Belinda van't Land

Klaske van Norren

Gregor Reid

Abstract

The intestinal immune system is severely affected by HIV and circulating microbial products from the intestinal tract that provide an ongoing source of systemic inflammation and concomitant viral replication. In addition, HIV-infected individuals can have a deregulated immune response which may hamper the anti-viral capacity of the host. Various probiotic organisms and prebiotic agents have been shown to enhance intestinal epithelial barrier functions, reduce inflammation and support effective Th-1 responses. As these characteristics may benefit HIV patients, this review aims to provide a theoretical framework for the development of probiotic and prebiotic interventions specifically for this population.

Intestinal defenses and homeostasis

The gastro-intestinal tract provides a range of habitats for microbes that have either co-evolved with the human species as symbionts or as potential pathogens. The different compartments of the tract host approximately 500 to 1000 bacterial species [1], totaling 10^{13} – 10^{14} cells. Collectively, these organisms represent at least 100 times more genes than the human genome. This complex microbial population influences an estimated 10% of all metabolites in our body [2], and could be regarded as 'the neglected organ'. In various eukaryotic species, including humans, the relation between bacterial communities and their host is mutualistic and symbiotic in nature [3]. The symbiotic benefits in humans include energy supply, nutrient metabolism and prevention of colonization by opportunistic pathogens [4].

In order to benefit from this symbiotic relationship, the immune system has to balance permissive, tolerogenic responses to food antigens and commensal microbes with potentially damaging, inflammatory responses to ward off pathogens. This delicate balance is maintained by the constant interplay between the microbiota, the intestinal barrier and the mucosal immune system and is a prerequisite for normal gut homeostasis. Imbalance of this system may lead to autoimmune inflammation or infectious pathology.

The first barrier against pathogenic infection and damaging inflammatory responses against commensal bacteria is a degree of physical separation between the intestinal bacteria and the host. An important component of this barrier are intestinal epithelial cells (IEC) that form a physical barrier on the body's largest surface area for interaction with microbes. The epithelium is also home to mucus-producing goblet cells and antimicrobial-peptide-producing Paneth cells [1]. Collectively, these cells produce a mucus layer that selectively limits the contact between bacteria and host cells, a mechanism which is thought to limit damaging inflammatory responses [5].

Despite this physical barrier, sampling and recognition of the intestinal content is a crucial function of the intestinal immune system that is necessary to mount appropriate immune responses. An important mechanism for IEC and immune cells to interact with

commensal and pathogenic bacteria is the recognition of microbe-associated molecular patterns (MAMP) by germline-encoded pattern recognition receptors (PRR). The best described PRR are Toll-like receptors (TLR), which have been found on a wide range of cell types. The TLR detect various conserved microbial structures such as lipoteichoic acid (LTA) (TLR-2), lipopolysaccharide (LPS) (TLR-4), flagellin (TLR-5) and CpG DNA (TLR-9) (Reviewed in [6]). Interaction of commensal microbes with TLRs appears to be essential for IEC integrity [7]. Other important PRR groups include the sugar-binding lectins and NOD protein families. NOD proteins are located in the cytoplasm of IEC and are activated upon invasion. While NOD-1 is located within all IEC, another variant, NOD-2, is only expressed in Paneth cells and plays a role in the synthesis of cryptidin and defensin [8].

Specialized structures for sampling intestinal content are present in the Gut Associated Lymphoid Tissues (GALT). These include Peyer's patches (PP) in the small intestine and lymphoid follicles in the colon which are covered by follicle-associated epithelium, containing non-mucus producing microfold (M-) cells. These cells are devoid of microvilli and are specialized in antigen transport into the PP, where the antigens are taken up by antigen presenting cells (APC) [9]. Dendritic cells (DC), the main APCs in PP, interact with T- and B-lymphocytes to induce suitable adaptive immune responses, depending on the type of stimulus. Therefore, PPs are major inductive sites of mucosal adaptive immune responses (reviewed in [10]). After activation, lymphocytes home to the lamina propria (LP) or intestinal epithelium to perform effector functions. The heterogeneous population of intestinal Intra-Epithelial Lymphocytes (iIEL) is thought to regulate the intestinal epithelial barrier integrity and regeneration, and reduce damage due to local immune responses [11]. Furthermore, the LP contains DCs that can sample luminal content by extending dendrites through the intracellular epithelial tight junctions, providing a mechanism to sample intestinal content outside of PP as well [12]. DCs are central regulators of adaptive immune responses, initiating either effector responses or inducing tolerance. The many different DC subpopulations that are present within the mucosal immune system each have different functional characteristics (reviewed in [13]). In the absence of inflammatory signals commensal microorganisms induce tolerogenic maturation of DCs,

leading to the induction of various types of regulatory T-cells (Treg), including $CD4^+CD25^+Foxp3^+$ lymphocytes [14], or hyporesponsive T-cells [15]. The maintenance of the Treg population is dependent on IL-2, IL-10 and TGF-β levels, which in turn is dependent on continuous background activation by commensal micro-organisms [16]. In addition, intestinal DCs are potent inducers of IgA synthesis in B-cells, which has anti-pathogenic effects but also prevents commensal bacteria from penetrating the host [17]. IgA accounts for >70% of our body's total immunoglobulin production. Several grams of secretory IgA (sIgA) are secreted in the intestinal lumen daily, which exerts considerable immunological pressure on the intestinal microbiota [18].

Reciprocally, the intestinal bacteria have a major influence the immune system as well. Studies comparing germ-free with microbially colonized mice have shown that the presence of microbes is crucial for the normal development of GALT as well as other secondary immune organs. In the GALT, the absence of bacteria leads to a multitude of effects, including limited development of Peyer's patches, reduced B-cell activation and IgA production and reduced numbers of intestinal intraepithelial lymphocytes (iIEL). Moreover, epithelial function is also affected, as evidenced by reductions in IEC turnover and changes in mucus production [19].

The effects of commensal bacteria on the immune system are dualistic in nature. On the one hand, mechanisms are induced that maintain tolerance and/or prevent inflammation. This includes IgA production, β-defensin production in the epithelium [20, 21], enhanced epithelial barrier integrity through TLR signaling [7], Treg induction and even immunosuppressive effects [22-24]. On the other hand, exposure to commensal bacteria induces the expansion of inflammatory lymphocyte populations, including cytotoxic intestinal Intra-Epithelial Lymphocytes (IEL) and IL-17 producing $CD4^+$ T-helper (Th17) cells [25, 26]. Especially Th17 and other IL-17 producing cells have been implicated in many inflammatory and autoimmune conditions [27-29], however, they have also been shown to play important roles in protective mucosal responses against extra-cellular bacteria and fungi (reviewed in [30]). It is interesting to note that the induction of Treg and Th17 populations share a dependency on TGF-β signaling. Furthermore, both populations are relatively abundant in the LP. Therefore, it is thought that both types of T-cells

are induced by signals from the intestinal bacteria and the balance between these opposing cell types is determined by the specific host-microbe interactions [31].

Commensal bacteria are able to influence the mucosal immune system not only through cell-cell interactions, but also through the secretion of immune-modulatory molecules. For example; adenosine triphosphate (ATP), which enhances the polarization of Th-17 T-lymphocytes [32]; polysaccharide A (PSA), which induces maturation of Th-17 cell populations [33]; and DNA, which induces IFN-α syntheses and favors IEC integrity [34]. Furthermore, the intestinal epithelium is also constantly exposed to inflammatory molecules such as LPS and peptidoglycans. Despite this continuous exposure, the intestinal immune system is unique in its ability to maintain tolerance in the presence of a multitude of immune triggers while minimizing the risk of systemic infection.

In summary, the net effects of the interplay between the commensal microbiota and the mucosal immune system are enhanced mucosal defense mechanisms, balanced by an inhibition of potentially damaging, inflammatory immune responses. In the absence of pathogenic stimuli, virulence factors or chronic inflammation, the host-microbe interaction leads to a predominance of tolerogenic mechanisms and intestinal homeostasis.
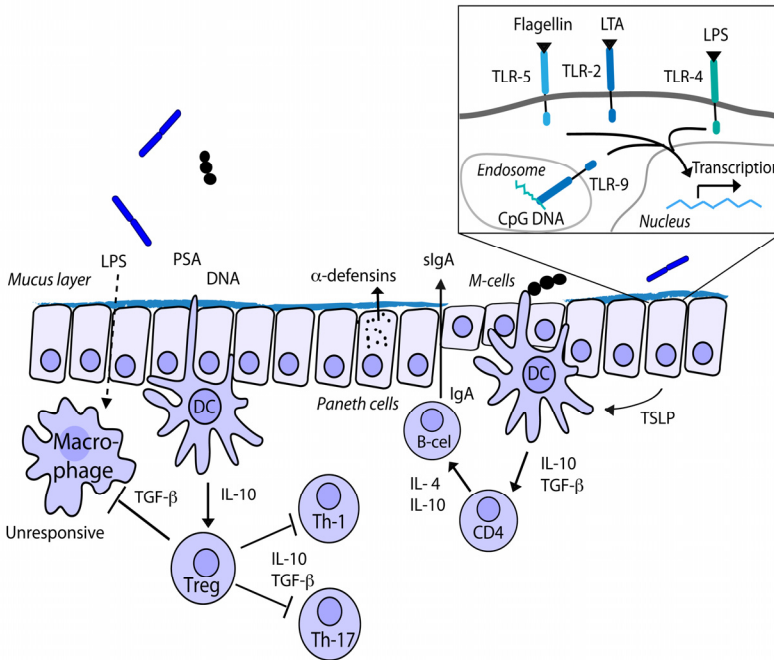
Figure 1. Normal mucosal defenses and homeostasis. The commensal microbiota induces a state of non-responsiveness through interaction with dendritic cells (DC) and subsequent induction of the T-regulatory phenotype and secretion of IL-10 and TGF-β. There is a limited uptake of bacterial antigens such as lipopolysaccharide (LPS), polysaccharide-A (PSA) and DNA, that induce intestinal defense systems such as the excretion of α-defensin and secretory IgA. Recognition of bacterial ligands also occurs by membrane bound toll-like receptors (TLR) and nod-like receptors (NLR) present in endosomes.

The result of HIV infection on the host-microbe interface

HIV infection has a disruptive impact on the physiological interplay between the commensal microbiota and immune system: CD4^{+} cells associated with the mucosal immune system are rapidly depleted after HIV infection [35, 36], including reduced numbers of DCs [37], a change in the composition of iIEL [38] and depletion and anergy of gamma-delta T-lymphocytes [39]. These detrimental changes on the mucosal immune system have severe consequences for the (immunological) function of the intestine and are associated with compromised epithelial repair mechanisms and enhanced epithelial permeability [40, 41]. The net result is an increased risk of gastro-

intestinal infections at all stages of HIV infection [42] and a high prevalence of gastro-intestinal disorders with unknown etiology [43].

Chronic immune activation and inflammation have long been described as characteristic features of progressive HIV disease, while the source of inflammation has remained unidentified. Indeed, increased B-cell activation, increased T-cell turnover, and increased pro-inflammatory cytokines are observed with HIV infection. In this pro-inflammatory state, the replication of HIV is markedly enhanced [44] and activation of the Nuclear Factor (NF) κB transcription factor plays a crucial role in this phenomenon [45]. Strikingly, the degree of systemic immune activation, indicated by the expression of the immune activation marker CD38 on CD8+ cells, is a better predictor of HIV progression than viral load or CD4$^+$ count alone [46]. Recently, it has been suggested that the gut might be a source of chronic inflammation. The hypothesis is that dysfunction of the mucosal immune response due to preferential depletion of intestinal mucosal immune cells including effector CD4+ cells and DCs [36, 37, 47] may affect systemic immune activation through the increased translocation of microbes and bacterial products from the intestinal tract [48]. The resultant pro-inflammatory environment [40] may then cause further damage to the gut barrier function, augmenting bacterial translocation and subsequently fuelling systemic inflammation. Evidence indeed suggests that bacterial translocation affects the activation state of the immune system, and in turn HIV progression.

Some HIV infected individuals, termed "non-progressors" have a low HIV viral load even without treatment and maintain a low degree of systemic inflammation [49, 50]. A mechanism that appears to contribute to the control of the virus is a capacity to maintain the integrity of the gut barrier and to mount an attenuated response to bacterial products and thus potentially reduce bacterial translocation [40, 51]. In non-progressors, serum LPS has been shown to be lower than those with progressive HIV infection [48]. One week of treatment with a 'gut sterilizing' antibiotic regimen markedly reduced serum LPS levels in macaques, concomitant with a reduction of fecal Gram-negative bacteria and inflammatory markers. However, after two weeks of antibiotics, plasma LPS had increased again, apparently due to growth of other bacterial species in the gut [48]. Although anti-retroviral treatment (ART) has been shown to enhance epithelial

barrier functions [52], the efficiency of CD4$^+$ recovery may still be compromised by bacterial translocation [53]. Future studies will need to focus on the role of the epithelial barrier and the microbiota composition along with translocation in the progression of HIV.

The intestinal microbiota has been shown to play important roles in other disease conditions. Bacterial translocation occurs during surgery [54], plays a role in alcohol induced liver cirrhosis [55], in exacerbation of graft versus host disease (GVHD) [56] and in inflammatory bowel disease (IBD) [57, 58]. Furthermore, correlations between the microbiota composition and disease have been shown for IBD [59] and obesity [60]. Strikingly, the transplantation of a gut microbiota from obese mice (ob-/ob-) to bacteria-free mice resulted in obesity in the recipients [61], suggesting that the microbiota may be a mediator of specific conditions. Lessons from such studies that focus on microbiota-disease interactions may help to provide more insight in the role of an aberrant microbiota in HIV-infected subjects.

The intestinal microbiota of HIV patients appears to contain higher levels of pathogens such as *Pseudomonas aeruginosa* and *Candida albicans* [62] and reduced or undetectable levels of *Bifidobacterium* and *Lactobacillus* species [63]. In the macaque model, colitis is very common after Simian Immunodeficiency Virus (SIV) infection, resulting in a reduced microbial diversity and an increased proportion of Campylobacteriaceae [64]. If the aberrant microbiota among HIV patients more easily translocates and provides an inflammatory stimulus for HIV replication, therapeutic modification of the gut microbiota might have a beneficial impact on HIV progression.

Table 1. Evidence implicating the intestinal microbiota and epithelial cell barrier as a factor in HIV progression

- Aberrant intestinal microbiota among HIV patients with fewer lactobacilli and bifidobacteria, and higher numbers of pathogenic *P. aeruginosa*, and *C. albicans* present (48, 49).
- Antibiotics can temporarily reduce bacterial translocation in SIV infected macaques (37).
- Increased bacterial translocation among HIV progressors and those not effectively responding to ARV (37, 39).
- Enhanced epithelial inflammation and scarring of GALT among HIV progressors (26, 33).
- Increased epithelial permeability with HIV at all stages of infection (27).

Several lines of evidence suggest that HIV modulates systemic immunity by skewing the Th1/Th2 balance towards Th2 responses. Recently, it was suggested that the induction of T helper-2 (Th-2) cytokine synthesis [65, 66] and the gradual increase of IL-4 and IgE [67, 68] observed after HIV infection, might be due to a Th-2 response to viral proteins such as gp-120, p24 and p17 [69]. It is well known that HIV patients suffer from high rates of allergies [70] and the ability of subgroups of HIV patients to maintain a vigorous Th-1 response and higher levels of IFN-γ is associated with increased survival [71, 72]. An HIV-triggered Th-2-skewed state of the immune system could compromise immunological control of HIV replication and lead to reduced protection to opportunistic infections. This immune imbalance may also aggravate inflammation and barrier dysfunction in the gut, as the   increase in IL-4 production can compromise the antimicrobial function of Th-17 cells [73] that line the intestine.

Probiotics

Probiotics, defined as "live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host" [74], have been studied in a myriad of conditions related to intestinal dysbiosis, including IBD, infectious diarrhea, allergy and surgery. Relevant research from these fields and studies on probiotic interventions among people living with HIV and safety considerations are now discussed.

Effects on gut barrier function

Supplementation of probiotic strains may enhance or restore the beneficial interactions between the commensal enteric flora and the host in health and disease conditions, leading to an enhanced barrier function and reduced bacterial translocation. Effects have been described in animal and human studies that may have relevance for HIV-infected subjects. For example, the Gram-negative probiotic strain *Escherichia coli* Nissle 1917 was shown to enhance the intestinal epithelial integrity via the induction of epithelial tight junctions proteins (ZO-1 and ZO-2) [75, 76]. Prior administration of candidate probiotic strains *Lactobacillus acidophilus* (ATCC19258) and

Streptococcus thermophilus (ATCC4356) to IEC has been shown to reduce the epithelial permeability induced by TNF-α and IFN-γ, suggesting that the impact of inflammation can be reduced [77]. It appears that these various protective mechanisms occur through the production of as yet uncharacterized proteins [78] or through direct interaction of microbes with IEC through TLR-4, TLR-5 and TLR-9 [34]. That the enhancement of the epithelial barrier might also translate to a reduction in bacterial translocation was shown in a murine model of enterohemorrhagic shock. In this model, prior challenge with *L. rhamnosus* LMG P-22799 reduced bacterial translocation and systemic inflammation [79], as did use of Bifidobacterium adolescentis in a murine model of burn wounds [80]. Furthermore, allergy-associated intestinal hyper-permeability [81] as well as alcohol-induced loss of gut barrier function has been found to be reversed by application of *L. rhamnosus* GG, resulting in less intestinal and liver inflammation in the latter [82].

In human clinical studies, probiotics have been applied to reduce bacterial translocation among different patient populations with varying degrees of success. A randomized controlled trial (RCT) among 65 critically-ill patients showed reduced rates of infections, sepsis and mortality with a combination of probiotics and prebiotics (Synbiotic 2000 Forte) [83]. In consecutive trials, the same product was shown to increase fecal IgA [84], reduce the incidence of bacteremia [85] and lower the rate of post-operative infections [86]. Two other RCT using different synbiotic preparations showed similar results. *L. casei* Shirota and *L. breve* Yakult given with Galacto-oligosaccharides (GOS) supplementation (15 gr/day) before biliary cancer surgery, resulted in reduced inflammatory markers and post-operative complications [87]. Another RCT with *L. acidophilus* La-5 and *B. lactis* Bb-12 combined with oligofructose (15gr/day) resulted in a reduced incidence of bacterial translocation [88]. However, the evidence is not conclusive as three other RCTs using *L. acidophilus* La-5 and *B. lactis* Bb-12 [89, 90] or *L. plantarum* 299v [91] reported no reduction in bacterial translocation. This suggests that there may be strain specific effects or that prebiotics are needed for efficacy.

Effects on mucosal and systemic immunity

The loss of tolerance in the intestine due to a defect in GALT homeostasis can have a detrimental impact on the gut barrier

function. Specific probiotic strains have been shown to enhance the recovery of GALT homeostasis. For example, administration of *Escheria coli* Nissle 1917 in wild type mice, but not in TLR-2 knock-out mice, ameliorated experimental colitis and reduced pro-inflammatory cytokine expression, suggesting a TLR-2 dependant pathway [92]. This response can be mediated by specific components of probiotic organisms. DNA from probiotic organisms modulates TLR-9 and elicits a different response from immune and epithelial cells than DNA from pathogenic organisms [93]. In HT-29 cells subjected to pro-inflammatory cytokines, challenge with DNA from the VSL#3 probiotic mixture could reduce the expression of the pro-inflammatory IL-8 cytokine and delayed NF-κB activation [94]. In IL-10 deficient mice, the administration of the probiotic mixture VSL#3 led to a reduction in mucosal TNF-α and IFN-γ release and improved the histological disease in a TLR-9 dependent manner [94].

Another way of restoring GALT homeostasis is through the induction of regulatory mechanisms to down-regulate inflammation. Induction of regulatory mechanisms by specific probiotics appears to be partly dependent on modulation of DCs [95]. Some probiotic organisms induce DCs to express a regulatory T-cells (CD4+FoxP3+) inducing phenotype [16, 96]. This induces tolerance that is partly mediated through IL-10 and TGF-β production. Recently, it was demonstrated that ingestion of a specific probiotic mixture (IRT-5) could induce CD4+FoxP3+ cells in mesenteric lymph nodes. Interestingly, probiotics alone, without the presence of DCs could not convert this effect. Administration of the probiotic micture also induced both T-cell and B-cell hypo responsiveness, down-regulated both Th-1 and Th-2 functions, and reduced the secretion of pro-inflammatory cytokines in GALT [97]. The biological relevance of these changes was verified in an IBD model. Administration of probiotics in this model was shown to enhance GALT homeostasis and severity of disease. The therapeutic effects were associated with an enrichment of CD4+Foxp3+ T-cells in inflamed regions [97]. O'mahony *et al.* demonstrated that challenge with *Lactobacillus salivarius* or *Bifidobacterium infantis* of DCs from mesenteric lymph nodes induced secretion of IL-10. This was in contrast to challenge with *Salmonella* strains, which induced the secretion of pro-inflammatory IL-12. Strikingly, DCs from peripheral blood did not show a differential response to lactobacilli or *Salmonella* strains [98],

suggesting that the response of DCs depends on their immunological compartment. In the intestine, tolerogenic effects of T-regulatory cells and anti-inflammatory effects may improve barrier function and intestinal homeostasis. Furthermore, a reduction in the chronic inflammatory state may reduce immune activation and potentially affect disease progression [48].

Systemically, specific probiotics have also been shown to induce a T-regulatory phenotype and counter-balance a Th-1 or Th-2 dominant state *in vivo* and *in vitro* [95, 99, 100]. The induction of a T-regulatory phenotype frequently occurred together with increased levels of anti-inflammatory IL-10 [101, 102]. A prime example of the clinical effects of IL-10 induction comes from an RCT of 77 adults with an abnormal IL-10/IL-12 ratio and concurrent irritable bowel syndrome (IBS). *B. infantis* 35624 was shown to normalize the IL-10/IL-12 ratio in parallel with a reduction in clinical symptoms [103]. In Crohn's disease, a Th-1 mediated condition, a strain of *L. rhamnosus* decreased after 2 weeks of supplementation both the syntheses of the Th-1 parameter IFN-γ and IL-2, which is essential survival and proliferation factor for effector T-cells [104]. Remarkably was that IL-4, a potent Th-2 cytokine, was also reduced with probiotic supplementation. Hence, the effects of probiotic supplementation were unlikely due to mere Th-1/Th-2 skewing and are best explained by the induction of a regulatory DC phenotype with the ability to induce a general hypo-responsiveness. In a study of children with atopic dermatitis, a Th-2-dominant condition, an up-regulation of IL-10 was noted after supplementation with *L. rhamnosus* GG [105]. These findings might be valuable in relation to HIV management, as both the induction of T-regulatory cells and anti-inflammatory effects can potentially be of benefit to HIV patients.

In vitro evidence indicates that several probiotic candidate strains can down-regulate the production of Th-2 cytokines and chemokines [106, 107] and modulate DCs to skew T-cell polarization toward a Th1-response [108]. The ability of probiotics to skew the immune system away from a Th-2 dominant state has been studied to some extent in humans, albeit in HIV uninfected subjects. An RCT of 230 children with cow's milk allergy showed that L. rhamnosus GG ingestion could reduce symptoms of atopic eczema and dermatitis [109] and up-regulate IFN-γ, indicative of a more pronounced Th-1

response [110, 111]. Moreover, L. rhamnosus GG was shown to up-regulate IL-6, involved in the mucosal response to stress [112] but also epithelial IgA production and mucosal protein syntheses [113], suggesting a direct effect of L. rhamnosus GG on IECs. Two other RCT's have shown similar results [114, 115] but one study in children did not confirm this outcome [116]. In addition to the ability of probiotics to improve barrier function and aspects of intestinal homeostasis, specific probiotic strains may therefore be able to skew away from a HIV-induced Th-2 predominance

Effects on intestinal microbiota and infections

Probiotics can interfere with the function and proliferation of pathogens in the gastro-intestinal tract in various ways. They can enhance the secretion of pathogen-specific IgA [117], induce β-defensin secretion [118] or secrete bactericidal proteins [119] and reduce the adhesion and invasion of pathogens [120, 121]. Antibiotic-like compounds such as reuterin produced by *L. reuteri* exhibit broad spectrum effects against Gram-positive, Gram-negative bacteria as well as fungi, yeast and protozoa [119], while non-reuterin producing strains such as *L. reuteri* RC-14 produce signaling molecules that down-regulate *Staphylococcus aureus* toxin production [122]. These characteristics could be beneficial for Acquired Immune Deficiency Syndrome (AIDS) as *L. reuteri* was shown to prevent cryptosporidiosis in a murine AIDS model [123].

Use of probiotics can lead to at least temporary modification of the intestinal microbiota. For example, in an RCT of 69 preterm babies, *B. lactis* Bb-12 significantly increased the levels of bifidobacteria and lactobacilli while reducing the numbers of enterobacteria and clostridia [124]. In 36 adults receiving triple therapy for *Helicobacter pylori* infection, the addition of *L. acidophilus* CUL60 and CUL21 and *Bifidobacterium* spp. decreased the intestinal load of *C. albicans*, facultative anaerobes and enterobacteria [125]. Moreover, genomic and metabolic studies suggests that probiotic microbes change the behavior of the intestinal microbiota [126].

The application of probiotics for the prevention and treatment of gastro-intestinal infections has been well established and might be especially useful among people living with HIV. A Cochrane review concluded that probiotics are a useful adjunct to lower the occurrence and reduce the length of episodes of infectious diarrhea (reviewed in

[127]). Synbiotics (probiotics combined with prebiotics) have also been shown to reduce diarrhea associated with ARV use [128] but these findings could not be confirmed by a cross-over study [129]. Although the application of probiotics to prevent gastrointestinal infections and the concomitant inflammatory state among HIV patients bears promise, no studies have so far been conducted to assess its potential.

Probiotic interventions in HIV

A limited number of studies suggest that the probiotic benefits could be translated to people living with HIV. One RCT of 77 children in Brazil showed an increase of 118 CD4$^+$cells/µl among those receiving *B. bifidum* and *S. thermophilus* for two months compared to a decrease of 42 CD4$^+$cells/µl among the placebo group [130]. An RCT of 24 HIV patients in Nigeria showed after four weeks of *L. rhamnosus* GR-1 and *L. reuteri* RC-14 an increase of 6.7 CD4$^+$cells/µl compared to a decrease of 2.2 CD4$^+$cells/µl among the placebo group [131]. A large RCT in Malawi (n = 795) testing the effect of SynbioticForte 2000 on malnutrition also included a proportion of HIV infected children (n = 361) [132]. Although there was no improvement in nutritional cure, there was an overall reduction in outpatient mortality including a trend towards a reduced mortality among the subgroup of HIV infected children.

Safety

Among HIV patients, several studies have been conducted to assess the safety of probiotic interventions. When treated with *L. reuteri* SD2112, no safety concerns arose among moderately immune compromised HIV patients (>350 CD4+cells/µl) [63]. In another study of severe immune compromised HIV patients (<200 CD4+ cells/µl) no safety concerns were detected with use of *L. rhamnosus* [129]. To date, five case studies of lactobacillemia have been reported in end-stage AIDS patients. Of these, three patients were reported to have central venous catheters and one patient to have pneumonia, all of whom had extremely low CD4 counts (<55 CD4+cells/µl) [133-135]. Currently, no indication exists to avoid oral probiotic use in HIV populations, but close monitoring of safety parameters is recommended.
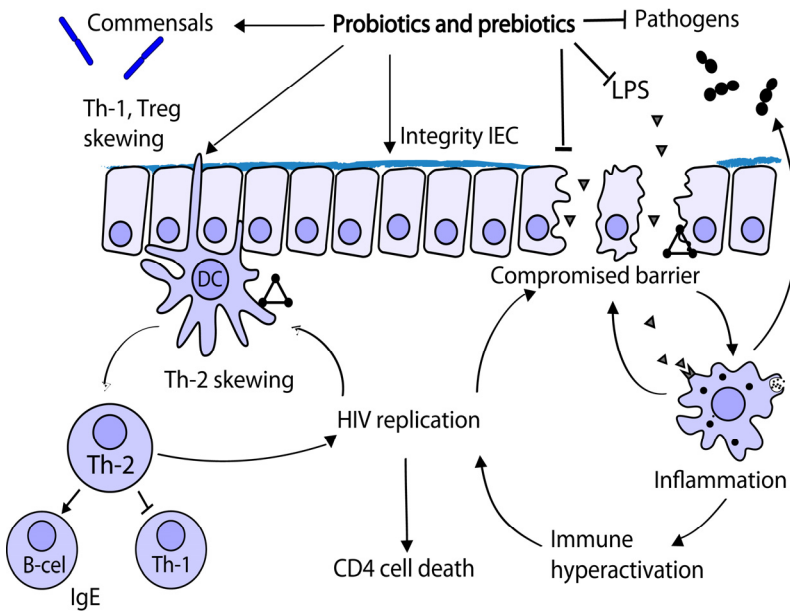
Figure 2. Potential benefits of probiotics and prebiotics in HIV-induced intestinal pathogenesis. HIV infection induces effects and positive feedback mechanisms that induce a loss of intestinal homeostasis and promote replication of the virus (triangles). Pro- and prebiotics may ameliorate the HIV-induced intestinal problems through effects on the microbiota and its metabolism, on various cells of the immune system (as represented by the arrow pointing at the sampling DC) and on intestinal epithelial cells.

Prebiotics

Altered microbe-host interaction

Prebiotics were defined most recently as "a non-viable food component that confers a health benefit on the host associated with modulation of the microbiota" [136], although the term is often used less strictly for components that modify the composition or metabolism of the intestinal microbiota. Prebiotics can modify host-microbe interactions via the microbiota and its metabolism, host epithelial and other cells, as well as by modifying receptor expression and bacterial adhesion. As alluded to earlier, prebiotics are candidate agents to improve the intestinal homeostasis in HIV-infected individuals. Since prebiotics do not contain bacteria but provide substrate for the intestinal microbiota, their fermentation depends on

the organisms present in the host. Prebiotic fructans and galacto-oligosaccharides (GOS) increase the percentage of 'beneficial bacteria' through selective fermentation as shown in a variety of human target groups, including infants [137, 138], healthy volunteers [139] and seniors [140]. The fermentation of fructans and GOS increases the production of short-chain fatty acids (SCFA), lactate and other bacterial metabolites [141, 142]. SCFA are known to have a plethora of effects on the intestinal milieu, epithelial cells as well as local immune cells. Various prebiotics induce differential effects on SCFA production and the ratios of butyrate, acetate and propionate [143]. The degree of specificity of prebiotic agents enables the potential development of specific prebiotics optimized to target HIV-specific issues. Relevant potential benefits for HIV patients are discussed in the next sections, focusing both on the effects of prebiotic intervention and on the effects of purified bacterial metabolites such as SCFA.

Effects on barrier function

As described previously, decreased barrier function and increased bacterial translocation is described in HIV-infected subjects. Prebiotics have been shown to influence barrier function via various mechanisms. A combination of fermentable fibers has been shown to significantly reduce endotoxemia over a 30-day intervention period in an RCT of 55 cirrhosis patients [144]. Animal studies using alcohol-induced liver damage showed similar beneficial results preventing intestinal dysbiosis with an oat-based prebiotic intervention [145].

There appear to be several mechanisms whereby prebiotics can enhance intestinal barrier function. Recently, Cani and coworkers [146] have shown that a prebiotic-induced increase in glucagon-like peptide-2 (GLP-2) played an important role in the beneficial effects of prebiotic intervention in *ob/ob* mice on a high-fat diet. Increased intestinal barrier function and expression of tight-junction proteins were observed, leading to reductions of hepatic markers of oxidative stress and inflammation, as well as reduced levels of systemic inflammatory mediators and endotoxemia [146]. The production of SCFA during fermentation of prebiotic agents can also lead to an improved barrier function. Butyrate in particular is an energy source for intestinal epithelial cells and, through the modulation of intestinal prostaglandins it stimulates mucus production [147]. Recently,

butyrate was shown to enhance intestinal barrier function *in vitro* by regulating the assembly of tight junctions in Caco-2 cells [148].

Effects on gastrointestinal infections

A few human studies have shown that prebiotics can reduce gastrointestinal infections, a functional characteristic that may potentially be used to counteract the HIV-increased prevalence of gastro-intestinal infections [42]. Fructan supplementation for 30 days reduced diarrhea relapse rates in an RCT among 140 patients with *Clostridium difficile*-associated diarrhea [149]. In addition, fructan supplementation showed a tendency to reduce traveler's diarrhea in an RCT of 244 healthy volunteers [150]. In a 12-month open-label intervention trial among 342 infants, a formula containing a specific combination of GOS and a long-chain fructan induced significant reductions in gastroenteritis and acute diarrhea [151]. The same combination of oligosaccharides was shown to reduce the number of fecal pathogens and increase intestinal IgA production in infants; two mechanisms by which prebiotics could reduce intestinal infections [152, 153].

SCFAs can contribute though acidification of the intestinal content and growth inhibition of acid-sensitive pathogens [154]. Butyrate stimulates the production of antimicrobial peptides, such as cathelicidins, which are able to kill a variety of potential pathogenic bacteria [155].

Prebiotic oligosaccharides can have anti-pathogenic effects that are independent of the intestinal microbiota and its metabolism. Human milk oligosaccharides are known to exhibit receptor-decoy functionality, based on the molecular structure and sugar moieties of the oligosaccharides [156], leading to binding of oligosaccharides to potential pathogens and preventing their adherence to the intestinal lining. Similarly, GOS and pectin-derived oligosaccharides can inhibit adherence of specific pathogens to epithelial cells *in vitro*, demonstrating that this mechanism is not limited to oligosaccharides of mammalian origin [157, 158]. Studies on HIV-infected adults and infants are required to better determine the efficacy of prebiotics against diarrhea, especially in developing countries where such infections can be lethal.

Local anti-inflammatory and immunomodulatory effects

IBD is characterized by intestinal inflammation in which the microbiota plays an important role, a situation not dissimilar to the HIV-induced inflammation in the gut. Small-scale studies using fructan-based pre- and synbiotic intervention in ulcerative colitis (UC) patients have shown beneficial effects on histological inflammation scores, and on mRNA expression of inflammatory mediators in biopsy samples [159, 160]. Similarly, a small, open-label study showed anti-inflammatory effects of fructan supplementation in moderate Crohn's disease patients. In that case, lamina propria biopsies showed that the intervention modulated the phenotype of intestinal DCs, enhancing IL-10 production and expression of TLR-2 and TLR-4 [161]. This suggested that the anti-inflammatory effects are related to changes in microbe-host interactions.

Many preclinical data using prebiotics in IBD models show corresponding results (reviewed in [162]). In mechanistic studies using different chemically-induced inflammation models, it was shown that the beneficial effects of prebiotic intervention could be reproduced in part or completely by infusing lactic acid bacteria intragastrically and/or SCFA into distal parts of the large intestine.

Recent animal studies confirm that the expression of PRRs in epithelial and immune cells can be modified by prebiotics and by butyrate *in vitro* [163, 164]. However, the molecular mechanisms remain to be elucidated. Whereas butyrate was found to reduce LPS and TNF-α-induced NF-κB activation in a colonic epithelial cell line [165, 166] these results were partly contradicted in a different colonic cell line [163]. A recent study indicatedthat NF-κB may be modulated directly by unfermented oligosaccharides. Pectin derived acidic oligosaccharides reduced NF-κB in vitro and reduced HIV-1 viral production *in vitro* [167].

Another molecular target for SCFA-induced modulation of inflammation are the G-protein-coupled receptor 41 and 43 (GPR-43), that are most efficiently activated by acetate and propionate [168-170]. GPR-43 is expressed mainly in innate immune cells and is critically important in the resolution or reduction of inflammation in a variety of mouse models. These mechanisms highlight the potential of prebiotic anti-inflammatory capacities, which could lead to an

amelioration of chronic inflammation and possibly immune activation in HIV-infected subjects [48].

Modulation of systemic immunity

In addition to local effects of prebiotics in the gut, systemic immunomodulatory effects of prebiotics have been described that are relevant for HIV-infected individuals. A 10-week cross-over study with GOS in 44 healthy, elderly subjects showed simultaneous bifidogenic and systemic immunomodulatory effects. The phagocytosis capacity and natural killer cell activity of circulating white blood cells was increased, whereas the production of inflammatory cytokines was reduced [140]. Furthermore, specific prebiotic interventions have been shown to modulate the immunological balance, consistent with a shift away from a Th2-dominant state. For example, a specific combination of GOS and short-chain fructans was shown to reduce the incidence of atopic dermatitis and allergy-related symptoms in infants at risk for allergy [171]. Correspondingly, changes in the antibody class and isotype ratios suggestive of a Th1 shift were detected [172]. The ability to induce this shift is thought to be beneficial in HIV patients, as it might result in more effective anti-viral control and a better immunological defense against opportunistic pathogens [69, 173].

Effects of the specific combination of GOS and long-chain fructans in multiple mouse models are also consistent with a shift from Th2 to Th1 responses, as allergic responses were reduced and Th1-dependent vaccine-specific DTH responses were enhanced [174-176]. In addition to modifying the microbiota, prebiotics may also mediate effects via the carbohydrate structures on immune cells. Very low-level systemic bioavailability of short-chain fructans have been described in the urine of healthy volunteers [177], suggesting the potential for direct systemic effects through interactions with lectins, galectins or other sugar-binding molecules.

Prebiotic intervention in HIV-infected individuals

A limited number of studies that have used prebiotics in HIV-infected individuals indicate that the benefits described above may be relevant for this population. Recently, a prebiotic intervention study was performed to investigate potential microbiological and

immunological benefits among 57 HIV patients. A 12-week intervention with a specific mixture of GOS, long-chain fructans and pectin-derived oligosaccharides in HAART-naïve HIV-1 infected individuals resulted in increased bifidobacterial levels and reduced numbers of the pathogenic *Clostridium histolyticum* cluster. In addition, reduced levels in the pathogenic *E. rectale and C. coccoides* cluster were found [178]. The prebiotic intervention was associated with reduced CD4^{+} T-cell activation, measured as percentage of CD4^{+}/CD25^{+} T-cells. In addition, improved NK-cell cytotoxicity was observed [179]. The beneficial effects of this pilot study were confirmed in a multi-centre RCT, in which a one-year intervention was tested in HIV infected individuals not on ART. A total of 340 participants were included in the trial and received the intervention product or an isocaloric, isonitrogenous control product. The intervention product consisted of the same mixture of prebiotic oligosaccharides in combination with bovine colostrum, omega-3 polyunsaturated fatty acids, and *N*-acetyl cysteine. The intervention significantly slowed down the decline of the CD4 count as the decrease in the intervention group (-28 cells/µl) was lower than the decrease in the control group (-68 cells/µl) [180]. These findings are very promising and show the potential for nutrition-based strategies to become an integral part of disease management.

Conclusion and future prospects

The interaction between the gastrointestinal microbiota and the human host plays a crucial role in intestinal homeostasis and the health status of the host. The gut-associated immune system tightly regulates this interaction and, under normal conditions, prevents damaging inflammatory reactions by maintaining a tolerogenic state. HIV infection has a disruptive impact on the intestinal homeostasis as it directly affects the host and indirectly affects the intestinal microbiota. The loss of intestinal CD4^{+} T-cells, epithelial function and immune regulation, in combination with a pathogen-enriched microbiota composition, leads to an increase in intestinal permeability, bacterial translocation and an inflammatory state.

Pro- and prebiotics are modulators of both microbiota and host factors, making them potential agents to ameliorate the intestinal problems induced by HIV. Various beneficial effects of pro- and

prebiotic interventions have been demonstrated that may translate to applications in HIV. These effects include improved barrier function, reductions in the translocation of bacterial products, reductions in pathogenic load, local and systemic anti-inflammatory effects and immunomodulatory effects to restore a proper Th1/Th2 balance.

Various therapeutic applications of pro- and prebiotics are conceivable, such as before initiation of ART, where they could potentially help reduce HIV-induced intestinal inflammation, intestinal infection or diarrhea. In addition, by reducing systemic inflammation and associated immune activation, disease progression may be slowed down. Obviously, pro- and prebiotics should not be used as alternatives to ARV but might have a role as conjoint therapy especially among immunological non-responders to ARV.

An additional benefit of the pro- and prebiotic is the possible application in low-cost interventions of limited complexity, which might be especially useful in resource-limited countries [181]. Depending on the target group, the oral application of pro- and prebiotics makes it possible to combine the intervention with specific (micro-) nutrients to prevent specific deficiencies or to aim at multiple targets simultaneously. Applied as interventions to improve intestinal homeostasis in HIV-infected individuals, pro- and prebiotics have potential to contribute beneficially to integrated disease management.

In recent years, promising initial studies encompassing pro- and prebiotic interventions have been performed in HIV-infected individuals, showing the potential for improvement of intestinal homeostasis and potentially a reduction in the decline in CD4 count. However, a clear need remains for additional, well-designed double-blind, randomized studies to provide evidence for the efficacy of specific pro- and prebiotic interventions.

Acknowledgements

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4.

Probiotic yogurt consumption is associated with an increase in CD4 count among people living with HIV

Stephanie L Irvine*
Ruben Hummelen*
Sharareh Hekmat
Caspar W N Looman
J Dik F Habbema
Gregor Reid

*Equal contribution

Abstract

Goal: To evaluate the long term effect of yogurt supplemented with *Lactobacillus rhamnosus* Fiti on the immune function (CD4 count) of people living with HIV/AIDS.

Background: Gastrointestinal infections as well as the leakage of microbial products from the gut have a profound impact on the deterioration of the immune system among people living with HIV/AIDS. Among persons not infected with the virus, probiotics can prevent gastrointestinal infections and restore an effective gut barrier, suggesting they might have a beneficial effect on the immune function of people living with HIV/AIDS.

Study: We performed an observational retrospective study over a period of three years, with longitudinal comparison of the CD4 count within subjects (n=68) before and during probiotic yogurt consumption, and compared to a control group of subjects not consuming the yogurt (n=82).

Results: Among the yogurt consumers before use and the non-consumers an average increase in CD4 count was seen of 0.13 cells/ɥl/day [95% CI; 0.07 - 0.20, p= <0.001]. After commencing consumption, yogurt consumers experienced an additional increase of 0.28 cells/ɥl/day [95% CI; 0.10-0.46, p= 0.003]. When adjusting for length of time using ARV medication the additional increase explained by yogurt consumption remained 0.17 cells/ɥl/day [95% CI; 0.01-0.34, p= 0.04]. Treatment with ARV medication was associated with an increase of 0.27 cells/ɥl/day [95% CI; 0.17-0.38, p= < 0.001].

Conclusion: The introduction of probiotic yogurt, made by local women in a low-income community in Tanzania, was significantly associated with an increase in CD4 count among consumers living with HIV.

Introduction

Recent attention on HIV/AIDS care in Africa has focused on increasing access to antiretroviral (ARV) medication. Although this is important, efforts are also needed to provide safe and affordable interventions for those without access to ARV's or with CD4 counts too high to initiate antiretroviral therapy, yet whose quality of life is diminished by micronutrient deficiencies, diarrhea and other conditions associated with HIV infection. An increased micronutrient intake, most notably vitamin B-complex in combination with C and E , seems to be an effective intervention that has been associated with a reduced mortality and increased CD4 count (1-5). The WHO recommends that an "increased micronutrient intake can be best achieved through an adequate diet" (6), favoring food based interventions.

The gut is one of the most severely affected sites by HIV (7, 8). Inflammation results in damage to the epithelial barrier, leading to an increased leakage of microbial products into the bloodstream. Recently it was theorized that this may be an ongoing source of systematic immune activation that fuels HIV (9), although this association was less clear in an African population (10). Capsule proteins of HIV may further facilitate viral replication by eliciting a profound Th-2 activation which inhibits an effective immune response against the virus (11).

Probiotic bacteria, "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (12) can potentially restore an effective gut barrier (13, 14), thereby reducing systemic immune activation. Furthermore, probiotics have been shown to up-regulate T-regulatory lymphocytes (15, 16), potentially skewing the immune system away from a Th-2 dominant state (17). Probiotic usage has been shown to be safe among people living with HIV (18-20) and recent randomised trials in Brazil (21) and Nigeria (22) suggest that probiotic use can increase the CD4 count.

The most commonly used vehicle for supplying probiotics, yogurt, is a significant source of vitamin A, B-complex, zinc and high biologic-quality protein (23) and is therefore an excellent food-based intervention to improve micronutrient intake among people living with HIV/AIDS. Based on these notions, a community kitchen in Mwanza,

Tanzania, was established in 2004 to produce yogurt supplemented with *Lactobacillus rhamnosus* GR-1 (Fiti) to be distributed as an adjunct to the diet of people living with HIV. In order to evaluate the impact of probiotic yogurt supplementation on immune function (CD4 count), the following observational study was undertaken.

Materials and methods

Study design

An observational retrospective study was designed with the collection of CD4 count measurements from three years prior to the date of interview. This allowed longitudinal comparison of the development in CD4 count within subjects (before and during probiotic yogurt consumption) and between probiotic yogurt consumers and non-consumers. Participants were informed about the study and gave their written or thumb printed consent prior to participation, and all procedures were performed according to the Helsinki Declaration as revised in 2000. The study was performed during April and May 2008.

Setting

Both probiotic yogurt consumers and non-consumers were recruited from three probiotic yogurt distribution sites in the urban area of Mwanza city (Mabatini, Mahina and Bwiru). At the Mabatini site, regular yogurt was produced and supplemented with *Lactobacillus rhamnosus* Fiti which was grown from stock cultures stored and harvested at the laboratory of the National Institute for Medical Research, Mwanza. Yogurt was served in portions of 200 ml containing 10^9 cfu/ml of viable *Lactobacillus rhamnosus* Fiti.

Subjects

The subjects were invited to participate when they were able to show their clinical HIV record with at least one CD4 count measurement. All people consuming yogurt at least once a week were invited to participate while they were visiting one of the three sites to receive their portion of probiotic yogurt. People living with HIV who were not consuming yogurt but were visiting one of the sites for another nutrition intervention (monthly distribution of maize and

beans) were also invited to participate without further standardization of the control group. Local leaders or the hospitals had referred both yogurt consumers and non-consumers to these nutrition programs because of the subject's lack of adequate nutrition.

Data collection

During a structured interview, data was collected pertaining to demographics, socioeconomic status (SES), dietary intake through a 24-hours recall, medication, disease history. Furthermore participants were interviewed about the number of days they experienced febrile symptoms, diarrhea and cough as well as the average length participants were able to work per day. Also they were asked to rate the severity of their gastrointestinal symptoms (stomach pain, stomach gas, nausea, diarrhea) and its impact on daily life.

CD4 count measurements three years prior to the interview were recorded from the clinical file. The CD4 count measurements were determined blinded for probiotic yogurt consumption under the National AIDS Control Program at Sekou-Toure regional hospital and Weil Bugando university hospital in Mwanza using conventional flow-cytometry.

Analyses

Differences between yogurt consumers and non-consumers were detected by comparing characteristics between groups using a Chi-square test while micronutrient data were compared using a T-test. Trends in CD4 count measurements were analyzed, allowing for repeated measurements, by using generalized estimating equation methods with an Identity link, Poisson variance and an exchangeable correlation structure. To adjust for the length of time on ARV medication, a variable was created with the number of days since date of commencement of ARV treatment at the time of each CD4 count. This continuous variable was then used to adjust the overall trend in time of CD4 count (number of days since first recording in clinical file) the effect of yogurt consumption (number of days since start of consumption of yogurt), length of time living with HIV (number of days since HIV diagnosis) and length of time using ARV treatment (number of days since initiation of ARV treatment).

We were interested in the effect of short tem probiotic yogurt consumption (70 days) since a randomized controlled trial (Hummelen *et al.* submitted) had indicated different effects before and after this period. To allow for this we created a spline variable. Wald's test with robust standard errors was used to compare differences between groups. All tests were performed two-sided at the $\alpha = 0.05$ significance level with no adjustments made for multiple comparisons. Data was stored in a Microsoft Access database and analysed using SPSS version 15.0.

Results

A total of 68 yogurt consumers and 82 non-consuming controls participated in the study. Demographics, SES and years living with HIV were similar between both groups (Table 1). Furthermore the proportion of consumers being treated with ARV's (52 of 68, 76%), and non-consumers (57 of 82, 70%) was similar (p=0.3). Among yogurt consumers, 196 CD4 counts were recorded before yogurt consumption and 97 measurements were recorded during yogurt consumption. Yogurt was consumed for a median [range] of 357 [3 – 1062] days. Among yogurt consumers 62 of 68 (91%) subjects had at least one CD4 count before commencing yogurt intake. In the group of 82 subjects who did not consume the yogurt, a total of 233 CD4 counts were recorded. The probiotic yogurt group had an average higher daily intake of calories (2097 vs. 1887 kcal, p= 0.01), protein (55 vs. 49 g, p= 0.03), vitamin B_1 (1.7 vs. 1.5 mg, p= 0.002), vitamin B_2 (0.9 vs. 0.7mg, p= 0.03), calcium (490 vs. 421 mg, p= 0.04) and iron (18 vs. 15 mg, p= 0.001) the day before the interview. No differences in daily intake of vitamin B12, C, D, E, selenium and zinc were detected.

Table 1. Characteristics of probiotic yogurt- and control group

Characteristics		Control % (n)	Yogurt % (n)	p
Gender	Female	85% (70)	81% (55)	0.5
	Male	15% (12)	19% (13)	
Marital status	Widow/Divorced	66% (54)	54% (37)	0.1
	Single	13% (11)	9% (6)	
	Married	21% (17)	37% (25)	
Water source	Pipe	68% (56)	56% (38)	0.1
	Well	32% (26)	44% (30)	
BMI	<18.5	9% (7)	10% (7)	0.7
	18.5 +	92% (75)	90% (61)	
Age (years)*	<18	2% (2)	5% (3)	0.5
	18-45	73% (60)	64% (43)	
	45+	24% (20)	31% (21)	
Education*	No schooling	21% (17)	9% (6)	0.1
	Grade 1-7	69% (56)	79% (52)	
	Form 1-4 or higher	10% (8)	12% (8)	
Living with HIV*	< 1 year	24% (20)	13% (9)	0.1
	1+ year	76% (62)	87% (58)	
Antiretroviral medication	No	31% (25)	24% (16)	0.3
	Yes	70% (57)	77% (52)	

* Numbers don't add up due to missing values
BMI = Body Mass Index

Within consumer differences

Before starting consumption, the 68 yogurt consumers experienced an average increase in CD4 count of 0.16 cells/µl/day [95% CI; 0.06 - 0.27, p= 0.002]. This increased an additional 0.23 cells/µl/day [95% CI; 0.02 - 0.44, p= 0.04] after the start of yogurt consumption. After adjusting for ARV use which was associated with an increase of 0.30 cells/µl/day [95% CI; 0.07 - 0.16, p= <0.001], the increase observed before the start of yogurt consumption was fully explained, as no significant increase remained after adjusting (p= 0.6). Furthermore, commencing yogurt consumption also was not associated with an increase in CD4 count (p = 0.3) after adjusting for ARV use. However, without the development of CD4 count over time as a variable, commencing yogurt consumption was again associated with an increase in CD4 count of 0.16 cells/µl/day [95% CI; 0.0 -

0.31, p= 0.05]. This indicates significant co-linearity between the variables threatening their individual contribution; hence a control group was included that did not consume yogurt at any point in time.

Within consumer and between group differences

Taking into account the correlated nature of the repeated CD4 count measures within individuals, no differences were noted of CD4 count levels before the start of yogurt consumption between the yogurt consumers (mean 376 cells/µl) and non-consumers (mean 366 cells/µl, p = 0.8). Among the yogurt consumers before use and the non-consumers an average increase in CD4 count of 0.13 cells/µl/day [95% CI; 0.07 - 0.20, p= <0.001] was seen. After commencing consumption, yogurt consumers experienced an additional increase of 0.28 cells/µl/day [95% CI; 0.10-0.46, p= 0.003]. When adjusting for length of time using ARV medication, the additional increase explained by yogurt consumption remained 0.17 cells/µl/day [95% CI; 0.01-0.34, p= 0.04] and the increase seen before yogurt consumption and among the control group was fully explained by ARV treatment, as no significant increase remained after adjusting (p= 0.6) (Figure 1). Treatment with ARV medication was associated with an increase of 0.27 cells/µl/day [95% CI; 0.17-0.38, p= < 0.001]. Time living with HIV was not associated with CD4 count development (p = 0.3) nor was the medical centre at which treatment was received (p = 0.6). This indicates that no differences in treatment existed between the centers that significantly affected CD4 count development. When allowing for a different CD4 count development before and after 70 days, we found that before the start of consumption and among controls, an increase of 0.13 CD4 cells/µl/day [95% CI; 0.06-0.20, p= <0.001] occurred. Among consumers, after commencing consumption an additional increase of 0.73 CD4 cells/µl/day [95% CI; -0.02-1.50, p= 0.6] during the first 70 days was seen. After this initial period the CD4 count continued to rise an additional 0.2 CD4 cells/µl/day [95% CI; 0.03-0.4, p= 0.02]. Using a Log link with a Poisson distribution provided similar inferences as well as using a generalized linear model.
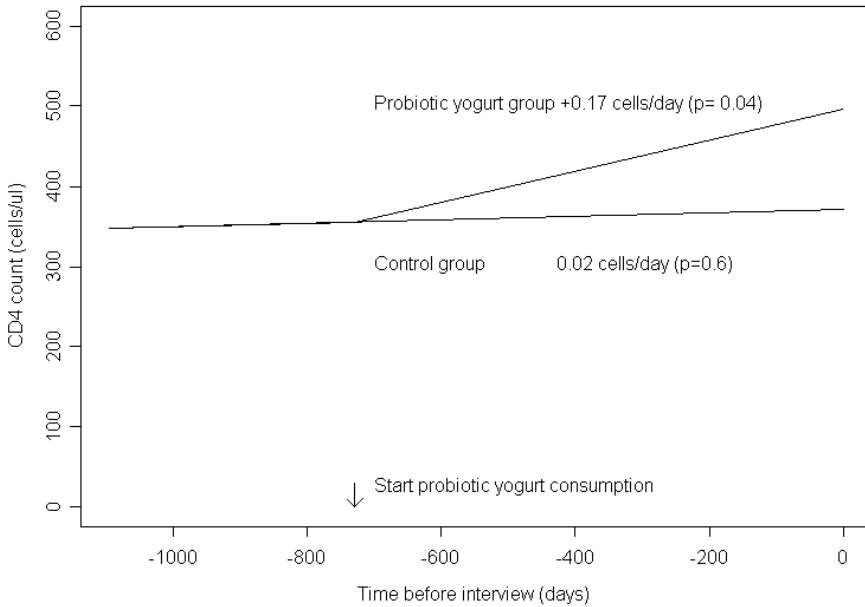
Figure 1. Increased CD4 count after commencing consumption of probiotic yogurt. Additional increase of 0.17 CD4 cells/μl/day [95% CI; 0.01-0.34, p= 0.04] after start probiotic yogurt consumption when adjusting for anti-retroviral treatment.

General health questionnaire

All participants, except one consumer, completed the interview. Probiotic yogurt consumers reported the ability to work 8 hours a day, compared to 6 hours reported by non-consumers (p= 0.01). Furthermore, consumers reported 1 day of fever a month compared to non-consumers who reported a median of 2 days a month (p= 0.01). Regarding gastrointestinal symptoms, among those consuming yogurt, 56 of 67 (84%) participants did not report any diarrheal symptoms; nor did 57 of 82 (69%) participants among the non-consuming group (p= 0.05). Also, 45 of 67 (52%) consumers did not reported any impact (intermediate or severe) of gastrointestinal symptoms on everyday life compared to 24 of 82 (39%) among the non-consumers (p= 0.004).

Discussion

The study site is typical of many low-income communities in the developing world where access to daily nutrition and medical care is limited, and HIV infection is highly prevalent (24). The introduction of a locally produced highly nutritious food (yogurt) supplemented with a probiotic strain, not only provides an economic stimulus for farmers and those producing the yogurt, but may also improve the immune function among those living with HIV and their ability to work.

The present study is, to our knowledge, the first to report the long-term effects of probiotic yogurt on CD4 count among people living with HIV/AIDS. The more pronounced increase of CD4 count during the first 70 days of consumption corresponds to another study at this site, in which intake of *Lactobacillius rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14 in capsule form increased CD4 count at 10 weeks moreso than at 25 weeks (Hummelen *et al.* submitted). The mechanism by which ingested lactobacilli can cause a boost in peripheral CD4 count is worthy of investigating as well as determining if the increase in CD4 count can be further stimulated by switching the type of lactobacilli used.

An important strength of this study is the long period (3 years) in which we assessed the development of the immune function, an asset that would have been difficult to achieve using a prospective study design. Also the observational nature of the study is a strength as we were able to assess the impact of probiotic yogurt at a grassroots level among a wide range of patients, as opposed to the perfect conditions created for a homogenous group of participants to access and consume an intervention in a clinical trial setting.

Although selection bias may have occurred due to the unstandardized sampling of the groups, the similarities between the groups indicates that this bias was limited. We were able to adjust for potential confounders such as length of time living with HIV and antiretroviral medication use, but not for co-trimoxazole use. When using the subject as his/ her own control, a significant increase in CD4 count was also found which validates the differences seen when including the control group in the analyses. Because the intervention in this study both included a probiotic strain and a nutritious carrier (micronutrients and high quality protein) we were limited in distinguishing the individual contribution of each component.

However, two randomized controlled trials have compared regular yogurt to a yogurt combined with probiotic strains and showed a significantly beneficial impact on the CD4 count when the yogurt was supplemented with additional probiotic strains (21, 22). Therefore, it is likely that the probiotic component of this intervention exerted beneficial affects on the immune system independently from its nutrient carrier.

Studies conducted in Africa have estimated the average annual increase in CD4 count of 90 cells/µl with ARV treatment (25) and an average decline of 20-50 cells/µl/year without ARV treatment (26, 27). In this study a similar rate of increase was observed of 99 cells/µl with ARV treatment (0.27 cells/ul/day), while no significant decrease was observed without ARV treatment. The results of this study indicate that probiotic yogurt consumption is associated with an overall increase in CD4 count of 62 cells/µl/year (0.17 cells/ ul/day). This could be due to an accelerated immune reconstitution after initiation of ARV treatment, thus shortening the time of severe immune deficiency, or, may be due to an increase in CD4 count among those not yet eligible for ARV treatment, which may potentially delay the need for ARV medication. This hypothesis will require future assessment as our sample size did not allow for this subgroup analyses.

The differences in symptoms experienced between the groups suggest that probiotic yogurt might alleviate gastrointestinal symptoms among people living with HIV. However, due to the subjective nature of a questionnaire these results need to be confirmed in a blinded setting. In conclusion; probiotic yogurt was significantly associated with beneficial long term effects on the CD4 count and may provide an effective intervention to delay the deterioration of the immune system or potentiate ARV treatment among those living with HIV.

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5.

Effect of 25 weeks probiotic supplementation on immune function of anti-retroviral naïve HIV patients

Submitted

Ruben Hummelen

John Changalucha

Nicodemus L Butamanya

Tara E Koyama

Adrian Cook

J Dik F Habbema

Gregor Reid

Abstract

Aim: To evaluate the safety and impact on the immune function of long term probiotic use among women with HIV.

Methods: A pilot study was performed with a double blind, placebo controlled trial design among 65 women with HIV who were naïve and non-eligible to anti-retroviral treatment. Women were randomized to receive oral capsules containing *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14 ($2x10^9$ colony forming units) or placebo twice daily for 25 weeks. CD4 count and biochemical parameters (Albumin, Alanine transaminase and creatinine) were measured and adverse events assessed at baseline, 10 and 25 weeks. The immune markers IgG, IgE, IFN-γ and IL-10 were measured at baseline and 10 weeks follow-up and the occurrence of diarrhea was reported daily.

Results: Ten participants were excluded post-randomization due to non-eligibility for inclusion when their status was re-assessed. Thirty participants were assigned placebo of whom 25 completed the study. Twenty-five participants were assigned probiotics of whom 19 completed the study ($p = 0.5$). Two participants did not return for any follow-up visit and were therefore not included in the analyses. From baseline to 10 weeks follow-up, the CD4 count declined on average with −3 CD4 cells/ɥl [95% Confidence Interval: -97; 91] in the placebo group versus an increase of 50 cells/ɥl [95% CI: -61; 162] in the probiotics group ($p = 0.5$). From baseline to 25 weeks, the CD4 count increased in the placebo group with 19 cells/ɥl [95% CI: - 90; 129] versus an average increase of 46 cells/ɥl [95% CI: - 100; 192] in the probiotics group ($p = 0.8$). The occurrence of diarrhea was low in both groups and no differences between the groups could be observed. Among the placebo group 10 of 29 participants reported at least one adverse event, versus 5 of 24 reporting an adverse event in the probiotics group ($p = 0.3$). The biochemical measures remained stable during follow-up and no differences were observed between groups.

Conclusion: *Lactobacillus* GR-1 and RC-14 may be safely consumed at $2x10^9$ CFU/day by moderately immune compromised HIV patients but this did not universally preserve immune-function.

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 lympoid 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 sub-group 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 does not have access to this therapy [13]. Moreover, ART is 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.

Materials and methods

Participants and procedures

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 [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 400mg 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 (2×10^9 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 IFN-γ, 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.

Results

A total of 229 women were screened of whom 65 were randomized to receive probiotics (n = 32) or placebo (n = 33) for 25 weeks (Figure 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, figure 2) [27].
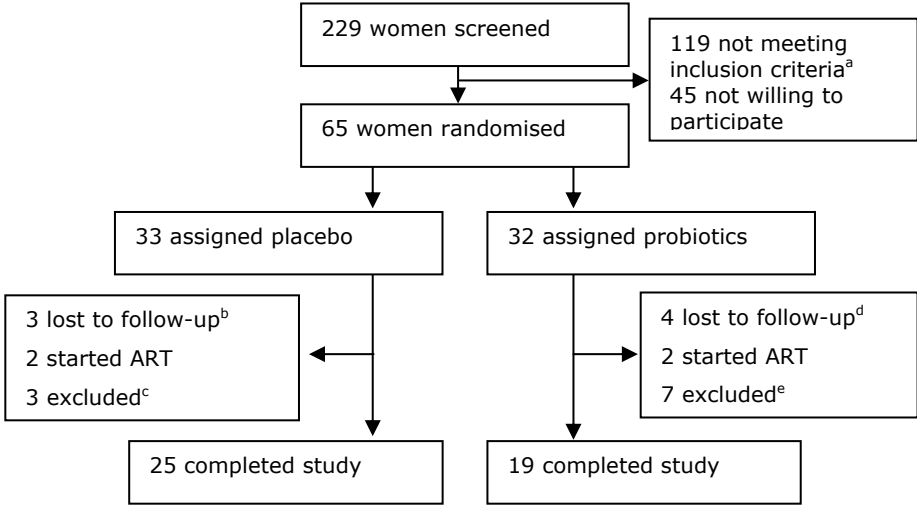

Figure 1. Trial profile

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) % (n)	Probiotic (n = 24) % (n)	p
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/цl)	< 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/m^2)[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)	

SD=Standard Deviation, 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.

From baseline to 10 weeks follow-up the CD4 count dropped on average with −3 CD4 cells/цl [95% CI: -97; 91] in the placebo group versus an increase of 50 cells/цl [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/цl [95% CI: - 90; 129] versus an average increase of 46 cells/цl [95% CI: -100; 192] in the probiotics group (*p* = 0.8). Changes in the immune parameters IFN-γ, IL-10, IgG and IgE did not differ between the groups (Figure 2).
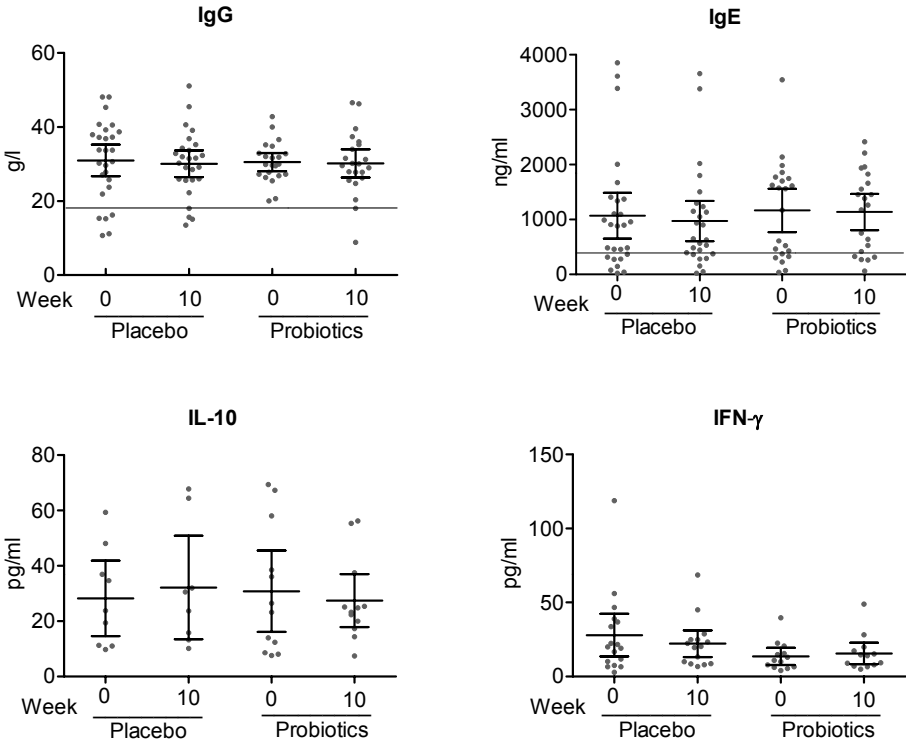
Figure 2. Immune parameters at baseline and 10 weeks follow-up. The horizontal lines in the IgG and IgE graphs indicate the upper reference values [28]. Individual values are shown as dots with the short horizontal line representing the mean and the 95% confidence intervals displayed as the error-bars.

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 odour 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).

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

Measure	Reference Value/unit	Placebo			Probiotics			
		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

[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 with *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 daily ingestion of 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 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. Of note, within our population, those who were moderately immune deficient at baseline (CD4 count 200 – 350 cells/ɥl) experienced an increase of 34 cells/ɥl [95% CI: -37; 105] in the placebo group versus a mean increase of 158 cells/ɥl [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/ɥl [95% CI: 26; 159] while the two placebo participants experienced a mean decrease of –69 cells/ɥl [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/ɥl) [28].

The immune markers IgE, IgG, IFN-γ and IL-10 did not show a significant impact of probiotic supplementation. Markers that are more specific for the intestinal barrier including plasma lipopolysaccharide (LPS), 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 colonisation (IgE), though this effect is likely to be limited as previous studies show levels do not significantly increase among HIV patients harbouring 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 either 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, 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.

Acknowledgements

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6.

Deep sequencing of the vaginal microbiota of women with HIV

Ruben Hummelen

Andrew D Fernandes

Jean M Macklaim

Russell J Dickson

John Changalucha

Gregory B Gloor

Gregor Reid

Abstract

Background: Women living with HIV and co-infected with bacterial vaginosis (BV) are at higher risk for transmitting HIV to a partner or newborn. It is poorly understood which bacterial communities constitute BV or the normal vaginal microbiota among this population and how the microbiota associated with BV responds to antibiotic treatment.

Methods and Findings: The vaginal microbiota of 132 HIV positive Tanzanian women, including 39 who received metronidazole treatment for BV, were profiled using Illumina to sequence the V6 region of the 16S rRNA gene. Of note, Gardnerella vaginalis and *Lactobacillus iners* were detected in each sample constituting core members of the vaginal microbiota. Eight major clusters were detected with relatively uniform microbiota compositions. Two clusters dominated by *L. iners* or *L. crispatus* were strongly associated with a normal microbiota. The *L. crispatus* dominated microbiota were associated with low pH, but when *L. crispatus* was not present, a large fraction of *L. iners* was required to predict a low pH. Four clusters were strongly associated with BV, and were dominated by *Prevotella bivia*, *Lachnospiraceae*, or a mixture of different species. Metronidazole treatment reduced the microbial diversity and perturbed the BV-associated microbiota, but rarely resulted in the establishment of a lactobacilli-dominated microbiota.

Conclusions: Illumina based microbial profiling enabled high though-put analyses of microbial samples at a high phylogenetic resolution. The vaginal microbiota among women living with HIV in Sub-Saharan Africa constitutes several profiles associated with a normal microbiota or BV. Recurrence of BV frequently constitutes a different BV-associated profile than before antibiotic treatment.

Introduction

The human vaginal microbiota plays an important role in the maintenance of health of a woman, partner or newborn [1]. Until very recently, the composition and dynamics of these organisms was poorly understood due to limitations of culturing methods.

The advent of PCR based techniques and pyrosequencing has made it possible to further examine this complex microbial niche (reviewed in [2]). Herein, we describe the development and utilization of a novel metagenomic approach based on Illumina sequencing to profile the vaginal microbiota. Illumina has fewer errors than 454 sequencing [3] and therefore we hypothesized that it could provide a higher phylogenetic resolution than 454 based approaches [4]. Furthermore, the advantage of Illumina to provide 30-times more reads would enable us to perform in depth sequencing of hundreds of samples in one run at a fraction of the costs, making it an excellent tool for large microbiome studies. We chose to sequence the V6 variable region of the 16S rRNA gene as it was adequate to resolve the majority of the expected organisms in the vaginal microbiota to the genus level [5] and provided resolution for a number of organisms in our samples to species and in some cases to strain level (Gloor *et al*. submitted). In selecting the population to study, we were struck by the extremely high prevalence of bacterial vaginosis (BV) in women living with HIV in Sub-Saharan Africa [6–9]. This condition is generally characterized by a depletion of lactobacilli and overcolonization by a range of anaerobic bacteria [10]. The apparent absence of lactobacilli and their by-product lactic acid increases vaginal pH, thereby disrupting physiological mechanisms that might inactivate or contain the virus [11–14]. These factors presumably result in an increased risk of transmission to a newborn child or partner associated with BV [15]. Knowledge of the microbiota profiles among women living with HIV is important as different microorganisms may be indicative of distinct risk-profiles in the transmission of HIV and severity of BV. Another consideration for selecting this study population was that BV appears to be more recalcitrant to antibiotic treatment in HIV patients and treatment is ineffective in reducing HIVshedding from the vaginal tract [6,16]. Assessing the response of the BV-associated microbiota to antibiotic

treatment may yield further insight in factors contributing to the failure of treatment. Therefore we sought to define (1) the microbial communities of women living with HIV, (2) their association with the vaginal pH and (3) their relation with the Amsel criteria and Nugent scoring system commonly used to diagnose BV [17,18].

Results

A total of 272 samples were sequenced using a single lane of a paired-end Illumina run resulting in >18 million reads of 76 nucleotides. Of these >12 million bar-coded V6 16S rRNA sequences were identified that completely covered the V6 region and that had intact barcodes and primer sequences (see Gloor *et al*, submitted, for a complete description of the workflow used to extract these sequences). Individual samples were covered with a median of 40,931 reads (range 7,018–129,054). The Chao1 measure of biodiversity estimation was the most conservative estimate and predicted that the reads covered >90% of the expected diversity in 235 of 272 samples (Table 1). After clustering in operational taxonomic units (OTUs) based on a >95% sequence similarity cut-off, which permitted up to 3 substitutions per V6 sequence, a total of 60 OTUs representing phylogenetic groups were identified (Tables S1, S2, Figures S1, S2). Of those, 30 were assigned to specific species based on sequence identity to a well-annotated organism and 18 to a genus. Strikingly, within some OTUs distinct strains could be separated. For example, within OTU 1 the sequences could be annotated as *Gardnerella vaginalis* 409-05, *G. vaginalis* NML060420, and as two unique uncultured *G. vaginalis* sequences. All four of these sequences differed from each other by a single diagnostic nucleotide.

Table 1. Coverage estimates

Method	N>0.9	N>0.95
Rarefaction	272	255
Ace	241	169
Chao1	235	181

The number of samples in which >90% and 95% of the expected diversity was sampled using rarefaction, Ace and Chao 1 estimates [34, 35]. Analyses were performed using the VEGAN package for biodiversity analyses [36].

L. iners and G. vaginalis constitute core members of the vaginal microbiota

Figure 1 shows the composition of the microbiota clustered by the similarity of the relative abundance of the organisms. We found that 207 of the 272 samples could be grouped into 8 major clusters with relatively uniform microbiota compositions. Across all clusters, *G. vaginalis* and *Lactobacillus iners* were detected in each sample, constituting core members of the microbiota. Two major clusters were associated with a normal vaginal microbiota, one dominated by *L. iners* and one dominated by *L. crispatus*. Four clusters were strongly associated with BV, and were dominated by *Prevotella bivia* or *Lachnospiraceae*, or comprised a mixture of different species. The remaining clusters were notable in that they contained normal, intermediate and BV samples and were composed largely of *G. vaginalis* and *L. iners* and a mixture of other species.
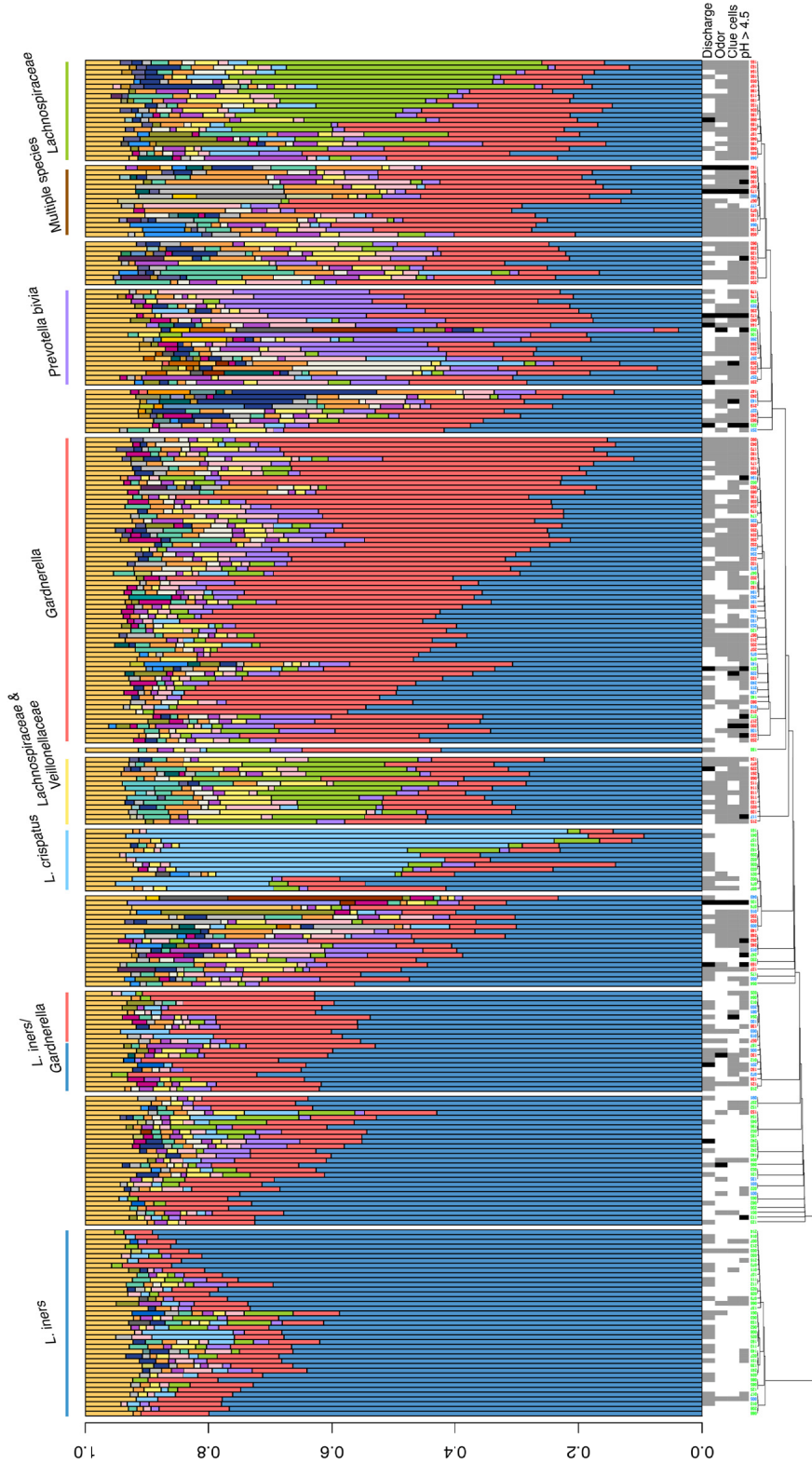
Figure 1. Relative abundance of taxa across samples. The composition of the microbiota was visualized by bar-plots. Samples were clustered by the similarity of the relative organism abundance and their similarity was visualized using a neighbor-joining tree. The major clusters (>10 samples) are named after a taxa with a relative even abundance across samples in the cluster. The sample numbers are coloured according to Nugent categories with BV = red, intermediate = green, normal = blue. Amsel criteria are shown for each sample with present = grey, absent = white and missing data = black. The complete legend is given in figure S2.

- Rare OTUs
- Corynebacterium ammoniagenes, OTU 102
- Uncultured clone (97%), OTU 76
- Uncultured Porphyromonas, OTU 70
- Novel, OTU 68
- Haemophilus, OTU 65
- Bifidobacterium bifidum, OTU 64
- Campylobacter, OTU 63
- Bifidobacterium saeculare (97%), OTU 61
- Gammaproteobacteria, OTU 60
- Mycoplasma pirum (97%), OTU 59
- Uncultured Methylocaldum tepidum, OTU 57
- Fusobacterium nucleatum, OTU 56
- Uncultured Lachnospiraceae (85%), OTU 52
- Peptoniphilus lacrimalis, OTU 50
- Uncultured clone (88%), OTU 48
- Uncultured clone, OTU 47
- Gardnerella vaginalis (97%), OTU 46
- Prevotella, OTU 45
- Mobiluncus curtisii, OTU 44
- Uncultured Sneathia (92%), OTU 42
- Uncultured Fusobacterium (98%), OTU 41
- Arcanobacterium (94%), OTU 39
- Uncultured Prevotella (95%), OTU 38
- Uncultured TM7 division, OTU 37
- Actinomyces turicensis, OTU 36
- Enterobacteriales, OTU 35
- Gemella, OTU 34
- Pasteurellaceae, OTU 33
- Uncultured Prevotella (98%), OTU 32
- Lactobacillus jensenii, OTU 31
- Enterobacteriaceae, OTU 30
- Anaerococcus tetradius, OTU 29
- Lactobacillus, OTU 28
- Gemella, OTU 27
- Porphyromonas, OTU 26
- Clostridiales, OTU 25
- Actinomycetaceae, OTU 24
- Clostridiales BVAB-3, OTU 23
- Peptostreptococcus anaerobius, OTU 22
- Streptococcus, OTU 21
- Streptococcus, OTU 20
- Streptococcus, OTU 19
- Prevotella melaninogenica, OTU 18
- Uncultured Prevotella (90%), OTU 17
- Dialister micraerophilus, OTU 16
- Atopobium rimae, OTU 15
- Veillonella montpellierensis, OTU 14
- Lactobacillaceae, OTU 13
- Dialister propionicifaciens, OTU 12
- Uncultured Peptococcaceae (87%), OTU 11
- Prevotella amniotica, OTU 10
- Prevotella timonensis, OTU 9
- Atopobium vaginae, OTU 8
- Sneathia, OTU 7
- Uncultured Veillonellaceae, OTU 6
- Leptotrichia amnionii, OTU 5
- Lactobacillus crispatus, OTU 4
- Uncultured Lachnospiraceae, OTU 3
- Prevotella bivia, OTU 2
- Gardnerella vaginalis, OTU 1
- Lactobacillus iners, OTU 0

The vaginal microbiota and pH

Figure 1 shows that all 13 samples within the *L. crispatus* cluster, but only 29 of 39 samples in the *L. iners* cluster had a pH ≥4.5 (Table 2). We used two independent methods to examine the strength of this association. Table 2 shows that the proportion of *L. iners* alone was only weakly associated with the vaginal pH. The strength of this association increased when the proportion of *L. crispatus* was added to the model. Figure 2A shows that *L. crispatus* is associated with a low pH, but if *L. crispatus* is not present, a large fraction of *L. iners* is required to predict a low pH. However, lack of both *L. iners* and *L. crispatus* is strongly predictive of high pH.

Table 2. Correlation Amsel and Nugent score with microbiota clusters.

Organisms	n	*p*H		Clue cell		Odor		Nugent score		
		≤4.5	>4.5	n	y	n	y	0-3	4-6	7-10
L. iners	39	29	10	33	6	35	4	38	1	0
L. iners & G. vaginalis	21	9	12	11	9	16	4	7	8	6
L. crispatus	13	13	0	11	2	10	3	13	0	0
Lachnospiriceae & Veillonellaceae	14	1	12	4	10	1	13	0	1	13
G. vaginalis	64	9	50	16	46	35	29	9	19	36
Prevotella bivia	20	0	18	4	14	7	11	3	4	13
Multiple species	15	2	10	2	11	3	10	0	3	12
Lachnospiraceae	21	0	21	0	21	5	16	0	1	20

This table summarizes the number of samples for each cluster (corresponding to Figure 1) with a specific Nugent or Amsel diagnostic characteristic. Categories do not always count up due to missing data (Figure 1).

The vaginal microbiota and the Amsel criteria

Table 3 shows that the various components of the Amsel criteria vary in their strength of association with the vaginal microbiota. In particular, the presence of a milk-like discharge did not appear to have any correlation with the resident microbiota and was a

confounding factor within the set of four Amsel criteria (Tables 3,S3). The presence of odor was moderately associated with the vaginal microbiota. Of note, two *Lachnospiriceae clusters*, and the multiple species cluster appeared to be most strongly associated with the presence of an amine odor (Figure 1 and Table 2). Figure 2B show that *Atopobium vaginae* and *Veillonellaceae* were most clearly associated with odor across clusters.

Table 3. Correlation proportion of lactobacilli with Amsel criteria and Nugent score.

Variable	Proportion	Pairs	R[1]	p	Strength[2]
pH	*L. iners*	254	-0,2469	0,0000	83
pH	*L. iners* & *L. crispatus*	254	-0,2311	0,0000	35
Nugent score	*L. iners* & *L. crispatus*	272	-0,2423	0,0000	34
Amsel score	*L. iners* & *L, crispatus*	242	-0,1531	0,0012	31
Odor	*L. iners* & *L. crispatus*	265	-0,1473	0,0034	231
Clue cells	*L. iners* & *L. crispatus*	262	-0,1927	0,0001	55
Discharge	*L. iners* & *L. crispatus*	259	0,0514	0,3131	418

[1] Kendall rank correlation coefficient and p-value.
[2] Strength of the association is indicated by estimating the number of subjects that would be required to show the correlation between the variables with ≥ 95% confidence.
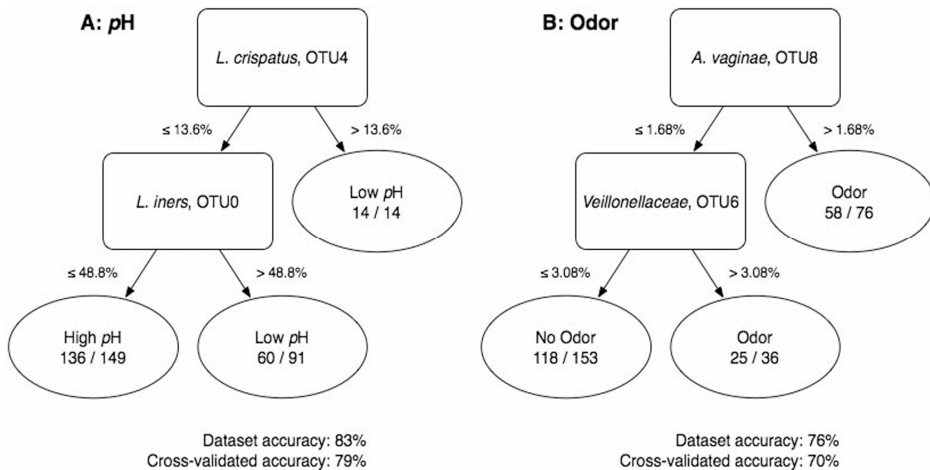
Figure 2. Association of vaginal pH and odor with members of the vaginal microbiota. The flow chart explaining the relationship between microbiota and diagnostic criteria were generated using data mining techniques (Materials and Methods). To obtain the prediction about a clinical sample, follow the arrows like a flow chart from the organisms (rectangular boxes) to the prediction (ovals). Each decision in the tree is split on the proportion of the organism above; thus, if a clinical sample has proportions less than 13.6% *L. crispatus* and less than 48.8% of *L. iners* it is predicted that the sample is taken from a vaginal environment with a pH≥4.5. The accuracy of the decision tree is reported for the entire dataset and for 10- folds cross validation, which is an attempt to estimate the accuracy of the decision tree on new clinical samples.

Impact of Metronidazole treatment

Of the 132 women enrolled at baseline, 47 had a normal microbiota, 22 intermediate and 67 had BV as defined by the Nugent score [17]. The microbiota of BV patients were composed a larger diversity of taxa than normal subjects (Figure 3). All subjects with BV were treated with metronidazole and 39 subsequently returned for follow-up visits. After 2 weeks, 57% of these patients still had BV. Treatment with metronidazole reduced the diversity at two weeks but the diversity increased slightly at later follow-up time-points indicating a tendency to return to a more diverse microbiota (Figure 3). Metronidazole frequently caused a shift from one BV-associated microbiota profile to another, but rarely resulted in a lactobacilli-dominated microbiota (Figure 4). *L. iners* was the only organism with an increase in relative abundance (from a median of 25% to 46%) after metronidazole intervention (p =,0.0001, Wilcoxon rank test).

The effect of metronidazole on two patients is presented in Figure 5, to illustrate the dynamics of microbiota changes with antibiotic intervention.
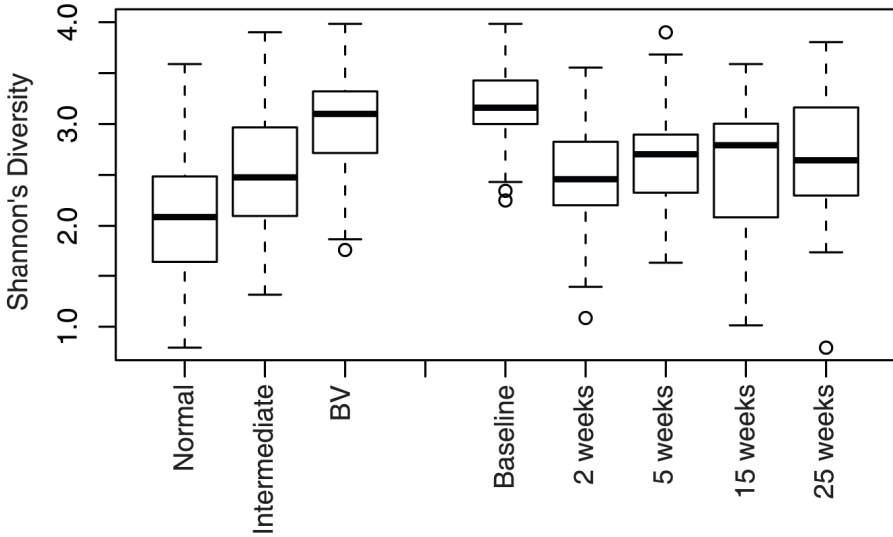

Figure 3. Diversity of the vaginal microbiota and the impact of antibiotics. The species diversity was measured by calculating Shannon's Diversity index [31]. The data was partitioned either by Nugent category [17], or by treatment time-point. Metronidazole treatment (week 0) reduced the diversity at two weeks but a slightly higher diversity was observed at later follow-up time-points indicating a return to a more diverse microbiota.
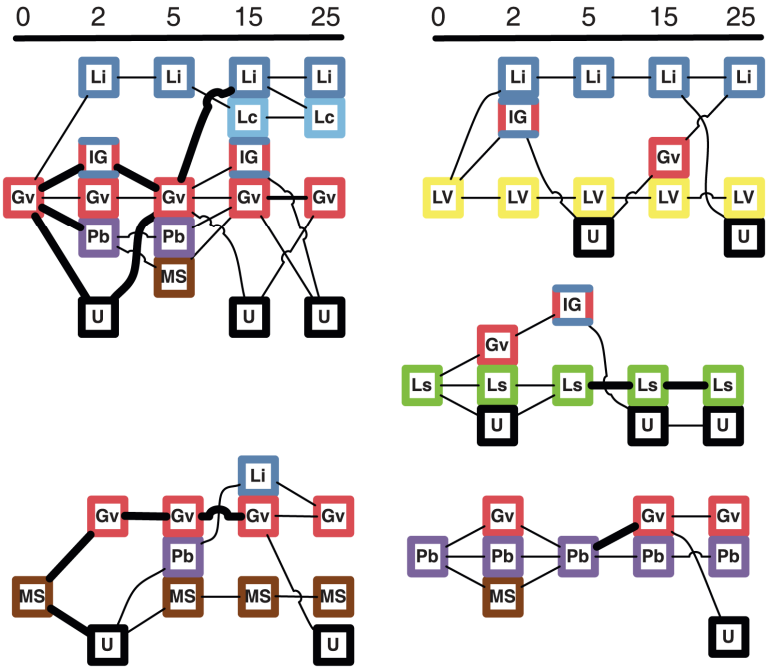
Figure 4. Changes in microbiota profile after antibiotic intervention. The network depicts the profile of the vaginal microbiota before (time = 0) and after metronidazole intervention. A thin line represents one subject while a thick line represents two. Only women were included with a complete follow-up and when a microbiota was present similar to the major profiles at baseline (Figure 1). Gv indicates G. vaginalis, MS = Multiple species, LV = *Lachnospiraceae* & *Veillonellaceae*, Ls= *Lachnospiraceae*, Pb= *P. bivia* and U = minor clusters.
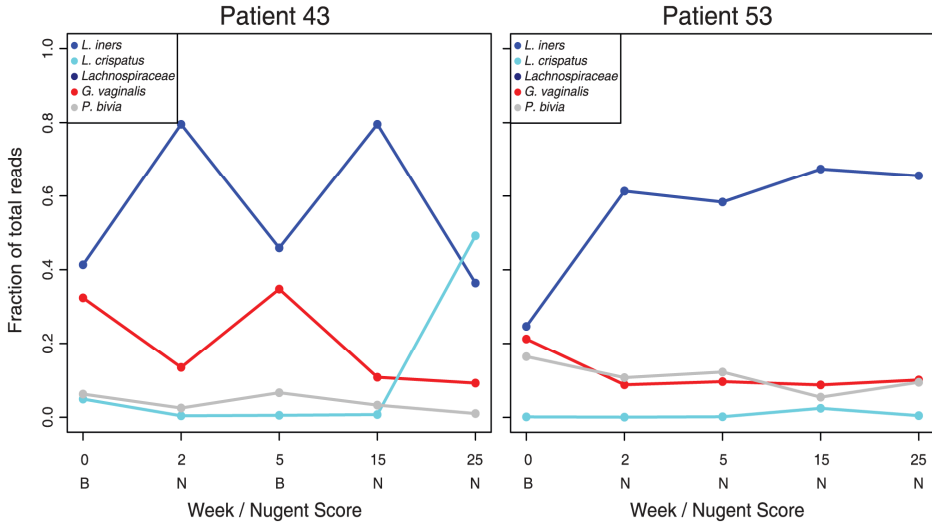
Figure 5. Dynamics of the vaginal microbiota after antibiotic intervention. The line graphs depict the changes in relative abundance for a select number of species after metronidazole intervention (week 0). After two weeks both patients are cleared of BV along with an increase in the relative abundance of *L. iners* at two weeks. However patient 43 experiences a recurrence and receives another course of metronidazole at 5 weeks. Of note, after an *L. crispatus* profile is established, BV does not recur in this subject.

Discussion

To our knowledge, we report the first successful attempt to nearly completely sample the diversity of the vaginal microbiota. Compared to 454 sequencing, the Illumina method provided 30-fold more reads with a 10-fold reduction in costs, enabling hundreds of samples to be processed in one run. This method could be adapted to sequence the V3 or V5 region suitable for uncovering the intestinal microbiota [5]. The reduced costs of microbial profiling ($10/sample) might enable the use of this technology in a diagnostic setting and allow large studies to be carried our on the human microbiota.

Based on similarity of the relative abundance, we have demonstrated in an HIV population of African women, that there are several microbiota profiles associated with BV, and two with a normal, healthy status. For BV cases, there was a dominant abundance of *P. bivia* or members of the order *Clostridiales* and family *Lachnospiraceae*. Interestingly, in a culture based study of African women, *P. bivia* was also found to be the dominant organism

[7], unlike Caucasian and black women from North America, in whom *Atopobium vaginae* and genera of the order *Clostridiales*, such as *Megasphaera sp.*, were dominant [10,19].

A recent 454 pyrosequencing study of a healthy cohort of American women including African Americans, showed the *Prevotella genus* to be the most abundant in one of five clusters [20]. *Prevotella bivia* is a well known pathogen that has been reported to invade epithelial cells, cause inflammatory responses, endometritis, pelvic inflammatory disease, and peri-rectal abscesses [21]. Its presence in the vagina of HIV patients warrants efforts to treat and eradicate it; however, this was not achieved by the use of metronidazole in the present study.

The common finding of *Clostridiales* organisms, presumably originating from the gut, indicates an ability to adapt easily to the vaginal environment. The two clusters in which *Lachnospiraceae* were heavily represented, were associated with the presence of an amine odor. Although many cases of BV can be asymptomatic, odor is a major reason for women deciding to seek treatment. It is unpleasant and adversely affects quality of life [22]. The association of *Clostridialis* organisms and *A. vaginae* with odor warrants the evaluation of interventions specifically directed against these organisms.

The presence of *L. crispatus* or a high abundance of *L. iners* was clearly associated with a healthy (≥pH 4.5). The fact that *L. iners* was detected in all the vaginal samples, along with *G. vaginalis*, indicates they have some core functions or at least a well developed means to persist in the fluctuating environment of the vagina. It would be worthwhile examining a larger sample pool of women colonized by *L. crispatus*, to verify what appears to be an important role in lowering the vaginal pH. The ability of *L. crispatus*, unlike *L. iners*, to produce hydrogen peroxide may also be a factor in maintenance of health [23]. The low prevalence of *L. crispatus* and the virtual absence of *L. jensenii* relative to non-African populations [10,19,23–25] might in part explain the high occurrence and low cure rates of BV among this cohort of Tanzanian women with HIV.

Treatment with metronidazole was associated with a shift in microbial profiles without necessarily a recurrence of the same profile, and poor efficacy at establishing a lactobacilli-dominant microbiota. The treatment caused a shift in abundance of *L. iners*, but

did not induce a recovery of *L. crispatus*. This is interesting for several reasons. The emergence of BV causes a displacement or killing of *L. crispatus*, while it does not eradicate *L. iners*. The genome of the latter contains exogenously acquired elements that appear to aid persistence and survival (Macklaim *et al*. manuscript submitted), but how they achieve this when confronted by biofilms of BV pathogens, remains to be determined. If such mechanisms are identified, it may be possible to try and up-regulate them in probiotic strains such as *L. crispatus* CTV05, *L. rhamnosus* GR-1 or *L. reuteri* RC-14. By surviving BV and antibiotic treatment, *L. iners* may be able to proliferate (as shown by its increased abundance following metronidazole treatment) and create an environment conducive to later re-colonization by *L. crispatus* via the rectum and perineum. This remains to be verified.

In summary, we report a novel, inexpensive, highly practical technique for profiling the human microbiota at a high phylogenetic resolution. The study identified 60 OTUs in the vagina of African women with HIV and *L. iners* and *G. vaginalis* were found to be core members of the vaginal microbiota. Four bacterial clusters were associated with BV, and two clusters with a normal vaginal microbiota. *L. crispatus* was more strongly associated with a low vaginal pH than *L. iners*, suggesting a different ecological function. Treatment failed to eradicate BV and only transiently reduced the diversity of the BV-associated microbiota.

Materials and Methods

Clinical study and sample collection

Women visiting the HIV clinic of Sekou-Toure Regional Hospital in Mwanza, Tanzania, were invited to participate in the study. Subjects were included when: their HIV infected status was confirmed, they had not yet initiated antiretroviral treatment, and they were between 18 and 45 years old. Participants were not included when pregnant, lactating, menstruating at time of screening, hypersensitive to metronidazole, had *Trichomoniasis* detected by saline microscopy, had budding yeast indicating *Candida* colonisation seen on Gram stain or had cervical inflammation noted during gynaecologic examination. The medical ethical review committee of Erasmus University Medical

Centre, The Netherlands, and the medical research coordinating committee of the National Institute for Medical Research, Tanzania, approved the study design and protocol. Subjects were informed of the purpose of the study and gave their signed informed consent before participation. The study was registered at clinical trials.gov NCT00536848.

A total of 132 women were screened of whom a sub-group of women (n =39) diagnosed with BV (Nugent score ≥7) were treated with metronidazole (400mg orally, twice daily for 10 days) (Aventis), and followed at 2, 5, 15 and 25 weeks. If symptoms and signs of BV in addition to a Nugent score of ≥7 were present at any follow-up visit from week 5, subjects were again prescribed metronidazole. At each visit, vaginal samples were collected for Gram staining, pH, saline microscopy and KOH preparation.

Samples for DNA extraction and PCR were obtained by brushing a polyester swab against the mid-vaginal wall after insertion of a non-lubricated speculum. The swabs were placed in a cryovial and stored at 22°C until analysis. BV was diagnosed at each visit by rating a gram stained smear by Nugent scoring [17] and Amsel's criteria [18]. A Nugent score of 1–3 was defined as normal, 4–6 as intermediate and 7–10 as BV. Also, the presence of *trichomoniasis* was diagnosed by saline microscopy and the presence of budding yeast by Gram stain to indicate *Candida* colonisation.

PCR amplification

The primers L-V6 (5'-CAACGCGARGAACCTTACC-3') and R-V6 (5'-ACAACACGAGCTGACGAC-3') were chosen to amplify the V6 hypervariable region of the 16S rRNA gene. The primers were tested in silico using a custom made database including all entries in the RDP with taxonomic information and longer than 1400 nucleotides. Of all those sequences the left primer matched over 96% and the right primer over 98% of the sequences in the dataset. The taxa *Sneathia*, *Leptotrichia*, *Ureaplasma* and *Mycoplasma* could only be amplified if up to 4 mismatches were allowed outside the first 5 nucleotides of the left primer, and therefore we suggest that our data are biased against these taxa. We developed different barcodes of 3–6 nucleotides long, to be attached to the 5' end of the primer that would give a relatively even mixture of the 4 nucleotides at the first several positions. The barcodes were between 3 and 6 nucleotides

long to reduce the likelihood that adjacent spots on the Illumina solid support would be scored as one spot during the sequencing of the amplification primers. For the left primer we developed 18 different barcodes (5'-3' CATGCG, GCAGT, TACGT, GACTGT, CGTCGA, GTCGC, ACGTA, CACTAC, TGAC, AGTA, ATGA, TGCA, ACT, TCG, GTA, CTA, TGA, GCTA) and for the right primer 16 different barcodes (5'-3' CGCATG, ACTGC, AGCTA, ACAGTC, TCGACG, GCGAC, TACGT, GTAGTG, GTCA, TACT, TCAT, TGCA, AGT, CGA, TAC, TAG). A-priori the primers and DNA extraction procedures were tested using the amplification conditions described below on *L. iners*, *L. rhamnosus*, *A. vaginalis* and *G. vaginalis*.

DNA extraction and amplification

Swabs were vigorously agitated in 1 mL of PBS (pH 7.5) to dislodge cells after which the DNA was extracted using InstaGene Matrix (Biorad #732-6030) according to the protocol of the manufacturer. After extraction, 10 µl of the Instagene supernatant was used as template for a 50 µl PCR reaction. The following PCR reaction conditions were used: 5 units Taq platinum: 1.7mM MgCl, 210 mM dNTPs and 640 nM of each primer. A touchdown protocol was employed with: initial denaturation 94^0C for 2 min; denaturation 94^0C; annealing starting at 61^0C and dropping with 1^0C over 10 cycles with the remaining 15 cycles at 51^0C; extension at 72^0C; all for 45 seconds and a final elongation step for 2 min. A negative control including all ingredients but with water instead of DNA template, and a positive control with a lower limit of detectable DNA, were performed alongside all test reactions. PCR-products were used when the negative control was free of PCR product and the positive control amplified. A constant volume aliquot of each amplification product was run on a 1.5% agarose gel to determine the approximate amount of product. The amount of product was scored on a 4 point scale and, based on this scale, between 2 and 40 µl of the PCR products were mixed together to give the final sample sent for Illumina sequencing at The Next-Generation Sequencing Facility in The Centre for Applied Genomics at the Hospital for Sick Children in Toronto. The library was prepared without further size selection. All new sequencing data have been deposited in GenBank under accession numbers: HM585291-HM585350.

Taxonomic classification of sequence reads

The number of reads and the short nature of the reads precluded the use of the data analysis pipelines currently in use for microbial community profiling by the 454 pyrosequencing method. We developed a data analysis pipeline and programs in-house (Gloor *et al* submitted). A shell script plus the Perl and R scripts able to run on OS X to recapitulate the analyses are available from the authors. Sequences present at >1% abundance in any sample were clustered by similarity to a seed sequence at 95% identity using UCLUST [26]. All clustering methods present a trade-off between sensitivity and specificity. The 95% identity cut-off was chosen because it was the best compromise between clustering of sequences derived from PCR-based sequencing errors and true taxonomic relatedness (See figure S1 for full justification of clustering cut-off). The most abundant sequence of each OUT was chosen as seed sequence. Each resultant seed sequence was first compared using mega BLAST against non-redundant bacterial nucleotide sequences in the NCBI database while excluding environmental samples. The best scoring hit was selected to represent the seed sequence if it displayed 100% identity and coverage. If multiple hits fulfilled these criteria, a common higher taxonomic rank was assigned. When no hits were present at >90% identity, uncultured samples were included in the search (Tables S1, S2, Dataset S1). Preference was given in taxonomic assignment to sequences derived from fully sequenced bacterial genomes, then to sequences derived from characterized organisms and finally to environmental isolates.

Association analyses

Kendall's Tau was used to test nonparametric rank association between lactobacilli fractions, the Nugent and Amsel criteria primarily for its simple interpretation [27,28]. The magnitude of association between the interval-scale *Lactobacillus* population fraction and ordinal-scale diagnostic criterion was quantified by a novel technique based on information theory [29]. To ensure invariance to distribution, population fractions were converted to ranks. Sampling variance was preserved by noting that the joint percentile distribution of all variables follows a Dirichlet distribution [30]. The Kullback-Leibler divergence of the uniform density to the empirical density was

used to estimate the expected accuracy for distinguishing populations. To facilitate interpretation, the information divergence was converted to an estimate of the number of patient evaluations required to determine with ≥95% confidence whether or not those patients came from a population where no association was present. The more patients needed, the smaller the association between variables.

Hierarchical clustering among samples

Samples were clustered for similarity of relative abundance by calculating a generalized angle between proportions, as given by their Euclidian inner product [31]. Such a distance is equivalent to minimum Fisher information connecting two points. From a biological perspective, this angle can be loosely interpreted to measure how much a mixture of the two populations would have in common with each of its components. Small angles imply that two samples are virtually identical, while large angle imply thatsamples are maximally distinct.

Diversity estimations

Diversity was calculated using Shannon's Diversity Index which calculates the entropy or variability of the system [32]. It is calculated as follows: $H_s = -\Sigma n_i \, ln \, n_i$ where H is the entropy, and we are summing the species proportions multiplied by the log of those fractions. The diversity index has a minimum value when there is only one species and a maximum when all species are equally and identically distributed.

Decision trees

Decision trees were generated using the j48 implementation of the C4.5 decision tree algorithm from the Weka machine learning workbench [33]. The C4.5 algorithm attempts to create the most effective decision tree to create accurate predictions by using information theory. At each step in the process, the program splits the dataset on an attribute (eg. the relative abundance of an organism) to maximize the information gained about an important predicted variable (eg. the likelihood of a pH >4.5); the split is then entered into the decision tree like an entry in a flowchart and the

process is repeated until no more information can be gained. The decision trees were created using default C4.5 parameters and were pruned to prevent over-fitting the training set. The C4.5 decision trees were validated using 10-folds cross validation.

Acknowledgments

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7.

Microbiome profiling by Illumina sequencing of combinatorial sequence-tagged PCR products

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Gregory B Gloor

Ruben Hummelen

Jean M Macklaim

Russell J Dickson

Andrew D Fernandes

Roderick MacPhee

Gregor Reid

Abstract

We developed a low-cost, high-throughput microbiome profiling method that uses combinatorial sequence tags attached to PCR primers that amplify the rRNA V6 region. Amplified PCR products are sequenced using an Illumina paired-end protocol to generate millions of overlapping reads. Combinatorial sequence tagging can be used to examine hundreds of samples with far fewer primers than is required when sequence tags are incorporated at only a single end. The number of reads generated permitted saturating or near-saturating analysis of samples of the vaginal microbiome. The large number of reads allowed an in-depth analysis of errors, and we found that PCR-induced errors composed the vast majority of nonorganism derived species variants, an observation that has significant implications for sequence clustering of similar highthroughput data. We show that the short reads are sufficient to assign organisms to the genus or species level in most cases. We suggest that this method will be useful for the deep sequencing of any short nucleotide region that is taxonomically informative; these include the V3, V5 regions of the bacterial 16S rRNA genes and the eukaryotic V9 region that is gaining popularity for sampling protist diversity.

Introduction

Microbiome profiling is used to identify and enumerate the organisms in samples from diverse sources such as soil, clinical samples and oceanic environments [1–3]. This profiling is an important first step in determining the important bacterial and protist organisms in a biome and how they interact with and influence their environment.

Microbiome profiling is usually achieved by sequencing PCRamplified variable regions of the bacterial 16S and of the protistan small subunit ribosomal RNA genes [4,5]. Other sequences, such as the GroEL genes may also be targeted for independent validation [6]. The microbial profile of a sample may be determined by traditional Sanger sequencing, by terminal restriction length polymorphism analysis or by denaturing gradient gel electrophoresis (reviewed in [7]). The recent introduction of massively parallel 454 pyrosequencing has resulted in a radical increase in the popularity of microbiome profiling because a large number of PCR amplicons can be sequenced for a few cents per read [4,8]. However, while constituting a tremendous improvement over previous methods, pyrosequencing is constrained by cost limitations and a relatively high per-read error rate. The high error rate has led to some discussion in the literature about the existence and importance of the 'rare microbiome' [9]. New methods for analyzing pyrosequencing output suggest that much of the raremicrobiome is composed of errors introduced by the sequencing method [10].

Until recently, the Illumimna sequencing-by-synthesis method of parallel DNA sequencing was thought to be unsuitable for microbiome profiling because the sequencing reads were too short to traverse any of the 16S rRNA variable regions. This can be partially circumvented by identifying maximally informative sites for specific groups of organisms (eg. [11]). A recent report demonstrated that short sequences derived from Illumina sequences could be used for robust reconstruction of bacterial communities. This group used Illumina sequencing to determine the partial paired-end sequence of the V4 16S rRNA region in a variety of samples using single-end sequence tagged PCR primers [12].

Here we report the methods used to perform microbiome analysis of the V6 region of 272 clinical samples using the Illumina sequencing technology. We used paired-end sequencing in combination with unique sequence tags at the 5' end of each primer. The overlapping paired-end reads gave us complete coverage of the V6 region. The combination of sequence tags at each end of the overlapped reads allowed us to use a small number of primers to uniquely tag a large number of samples. The Illumina sequencing method generated >12 million useable reads at a cost of ~0.03 cents per read, an approximate order of magnitude cheaper than the per-read cost of pyrosequencing. The cheaper per-read costs allows economical experiments on large numbers of samples at very large sequencing depths. Since Illumina sequencing is now capable of ~100 nt long reads from each end of a DNA fragment, the methods described here can be easily adapted for paired-end sequencing of the microbial V3, V5, V6 and the eukaryotic V9 rRNA regions. Similarly to others [12], we found that methods used to analyze pyrosequencing microbiome data were often unsuitable for reads generated by Illumina sequencing and we present a workflow that can be used for rapid and robust generation of the relative abundance of organisms in each sample.

Importantly, we found that the Illumina sequencing method has an exceedingly low error rate and that the majority of errors arise during the PCR amplification step. We argue that the error profile has profound implications for choosing the appropriate seed sequence for clustering using the data generated by Illumina sequencing.

Results

Description of the Data

The DNA samples analyzed by this method were derived from a study designed to examine the vaginal microbiota in HIV+ women in an African population. A separate manuscript details the clinical findings of the study [13]. In all we analyzed 272 clinical samples by a single Illumina paired-end sequencing run.

The Illumina sequencing platform is currently restricted to read lengths of ~100 nucleotides from each end of a DNA fragment, and was limited to ~75 nt at the time of experimental design. Thus, a

paired-end sequencing run could only traverse the short 16S variable regions: V3, V5 and V6. The expected distribution of amplified fragment sizes, including the primer, for each variable region is shown in Figure 1. We decided to use the V6 region for two main reasons. First, the V6 region was expected to produce amplified fragments between 110 and 130 bp, ensuring that the majority of paired-end reads would overlap. Secondly, the V6 region provided resolution for a number of organisms of interest in our samples down to the species and in some cases the strain level [14]. The Illumina platform currently provides reads long enough to overlap in either the bacterial V3 [1,14] or V5 regions or in the eukaryotic V9 region [5]. We suggest the region(s) chosen for sequencing should be characterized for the resolution of taxa of interest, and several studies have examined this in detail [7,11].
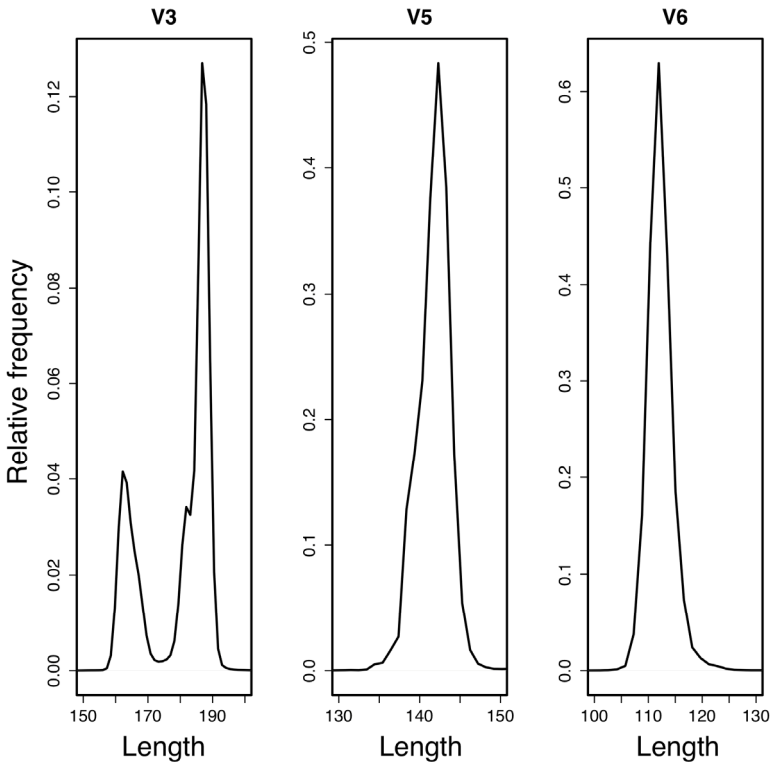

Figure 1. Expected amplified product size using constant regions flanking eubacterial variable regions.

PCR Primer Characterization

The primers were located within two conserved 16S rRNA segments that flanked the V6 region. The left and right primer sequences mapped to the 967–985 (CAACGCGARGAACCTTACC) and 1078–1061 (ACAACACGAGCTGACGAC) using the coordinates on the *Escherichia coli* 16S rRNA segment, and were chosen to amplify the majority of species expected to be found in the vaginal environment. The potential ability to amplify the regions flanking the V6 region was tested computationally by two methods. First, the primers were assessed using the probematch service from the Ribosomal Database Project [15]. The forward and reverse primers were found to match 96.8% and 99.3% of the good quality, long 16S rRNA sequences with 2 or fewer mismatches. The forward primer was strongly biased against amplification of sequences in the *Tenericutes* and *Thermotogae* phyla, amplifying 201/1438 and 8/82 in these groups. The reverse primer was unbiased. Secondly, we used a method similar to Wang and Qian [16]. Unaligned 16S rRNA sequences were downloaded from the Ribosomal RNA Database Project [15] and the 187260 sequences longer than 1400 nucleotides were extracted. Sequences of this length are nearly-full length and are expected to contain the V6 region. These sequences were filtered to remove entries where the only entry on the annotation line was 'unidentified bacterium' or 'uncultured bacterium', leaving 97987 entries. Approximate string matching (agrep) with the TRE regular expression library [17] was used to determine that the left and right primers matched 94101 and 96432 of 97987 sequences with the requirements of perfect matching at the 5 nucleotides at the 39 end and up to 2 mismatches in the remainder of the primer. Using this measure, the left primer matched over 96% and the right primer over 98% of the sequences in the dataset. However, as shown in Table 1, either the left or right the primers did not match the majority of sequences annotated as *Sneathia*, *Leptotrichia*, *Ureaplasma* or *Mycoplasma*. We found that relaxing the parameters somewhat resulted in matching to the majority of species in these groups (Table 1). We suggest that these primers would allow amplification of the majority of species in each of these groups, but that amplification may occur at lower efficiencies in some groups.

The primers were tested for their ability to amplify the 16S rRNA V6 region of *Lactobacillus iners*, *Lactobacillus rhamnosus*, *Gardnerella vaginalis* and *Atopobium vaginae*. All were amplified equivalently using the following PCR parameters: denaturation 94^0, annealing 51^0, extension 72^0 all for 45 seconds over 25 amplification cycles.

Table 1. Number of species matching each primer in a RDP dataset.

Taxon	Total species	Left[1]	Right[a]
Escherichia	322	320	318
Citrobacter	113	111	110
Bacteroides	275	265	270
Streptococcus	1249	1243	1244
Staphylococcus	704	696	694
Lactobacillus	1922	1908	1910
Lachnospiraceae	82	82	82
Peptostreptococcus	28	28	28
Anaerococcus	29	29	29
Megasphaera	38	38	38
Dialister	21	21	21
Candidatus	579	377	566
Mobiluncus	25	25	25
Propionibacteriaceae	12	12	12
Bifidobacterium	146	145	143
Porphyromonas	111	109	111
Prevotella	269	264	264
Fusobacterium	103	102	103
Sneathia	4	4	0(4)[b]
Leptotrichia	60	60	1(58)[b]
Gardnerella	3	3	3
Ureaplasma	36	0(34)[c]	36
Mycoplasma	414	95(331)[d]	336

[a]number of hits with identity at the 3' 5 nucleotides and up to 2 mismatches in the rest of the primer:
[b]number of hits requiring identity at the 3' 4 nucleotides:
[c]number of hits allowing 3 mismatches and identity at the 3' 5 nucleotides:
[d]number of hits allowing 4 mismatches and identity at the 3' 5 nucleotides.

Sequence Tag Choice

The Illumina sequencing platform uses dye-terminated primer extension to sequence DNA [18] and the base-calling algorithm uses the intensities from the first several nucleotides incorporated to normalize the fluorescent signal from subsequent nucleotide incorporation events [19,20]. Thus, we chose sequence tags to ensure all 4 nucleotides were represented in each of the first four positions of the primers using parameters similar to those in the barcrawl program [21]. This was achieved, in part, by varying the length of the tags between 3 and 6 nucleotides long. The tag length variation was expected to reduce the likelihood that adjacent spots on the Illumina solid support would be scored as one during the sequencing of the amplification primers [19,20]. All sequence tags were checked with a primer design program to ensure that they would not induce primer-dimer formation [22]. The sequence tags are given in Table 2. The right-side sequence tags can be uniquely identified if they are full-length, or if they are truncated by 1 nucleotide, as commonly occurs during oligonucleotide synthesis. Three of the left-side sequence tags (GTA, CTA, TGA) could derived from three longer left-side sequence tags (AGTA, GCTA, ATGA) by N-1 truncation. Only reads with full-length sequence tag sequences were used in this analysis. The three nucleotide-long sequence tags have been redesigned for subsequent experiments to remove any ambiguities that arise from N-1 truncation. The sequence tags were incorporated at the 5' end of the PCR primers.

Table 2. Sequence tag and primer sequences.

L-tag	Name	R-tag	Name
catgcg	0-v6L	cgcatg	0-v6R
gcagt	1-v6L	actgc	1-v6R
tagct	2-v6L	agcta	2-v6R
gactgt	3-v6L	acagtc	3-v6R
cgtcga	4-v6L	tcgacg	4-v6R
gtcgc	5-v6L	gcgac	5-v6R
acgta	6-v6L	tacgt	6-v6R
cactac	7-v6L	gtagtg	7-v6R
tgac	8-v6L	gtca	8-v6R
agta	9-v6L	tact	9-v6R
atga	10-v6L	tcat	10-v6R
tgca	11-v6L	tgca	11-v6R
act	12-v6L	agt	12-v6R
tcg	13-v6L	cga	13-v6R
gta	14-v6L	tac	14-v6R
cta	15-v6L	tag	15-v6R
tga	16-v6L		
gcta	17-v6L		

Extracting Sequence Reads and Sample Assignment

As stated by others [12] the large number of sequences and the short sequence reads present a challenge. The number of and the short length of the reads prevented the application of many common pyrosequencing data analysis pipelines. We therefore developed the data analysis pipeline shown schematically in Figure 2. A full description of each step is given below. All programs to extract the sequence reads and to index them into individual sequence units (ISUs) were developed in-house. A Bash shell script referencing C, Perl and R programs and scripts that are able to recapitulate this analyses on OS X are available from the authors.

We received 18047860 reads that were 76 nt long from each end of the PCR amplified region. Of these, 6236435 and 5491692 reads contained one or more low quality positions in the left and right end reads (defined as having the lowest base quality scores). However, there were only 53598 and 88498 reads that contained one or more 'N' character in the sequence calls.

A custom program was written in C to identify the overlapping segments of the forward and reverse reads. The program first identifies perfect overlaps between the two reads, and then finds reads that overlap if a single mismatch is allowed. The quality score is used to identify the most likely nucleotide in the overlapped segment, and a new fastq formatted file is generated for the combined reads. With this strategy 14960488 reads were obtained that had a perfect overlap between 10 and 50 nucleotides and an additional 1922084 reads had a single nucleotide mismatch in the overlapped region. The extraction of overlapping reads with proper primer sequences and correct sequence tags was performed with a custom Perl program. We found that 12035329 sequences contained two valid sequence tags and both primer sequences; allowing up to 3 mismatches per primer. The sequences derived from the perfectly overlapping reads form the basis of the remainder of the analysis.
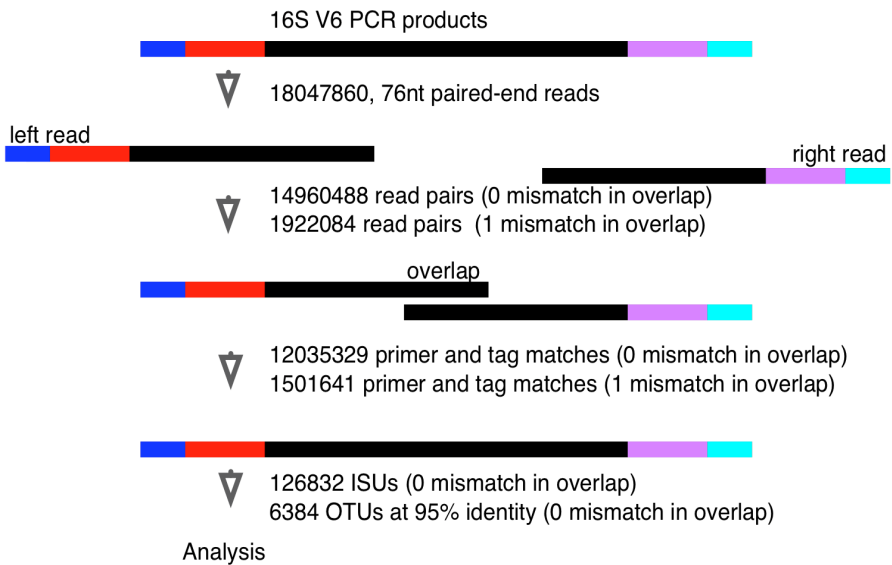
16S V6 PCR products

18047860, 76nt paired-end reads

left read

right read

14960488 read pairs (0 mismatch in overlap)
1922084 read pairs (1 mismatch in overlap)

overlap

12035329 primer and tag matches (0 mismatch in overlap)
1501641 primer and tag matches (1 mismatch in overlap)

126832 ISUs (0 mismatch in overlap)
6384 OTUs at 95% identity (0 mismatch in overlap)

Analysis

Figure 2. Conceptual workflow of the data analysis. PCR products derived from the eubacterial V6 rRNA region were sequenced on a single paired-end Illumina run. Reads were filtered for quality, overlapped and clustered as outlined in the text. Only reads with 0 mismatches in the overlapping region were used for further analysis.

Inspection of sequences with incorrect sequence tags showed that the single largest contributor to the difference between the number of reads with proper primer sequences and the number of

reads with proper sequence tag sequences was an N-1 truncation of the sequence tag, which presumably arose during the primer synthesis. The next largest class of sequence tag error was complete lack of the left or right end sequence tag. Together, these classes account for slightly more than half of the missing reads. The remaining missing reads are composed of a large number of classes of sequence tag sequences each containing small numbers of errors including additional 5' bases, misincorporated bases or difficult to classify errors that presumably arose during the PCR amplification.

Sequence Clustering

Clustering was used to group identical sequences into ISUs, and these ISUs were further clustered into operational taxonomic units (OTUs). The variation in an OTU can come from sequence differences between closely related taxa in the underlying population, through errors introduced into the amplified fragment from the PCR amplification, or from DNA sequencing errors.

ISUs were produced by collecting and collapsing identical sequences located between the primers and collapsing. A custom Perl program was written that associated each ISU with the number of identical sequences in it, that indexed each read to the appropriate ISU and, later the OTU. The 12035329 reads were collapsed into 126832 ISU sequences, with the most abundant ISU containing 4321348 identical reads.

The occurrence of chimeric sequences was examined using UCHIME, a part of the UCLUST package. Chimeric sequences can arise during PCR [23] or because of overlapping spots on the solid support when imaged during DNA sequencing [18]. The default settings of UCHIME identified 5211 putative chimeric ISUs, containing 18614 reads. Thus, 5.6% of the ISUs were putative chimeric sequences, but these composed only 0.15% of the total reads. There were 21 abundant putative chimeric ISUs that contained >100 reads; the most abundant contained contained 1271 reads.

Each of the abundant putative chimeric ISUs were tested for chimerism with BLAST using the ISU sequence as the query and using the bacterial subset of nucleotide sequences at NCBI as the database. We found only two putative chimeric sequences had sequence derived from two different species and had a UCHIME chimera score >10, the other 19 putative chimeric ISU sequences matched multiple

independent sequences in the dataset with ≥98% identity for their entire length. Thus, the occurrence of chimeric sequences was re-evaluated using a chimera score cutoff of 10 and only 497 ISUs containing 1834 total reads (0.015% of the dataset) were above this threshold. We concluded that chimeric sequences composed a very small subset of the total number of ISUs, probably because the primers amplified across a variable region only. The dataset was used without further regard to chimeric sequences because putative chimeric sequences composed a minuscule fraction of reads.

The ordered ISU sequences were clustered into OTUs, operational taxonomic units, by UCLUST which clusters each ISU to a seed sequence at a fixed sequence identity threshold using sequences as seeds in the order they are encountered in the file. We ordered the ISU sequences from the most to the least abundant, under the assumption that read abundance correlated with the abundance of the sequences in the underlying population. Several lines of analysis were used to decide on appropriate clustering values.

It is expected that the abundance of sequence variants per OTU will decrease according to a power law if the variants are generated stochastically. However, if a variant represents a distinct taxon in the underlying microbial population, the frequency of the variant is expected to reflect the proportion of the bacterial DNA in the sample.

Figure 3 shows a plot of the number of reads in an OTU having n mismatches compared to the most frequent read in the OTU at a cluster percentage of 92%. For an OTU with a length between 72–80 bp this corresponds to ~5 mismatches with the seed sequence. The red line in Figure 3 shows the plot for the 37 bp concatenated left and right primer sequences, which are expected to have half the per-nucleotide PCR-dependent error rate as the sequence between the primers, because 50% of the sequence is not derived de novo but is contributed by the primer sequence. Because the concatenated sequence is about one-half the length of the sequence between the primers, the overall slope of the primer line should approximate the slope of a single-species OTU that includes errors arising only from the PCR and sequencing. Note that the line for the primer sequence is nearly linear and, in line with our expectations, the number of reads having additional differences with the seed sequence for the OTU is far less abundant than the reads with one fewer difference. Also plotted are the results for the 25 most abundant OTUs, with OTU 0,

the most abundant OTU comprising 51% of the total reads, shown in blue. The line for OTU 0, and several other OTUs closely follow the line for the concatenated primers until 4 or 5 differences with the seed sequence are included. The simplest interpretation is that one or more additional rare taxa having 4 or more mismatches with the seed sequence for OTU 0 are now being included at this level of clustering. The lines for 11 of the 25 OTUs show a similar pattern with a sharp increase at 4 or more mismatches. Only 3 of the OTUs show a continuous decline for all number of mismatches with the seed member of the OTU suggesting that clustering at 92% identity was including sequences not derived from PCR or sequencing error.
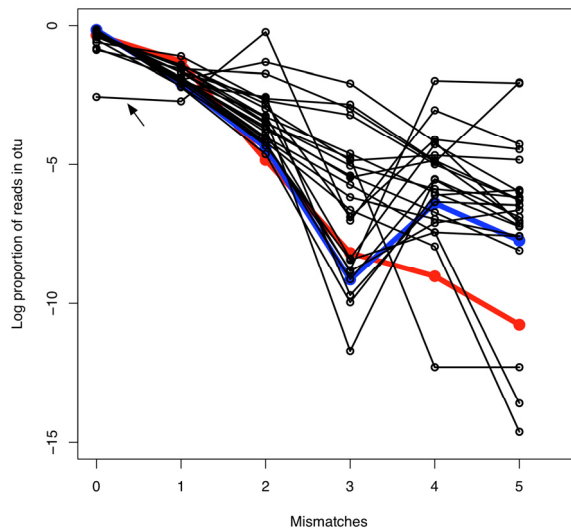

Figure 3. The proportion of reads in the 25 most abundant OTUs clustered at 92% identity as a function of the number of differences with the seed ISU. The red line shows the plot for the concatenated primer sequences, and the blue line shows the plot for the OTU containing the most abundant ISU.

We next calculated the Levenshtein distance — the minimal number of substitutions, insertions or deletions needed to convert one sequence into another — for all pairs of the 108 ISU sequences that occurred with a frequency of >1% in any of the 272 samples. Examination of the neighbour-joining tree drawn from these distances showed that there was a continuum of distances between ISU sequences, but that there seemed to be a natural distance cutoff of three substitutions in this dataset. This is illustrated in Figure 4 where

the branches sharing red nodes connect ISU sequences that clustered together at 95% identity, and branches sharing green nodes connect ISU sequences clustered at 92% identity. Several of these are instructive. The clade at 2 o'clock is anchored around ISU 0. The other ISU sequences in this clade differ from ISU 0 by one or two substitutions, and, as we show below, represent substitutions because of PCR error. All the members of this clade are wellseparated from all other clades. The other extreme can be illustrated by the clades at 4 and 6 o'clock. Here, as shown below, the grouping at 95% identity includes differences derived from PCR errors and from underlying sequence diversity in the microbial sample. However, grouping at 92% identity (Levenshtein distance of 5) clearly groups outlier clades with the main group. It is standard to assume that clustering at 97% identity represents species units [12]. However, taking the two extremes as examples, clustering at greater than 95% identity would result in splitting clades that contain differences derived only from PCR error (i.e. ISU 0 and associated ISUs) and clustering at less than 95% identity would group sequences that should be distinct.
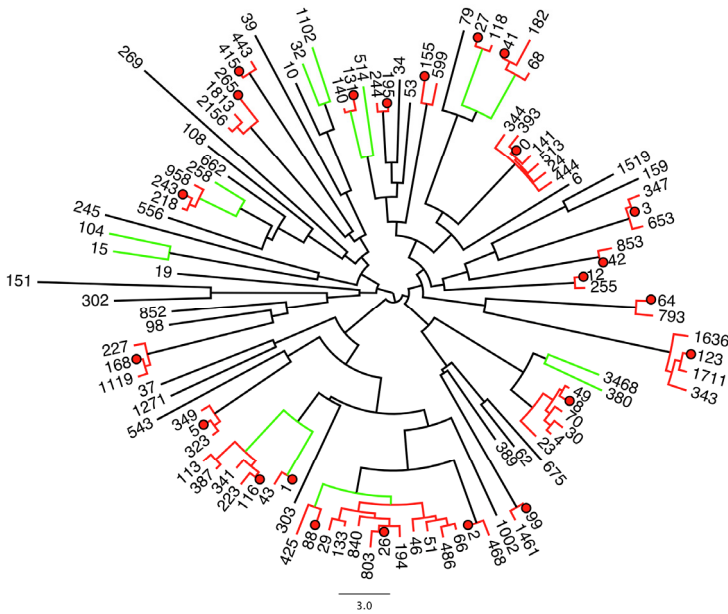
Figure 4. Neighbour–joining tree derived from Levenshtein distance between the 108 most abundant ISU sequences. ISUs clustered into OTUs at 95% identity are connected with red branches and ISU sequences clustered at 92% identity are connected with green branches. The seed sequence for each 95% identity OTU cluster is identified by a red dot.

Based on these analyses a cluster percentage of 95% was used for the analysis given below because it allowed up to 3 nucleotide differences with the seed sequence per OTU. At the 95% clustering threshold, 15 of the OTUs showed ISU mismatch frequency decay characteristics similar to that expected for errors introduced only via PCR or sequencing error; i.e., their abundance profiles decayed at a rate similar to that seen for errors in the primer sequences. This indicates that these 15 OUT sequences may be well differentiated from their neighbours at this level of clustering and may represent distinct sequence species in the underlying population. On the other hand, the most abundant ISU in several OTUs was outnumbered by clustered ISU sequences. In the most extreme cases, OTUs 46, 97 and 119, ISU species with 2 and 3 differences from the seed ISU outnumbered the seed ISU by 2–3 orders of magnitude. An example of this characteristic profile is labeled with an arrow in Figure 3. As shown below, these OTUs represent clusters of errors derived from very abundant organisms in the underlying population.

Assignment of OTUs to Taxonomic Groups

The tools used for taxonomic assignment are not designed to work with the short sequences derived from this type of analysis [15]. Therefore, similar to others we designed a simple classification scheme based on sequence comparison with BLAST [12,24] vs. eubacterial sequences (taxid 2), excluding uncultured and environmental samples, in the GenBank database [25]. In essence, sequences were identified at the species level if a fully-sequenced or classified type-species sequence matched the OTU with 100% identity and 100% coverage and no other sequence matched with >97% identity. Sequences that matched with less than 100% identity were classified at the genus level if another genus matched with a lower percent identity. Sequences with less than 95% identity were matched to the taxonomic level supported by the groups of reads. With these rules we were able to assign the 63 OTU sequences that were at an abundance of >1% in any of the 272 samples unambiguously. As discussed below, three of the OTUs were derived from PCR errors from the *G. vaginalis* strains and were classified accordingly. The classifications of these OTUs, and the supporting evidence for each is shown in Supplementary Table S1. The sequence of the seed ISU for each OTU has been deposited in Genbank with sequential accession numbers between HM585291–HM585350 inclusive.

Systematic Sources of Error

Recently, Quince *et al* [10] examined the effect of pyrosequencing errors on the classification of organisms in high-throughput microbiome analyses. They concluded that a large fraction of the 'rare microbiota' was composed of pyrosequencing errors and introduced a method to accurately cluster the reads based on their expected errors. Since the Illumina sequencing platform has a substantially lower error rate than does the 454 pyrosequencing platform, and the read length is deterministic rather than random [19] we were thus interested in identifying the sources of error in the ~13 million overlapping reads in our dataset. Most notably the Illumina platform is not susceptible to miscalling the number of nucleotides in homopolymeric regions; this type of base-call error is more

pronounced in pyrosequencing reads when sequence coverage is relatively low [19].

If the major source of error in the data came from DNA sequencing, we would expect that errors should increase as a function of distance from the sequencing primer until the region of overlap and that the errors should be much less frequent in the overlapped region. This hypothesis can be assessed by comparing the Q, or quality, score assigned by the Illumina base-calling algorithm in the overlapped 16S sequences with the error frequency per position. Figure 5 shows a box-plot of the Q scores for the reads of length 120 nt, which composed 24% of the ~12000000 overlapping reads. Similar results were obtained for reads between 113 and 126 nt, which together compose >99% of the overlapping reads. Two important conclusions can be drawn. First, as expected, the median Q score decreases and the range of scores increase as the distance from the sequencing primer becomes greater. Second, the Q scores, and the variability in these scores for the region of overlap are greater than for the region of single coverage.
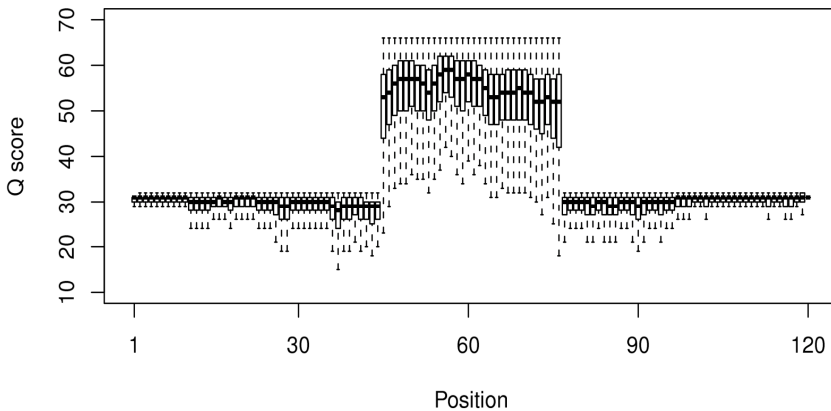

Figure 5. Quality scores for all overlapped 120 bp composite reads. The **Q** scores a log-odds score of the likelihood of error in the base call, higher **Q** scores represent lower likelihoods of error [40]. They are expected to decrease with distance from the left or right sequencing primer, and to be highest in the region of perfect overlap because **Q** scores are additive.

Initally, the concept of stochastic error contributing to sequence variation was examined by measuring the frequency of occurrence of each nucleotide in the left and right primers. Figure 6 shows a plot of the number of times that each nucleotide occurred at each position in

the left and right V6 primers. This figure illustrates several points. First, the most frequent variant at each position is usually a transition rather than a transversion, although several positions did not follow this pattern. Secondly, the frequency of the residues differing from the primer sequence are found in a relatively consistent range. Thirdly, position 9 in the left primer, which was synthesized as a mixture of G and A, shows a strong deviation from the background frequency. Thus the underlying nucleotide frequency in the population of molecules being amplified strongly affects the nucleotide frequency at the polymorphic position. Finally, the variation is constant across the entire length of the primers except for position 9 and is not dependent on the distance from the sequencing primer. These observations support the hypothesis that stochastic errors may contribute significantly to sequence variation in our dataset.

The relationship between the Q scores and the abundance of sequence variants for each OTU was examined by mapping the variants onto seed ISUs as was done for the primer sequences. All ISU sequences in each OTU were used to make a BLAST database for that OTU and the OTU seed sequence was used as the query sequence. An additional 6 nucleotides were added onto both ends of both the OTU sequence and the ISU sequences because of the edge effects in the BLAST algorithm [26]. These nucleotides were later trimmed for the analysis. The number of sequence variants at each position, weighted by the number of reads that the variant occurred in was tabulated and converted into graphical representations of nucleotide counts at each position in the OTU.
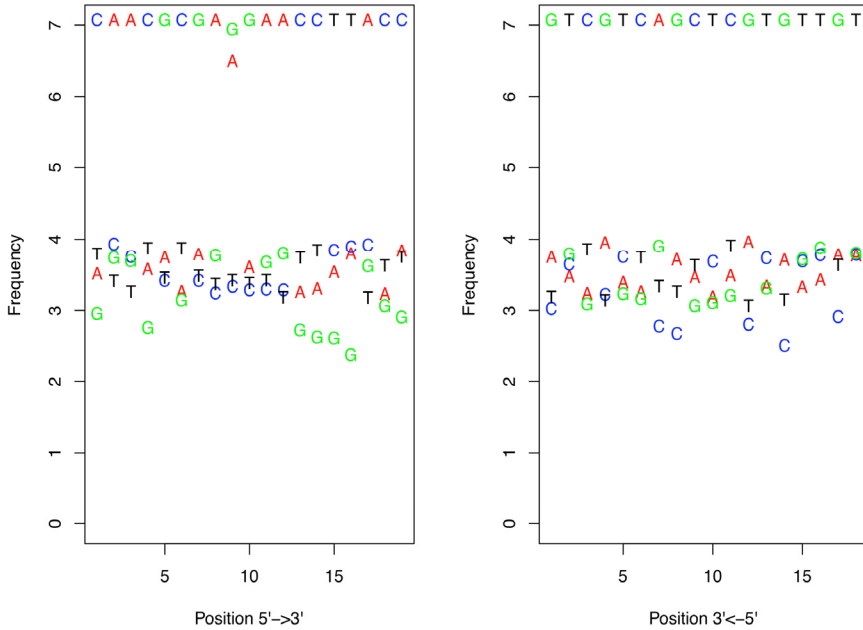
Figure 6. The frequency of each nucleotide observed at each position in the left and right primers derived from the Illumina dataset. There are >12 million sequences, and the difference in frequency between the correct and altered nucleotide is relatively constant. Note that the errors are at the same frequency at each end of the primers.

Two representative examples for the rRNA V6 region are given in Figure 7, and a summary of the distributions is given in Figure 8. Figure 7 shows the number of reads that contain an individual residue at each position plotted in color. The entropy of each position is plotted as open or filled diamonds; higher entropy values correspond to greater variability at the position. Both of these OTUs contain several million reads, and the predominant nucleotide corresponds to the OTU seed sequence. However, there are many variants that were clustered together in these OTUs.

Figure 8 shows a summary plot of the distribution of differences in the frequency across each OTU between the most commonly occurring residue and the other 3 residues at each position. The OTUs are arranged approximately from those with the most to the least number of reads. Several interesting observations can be made from these bar-plots. First, the frequency differential varies between 10^{-2} and 10^{-5} for the vast majority of sequence variants from the seed ISU sequence. Second, about half of the OTUs contain one or more

strongly outlying values. These correspond directly to the common variant residues seen in Figure 7. Compare, for example, the uniform distribution of variants in the top panel of Figure 8 (OTU 0) and the three outlying variants in the bottom panel (OTU 1) with the nucleotide distributions in Figure 7 for these OTUs. Third, the evidence for outlying positions becomes progressively weaker as the the number of sequences in the OTU decreases.
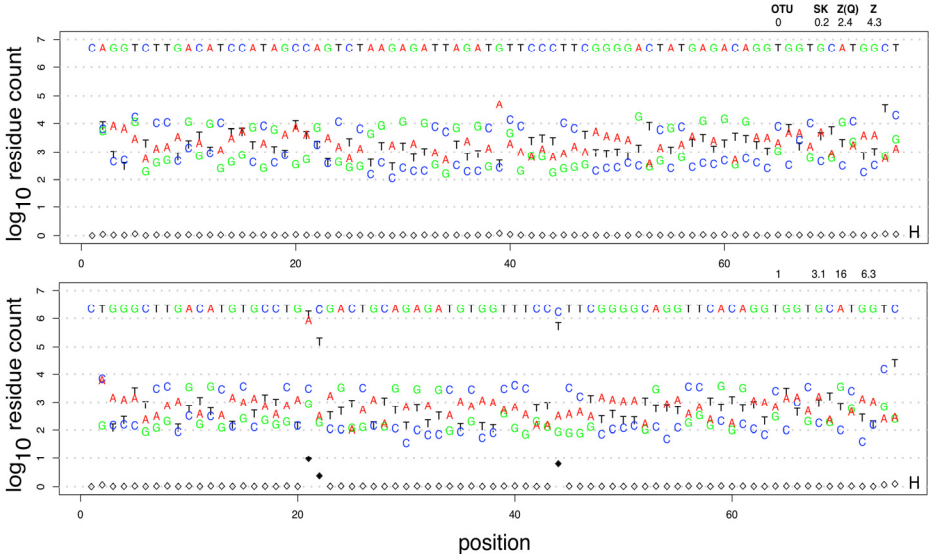

Figure 7. The sequence variation in OTU 0 and OTU 1. The plot shows the number of times that each nucleotide occurred at each position in two example OTUs.

The data in these two figures can be summarized numerically by examining the distribution of the entropy of the positions in each OTU. Skew in the entropy values is calculated by: $SK=H_{median}-H_{mean}x100$. The SK value tells us if the distribution of entropies is strongly skewed by the occurrence of highly variable positions. Values near or greater than 1 indicate a strongly skewed entropy distribution and represent a situation where several to many positions are highly variable.

Z and ZQ both measure how different the maximum entropy value is to the central tendency of the entropy distribution, and are calculated as follows: $Z=(H_{max}-H_{mean})/\sigma H$ and $Z_Q\sim(H_{max}-H_{median})/(H_{95thpercentile}-H_{median})$. Thus Z represents the number of standard deviations that the maximum entropy value is from the mean, and Z_Q is the number of 95 percentile deviations of the

maximum entropy value from the median. Both values are required since Z is not informative if a distribution has a large variance. Z_Q has extreme values in the instances of a skewed distribution with small number of extreme values. Inspection of the plots suggests that values of SK>1, Z>6 or Z_Q>6 represent situations where the nucleotide distribution for a suggests a mixed population of reads. Conversely, OTUs where all 3 values are less than these cutoffs strongly suggest that the variability seen in the OTU arose from stochastic errors inherent in the experimental protocol.
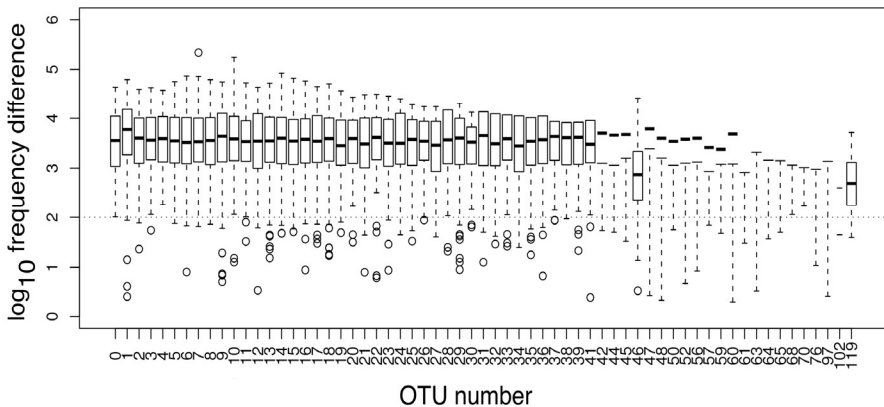

Figure 8. Boxplot summaries of the difference between the frequency of the most in common residue at each position and the frequency of each sequence variant. The OTU numbers are given at the top of the graph.

Supplementary Table S2 summarizes these statistics for each OTU. The complete set of Figures, shown in Supplementary Figure S1, and the associated summary statistics provide information about the potential mixture of sequences found in each OTU.

The information in Figure 7 and in 8 and the associated entropy information, allow us to classify the OTUs into groups that contain a homogeneous population of reads that differ from each other only because of variations introduced during the PCR step (eg. OTU 0) and OTUs that contain sequence variants derived from the underlying population (eg. OTU 1). As an example, the top panel in Figure 7 corresponds to OTU 0, and the seed sequence in this OTU is identical to the V6 region of *Lactobacillus iners* in both the RDP and NCBI nucleotide databases. The bottom panel corresponds to OTU 1, and the seed sequence is identical to one annotated as *Gardnerella*

vaginalis 409-05. The second most common sequence is identical to one annotated as *G. vaginalis NML060420*, and the third and fourth most common sequences are identical to sequences annotated as uncultured *G. vaginalis* sequences. All four of these sequences differ from each other by a single diagnostic nucleotide, and the variant counts match the counts of the 4 major ISUs. These 4 ISUs make up the 88.9% of the reads in OTU 1. Based on the analysis of these two OTUs and the similar analyses of the remaining OTUs, we conclude that OTUs that exhibit the pattern of variation seen in OTU 0 represent distinct sequence entities in the underlying dataset and that those exhibiting a pattern of variation similar to OTU 1 represent the grouping of sequence entities in the underlying dataset based on sequence similarity. In the case of OTU 0, no sequence in the RDP database [15] could be clustered with it without including at least 5 nucleotide substitutions, leading us to conclude that OTU 0 represents a distinct taxonomic group at the sequence level. In the case of OTU 1 there are several sequences, all annotated as different strains of the same species that are grouped together, and like OTU 0, all are well-separated from other V6 sequences. Thus, we conclude that OTU 1 is a cluster of distinct *G. vaginalis* strains.

OTUs 46, 97 and 119 in the dataset, had distinct distributions when plotted as in Figures 7 and 8. The nucleotide frequency difference between the seed sequence and the nucleotide variants in these three OTUs was much smaller than in the other 61 OTUs. Inspection of the sequences making up these OTUs showed that they were most similar to one or more of the G. vaginalis strains. We propose that these OTUs are composed of ISU sequences derived solely from PCR errors that failed to cluster with the seed sequence in OTU 1. We are currently working on a clustering procedure that explicitly accounts both for edit distance and read abundance to more accurately cluster sequences derived by very high throughput sequencing.

Organism Diversity and Data Reproducibility

We found that one right-end tag, GCGAG, was composed of a mixture with the ratio 69.5/30.5 of the full-length and the unique N-1 truncation-derived GCGA tag. This oligonucleotide synthesis error was exploited to determine the effect of the number of reads on within-

sample variability; in essence the N-1 truncated tag allowed an examination of the technical replication of the experiment. The GCGAC tag was used in 17 samples. The black-filled circles in Figure 9 show the number of reads from the full length GCGAC tag compared to the truncated GCGA tag in these samples. The red open circles in Figure 9 show an example of the read replication observed from independent samples. The replication of the read numbers in the full length and N-1 samples is extremely high for reads occurring at least 30 times in the fulllength tag set, and at least 10 reads in the N-1 tag set. As expected the read replication for independent samples is much poorer. The correlation coefficients for the 17 full-length and N-1 samples ranged from 0.97 to 0.99 when the N-1 sample contained at least 10 reads. Thus, we conclude that the number of reads in a sample is reproducible, if at least 10 reads are observed. Similar conclusions about the minimum read abundance have been drawn from RNA-seq experiments [27].
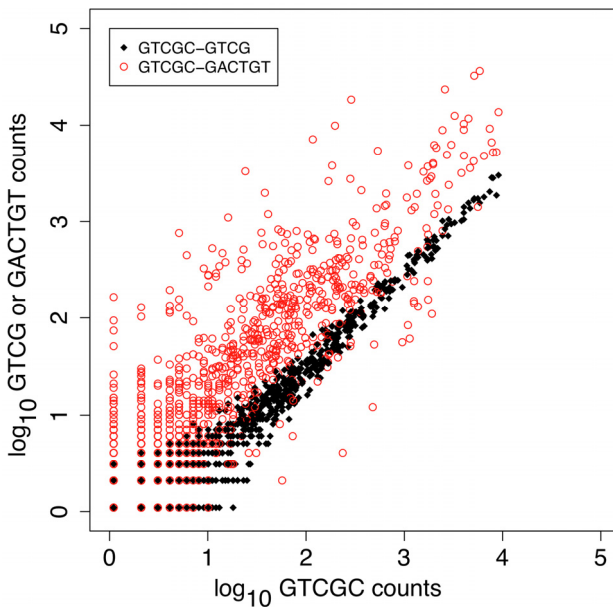
Figure 9. Plot of the reproducibility between and within samples. The black-filled circles plot within-sample variation, and the red circles plot the between-sample variation for the GTCGC tag. The count of sequences composing OTUs clustered at 95% identity for samples containing the GTCGC tag and the GTCG N-1 tag are in black. This shows the technical replication of the data when amplified from the same sample in the same tube. The open red circles plot the correspondence for between-sample OTU counts.

Rarefaction Curves

A second way to examine reproducibility is to generate rarefaction curves where the number of species sampled per unit of effort is estimated by resampling the dataset [28,29]. Rarefaction curves for the dataset from each sample were generated by performing 10000 random samples with replacement [30] on the complete set of OTUs or ISUs or by including only those OTUs and ISUs that occurred in a sample more than twice. The values for resampling without replacement will approach the observed value (i.e. will saturate) only if the sample is of sufficient size to encapsulate all possible diversity [30]. Thus, if the values do approach saturation when resampling with replacement, we can be confident that we have sampled most, if not all, of the available sequence species [30,31].

Figure 10 shows rarefaction curves generated for ISUs and OTUs in sample 1 using different protocols for a representative sample in our dataset; it is worth pointing out that this rarefaction curve is one of the few curves that does not reach saturation. The white-filled symbols show curves generated for unclustered ISUs in this sample, and the black-filled symbols are for OTUs generated at 95% sequence clustering. Here, the effect of removing rare sequence species is clear. The curve saturates when sampling only 50% of the reads if either rare ISU or rare OTU sequences are removed, but does not saturate for either the ISUs or OTUs even with the full set of reads. Inspection of the full set of rarefaction curves shows that this failure to reach the limit is commonly observed when the sample is dominated by one or a few species, which is the case in many of the microbiota samples in our complete dataset. Samples containing a broader range of species show rarefaction curves that generally reach the limit near 20000 reads, suggesting that this is an appropriate number of reads to sample the microbiome in the vaginal environment. Rarefaction curves for each of the 272 samples are given in Supplementary Figure S2.
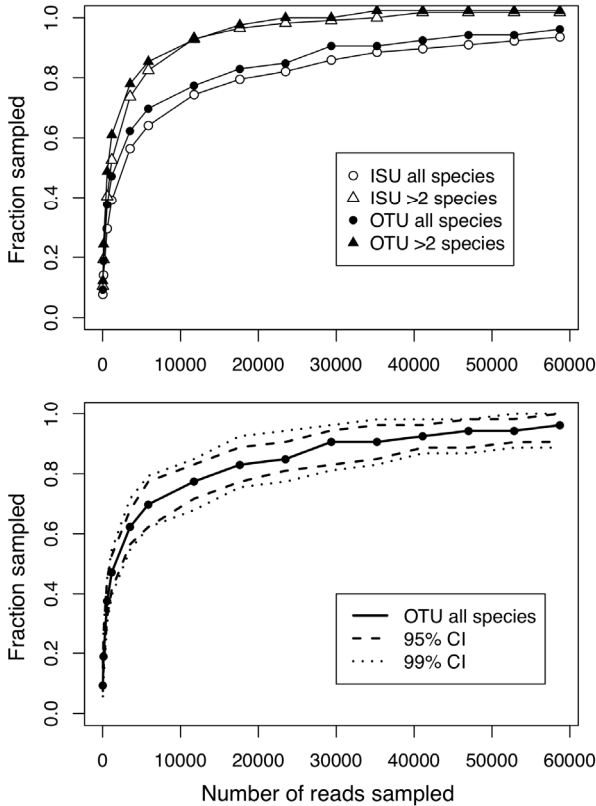
Figure 10. An example rarefaction curve. The top panel shows rarefaction curves generated for sample 1 by resampling with replacement either all OTUs or ISUs, or OTUs and ISUs where at least 3 reads were observed. The bottom panel shows the rarefaction curve and the 95% and 99% confidence interval for all OTUs in sample 1. Rarefaction curves for all 272 samples are given in Supplementary Figure S2.

Estimating Species Richness

Another method of examining species richness is to use the Chao1 or ACE methods to estimate the number of unseen species in the sample [32,33]. We used both methods to determine the number of species expected in each of the 272 samples with the VEGAN package for biodiversity analysis [34]. There were 37 and 31 of 272 samples where the Chao1 and ACE estimates indicated that we observed <90% of the real species. The correspondence between the Chao1 and ACE measures is plotted in Figure 11 and it is clear by these two measures that the vast majority of samples were expected to contain the majority of the available species. Included in this plot is the

fraction of species found when the rarefaction analysis was performed with the number of reads in the sample. Rarefaction with a saturating number of reads again showed that the 206 of 272 samples identified all or almost all of the available species.
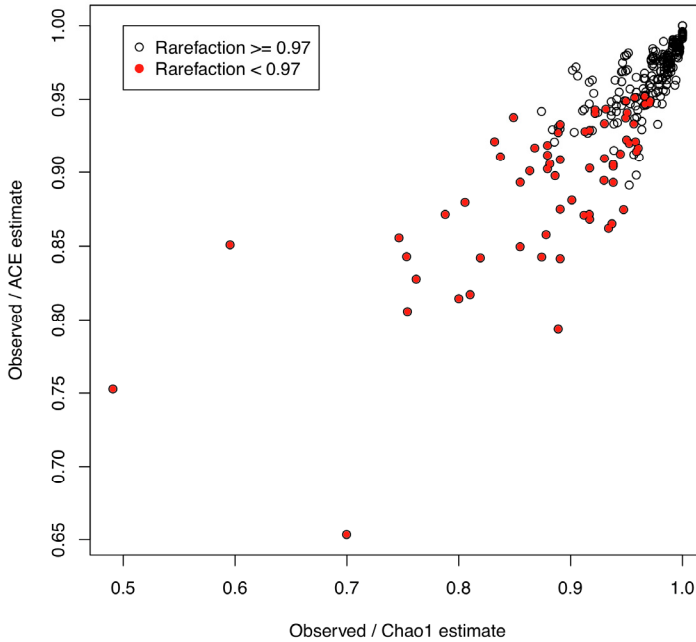
Figure 112. Correspondence between Chao1, ACE and rarefaction curves for the 272 samples. The X and Y axes show the fraction of species that were found in each sample for the two estimates. Red-filled circles highlight those samples where the limit rarefaction value was less than 0.97.

Diversity vs. Number of Reads

Finally, species richness can be examined as a function of the number of reads across all 272 samples. This is plotted in Figure 12 for ISU and OTU sequences. In this case the white-filled symbols represent populations derived from samples classified as 'normal', and are expected to be dominated by one or a few species, and the red or blue-filled symbols represent populations classified as bacterial vaginosis (BV), where there is expected to be a more even distribution of species [35,36]. There are strongly diminishing returns when more than 20000–25000 reads are obtained regardless of the diversity of the population; sampling more than 50000 reads was

sufficient to sample all the available OTU diversity in the samples. Interestingly, the number of distinct ISU sequences increases linearly with the number of reads, providing further evidence that increasing the number of reads increases the background number of ISUs that contain PCR-derived errors. Taken together with the rarefaction, Chao1, ACE data, we conclude that the number of reads obtained by this Illumina sequencing is adequate to sample nearly saturating numbers of species in this environment.
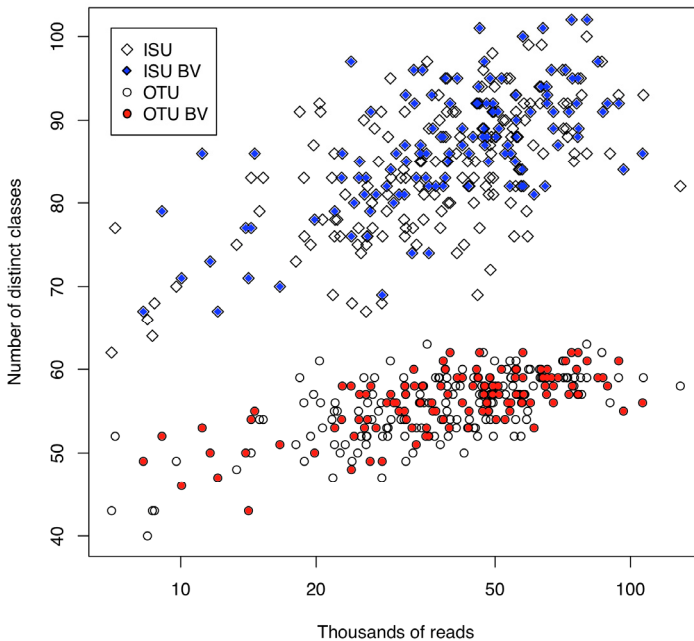
Figure 12. **Plot of the number of distinct ISU or OTU classes in each sample as a function of the number of reads.** The number of ISU classes increases with the number of reads, but the number of OTU classes becomes constant above 20000–30000 reads.

Comparison with DGGE

Results from Illumina sequencing were compared to those from dideoxy chain termination sequencing of bands isolated from following denaturing gradient gel electrophoresis (DGGE) analysis of amplified PCR products; a method traditionally used for the separation of bacterial species. A total of 20 samples were selected that were expected to have a diverse population of organisms by

extrapolation from the 272 samples sequenced by Illumina. DNA fragments from the bands were sequenced and each OTU sequence and each sequence from the DGGE bands were assigned to taxonomic groups by BLAST using the GenBank nucleotide database as described above. OTU sequences were assigned to species if they matched 100% of their length at 100% identity, and to genus or other groups as outlined in Supplementary Table S3. DGGE was found to detect only those bacterial species of greatest abundance in the sample, with a minimal Illumina read abundance of 11%. In two cases, one shown in Figure 13A-lane 89, a distinct band was excised and sequenced that had an Illumina abundance of between 2–3%. Figure 13B shows that a total of 8 organisms were detected through DGGE analysis, compared to 59 organisms detected through Illumina analysis in the same 20 samples, and that the organisms identified by DGGE analysis were a strict subset of those identified by Illumina sequencing.
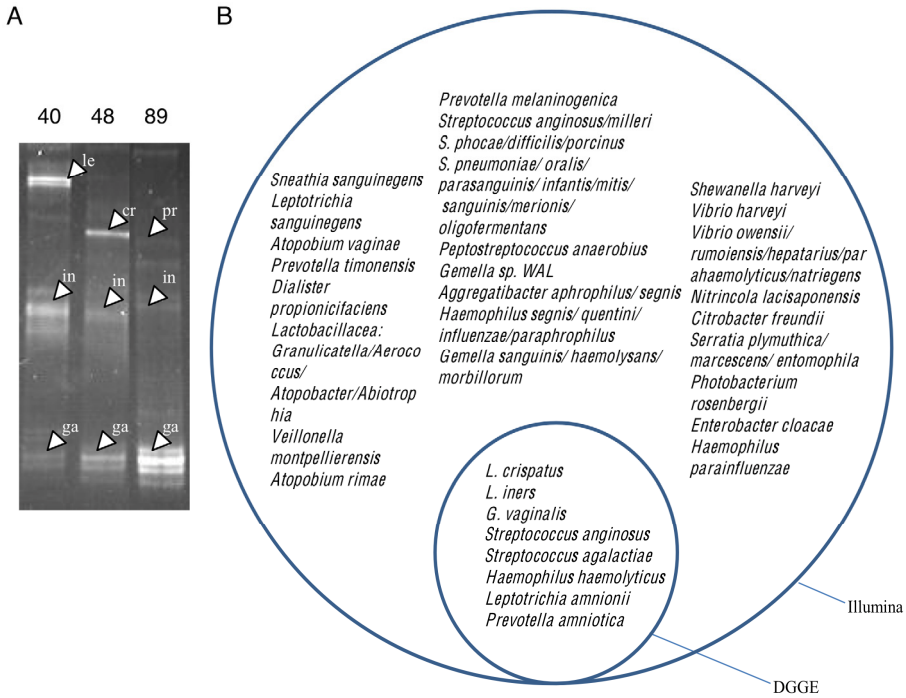
Figure 13. DGGE analysis of selected samples. Panel A shows representative PCR amplicons from 3 of 20 clinical samples (Subjects 40, 48 and 89) were electrophoresed on a denaturing gradient gel. Bands were excised, sequenced and identified as in the Materials and Methods. Bands are labeled as follows: le = *Leptotrichia amnionii*; in=*Lactobacillus iners*; ga=*Gardnerella vaginalis*; cr=*Lactobacillus crispatus*; pr=*Prevotella amnii* (also named *P. amniotica*). Panel B shows a Venn diagram of the organisms identified by Illumina sequencing of the V6 rRNA region and by sequencing DGGE bands amplified from the V3 rRNA region.

Discussion

We present and characterize a low-cost, high throughput method for microbiome profiling. The method uses combinatorial sequence tags attached to the 5' end of PCR primers that amplify the rRNA V6 region, but may be easily adapted for use in other bacterial and eukaryotic sequences. Illumina paired-end sequencing of the amplified sequences generates millions of overlapping reads. The combinatorial sequence tags allow the investigator to examine hundreds of samples with far fewer primers than is required for single-end bar-code sequencing. We propose that this method will be useful for the deep sequencing of any short sequence that is informative; these include the V3 and V5 regions of the bacterial 16S

rRNA genes and the eukaryotic V9 region that is gaining popularity for sampling protist diversity. The use of the V3 and V5 regions is currently being attempted by our group. Two other groups recently used strategies similar to ours. One collected overlapping paired-end reads, but without the sequence tags, to examine genomic DNA from mixed bacterial populations [37]. The other used a small number of sequence tags on one of the two amplification primers to examine microbial diversity using paired-end Illumina sequencing [12]. However, their method required three Illumina sequencing reads instead of two: two of the reads to sequence each end of the amplified product and a third using a custom primer to identify the sequence tags. In contrast, in our study, we attached very short sequence tags to both the left and right primers and read the sequence of the tags directly in the paired-end run. Furthermore, we used a simple set of rules to choose short sequence tags that balanced the nucleotide composition in the first 4 positions of the reads, that maximized the stagger in the primer sequences when attached to the solid surface and that minimized the possibility of primer-dimer formation. Using these simple principles and avoiding the N-1 generation of nonunique sequences, short sequence tags should be easily derived that are suitable for primers specific to any small region of interest. Sequence tags can be chosen automatically using the barcrawl program [21] or can be chosen by hand.

We observed very few chimeric sequences in our dataset. There are several reasons. First, we used a relatively small amount of input DNA and used a small number of PCR cycles for amplification [23]. Secondly, many chimeric sequences may have been removed because of the strict requirement for proper sequence tag and primer sequences on the left and right ends, and because of a requirement for long overlapping segments of a defined length. In this case, the deterministic read lengths of the Illumina protocol combined with our narrow window for overlapping segments would have resulted in many chimeric sequences being filtered out. Indeed, inspection of a fraction of the read pairs that failed to overlap, or that failed to pass the sequence tag and primer requirements showed that many of these were chimeric or deleted at one or both ends (data not shown). Thus, while the Illumina sequencing protocol is limited to short segments these can be combined into longer segments using the paired-end approach as long as there is a significantly overlapping

segment. The utility of the method is further demonstrated by the nearsaturating number of ISU and OTU sequences obtained from a large number of clinical samples. We used several lines of evidence to show that 20000 reads are sufficient to capture all or virtually all of the sequence diversity in the vaginal microbiome, and that obtaining over 50000 reads results in no new sequence species. Thus, assuming a requirement for 50000 reads, up to 200 samples can be combined into a single Illumina lane, while up to 500 samples are possible if only 20000 reads are required. This is much greater depth at a much lower cost than is possible with current pyrosequencing technology. Strikingly, we observed that none of our samples contained the full range of species in the microbiome as a whole, and that we found fewer species than in a recent report that used pyrosequencing in the same niche [38], despite averaging 20-fold greater sequence coverage. We suggest that the higher fidelity Illumina sequencing may have resulted in fewer taxa because of a lower error rate contributing to fewer 'rare microbiome' taxa.

Finally, we showed that the spectrum of errors could be examined for each OTU to help determine if the OTU was derived from a single underlying sequence in the sample population. The large number of reads presented a challenge for sequence-based clustering because sequencing millions of reads ensured that much of the read variation was derived from PCR-amplification. We show that sequence clustering of the large number of reads derived from Illumina sequencing would be more accurate if it took both the sequence variation and the underlying error rates into account. We are currently working on developing methods to cluster that use both sequence similarity and read abundance.

Materials and Methods

Ethics Statement

The medical ethical review committee of Erasmus University Medical Centre, The Netherlands, and the medical research coordinating committee of the National Institute for Medical Research, Tanzania, approved the study design and protocol. Subjects were informed of the purpose of the study and gave their signed informed

consent before participation. The study was registered at www.clinicaltrials.gov NCT00536848.

Sample Preparation and Amplification

DNA was prepared from clinical swabs as part of a clinical study [13]. Amplification was initiated with a 61^0 annealing temperature that dropped to 51^0 in 10 increments followed by 15 cycles of: denaturation 94^0, annealing 51^0, extension 72^0 all for 45 seconds with a final elongation for 2 minutes. A constant volume aliquot of each amplification was run on a 1.4% agarose gel for to determine the approximate amount of product. The amount of product was scored on a 4 point scale and, based on this scale, between 2 and 40 µl of the PCR products were mixed together to give the final sample sent for Illumina sequencing at The Next-Generation Sequencing Facility in The Centre for Applied Genomics at the Hospital for Sick Children in Toronto. The library was prepared without further size selection.

Denaturing Gradient Gel Electrophoresis Analysis

Clinical samples were amplified using eubacterial primers flanking the V3 region of the 16S rRNA gene: HDA-1 (5-ACTCCTACGGGAGGCAGCAGT-3) at position 339–357 (with a GC clamp located at the 5 end), and HDA-2 (5-GTATTACCGCGGCTGCTGGCA-3) at position 518–539, with an annealing temperature of 56^0C. PCR reactions were carried out in 50 µl reactions for 30 cycles using the profile: 94^0C, a gradient of annealing temperatures 71{510^C at 45sec each, elongation 72^0C all for 45sec.

Preparation of the 8% polyacrylamide denaturing gradient and gel electrophoresis was done according to the manufacturers instructions for the D-Code Universal Detection System (Bio-Rad) with a 30–50% gradient of urea and formamide. The gel was run in Tris-acetate buffer and pre-heated to 59^0C. The gel was run at 130V for 2 hours or until the xylene cyanol dye front reached the lower end of the gel. DNA was visualized by UV irradiation following stain with ethidium bromide. Bands were excised and reamplified, using the same primers and profile but without the GC clamp. This second PCR product was purified and sequenced with the HDA forward primer via

dideoxy chain termination. Analysis of results was carried out using the GenBank nucleotide database and BLAST algorithm [39].

Acknowledgments

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8.

Probiotic strategies for the treatment and prevention of bacterial vaginosis

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Roderick MacPhee
Ruben Hummelen
Jordan Bisanz
Wayne L Miller
Gregor Reid

Abstract

Importance of the field: Urogenital infections are on average the number-one reason for women to visit the doctor. Yet, treatment and preventive strategies have gone unchanged for close to 50 years. With prevalence rates for bacterial vaginosis at more than 29%, depending on the population, and similarly high incidences of vulvo-vaginal candidiasis and urinary tract infections, plus HIV, new therapies are urgently needed to improve the health of women around the world.

Areas covered in the review: This review discusses the vaginal microbiota, our improved understanding of its composition, and its role in health and disease. It also discusses the progress made in the past 10 years or so, with the development and testing of probiotic lactobacilli to improve vaginal health and better manage urogenital infection recurrences.

What the reader will gain: The reader will have an understanding of the clinical data obtained so far, and the potential mechanisms of action of probiotics. Despite the need for more clinical studies, the review illustrates a case for inclusion of probiotics as part of the approach to disease prevention, and as an adjunct to antimicrobial treatment. Challenges remain in optimizing clinical benefits, selecting new strains, preparing new products and having them tested in humans then approved with informative claims, and making products readily accessible to women in the developed and developing world.

Take home message: The vaginal microbiota is a complex structure that can change quickly and dramatically and significantly impact a woman's health. New health-maintenance and disease-treatment approaches are badly needed, and probiotics should be considered.

The vaginal microbiota

The vagina is a dynamic environment colonized by a wide range of microorganisms that are collectively referred to as the vaginal microbiota. The most important constituent of the microbiota in healthy women are members of the genus *Lactobacillus.*

A decade ago, *L. crispatus* and *L. jensenii* were viewed as the predominant vaginal species [1]; however, with recent advances in culture-independent community profiling, the nutritionally fastidious *L. iners* is emerging as a dominant organism, present in both healthy and lactobacilli-deficient aberrant vaginal environments [2]. Bacterial migration from the rectum, across the perineum to the vagina occurs naturally in women and is a source of both pathogens as well as lactobacilli. The latter are believed to offer protective effects against pathogens through displacement, modulating immunity, production of antimicrobial substances and lowering vaginal pH [3,4].

Estrogen levels confer a wide range of effects on the vaginal environment, including influencing the microbiota [5,6]. Estrogen stimulates glycogen production by vaginal epithelial cells, and the glycogen is then metabolized to glucose, which can then be fermented by lactobacilli as an energy source. Lactic acid, as a byproduct of metabolism, is released and functions to acidify the vaginal environment from a pH of 5 to 3.8 - 4.2. The lactic acid is a key antimicrobial agent in the vaginal fluid and has been shown to affect uropathogenic *Escherichia coli* surfaces [7,8]. During ovulation, an increase in lactobacilli numbers has been observed with a sub-sequent decrease during menses, resulting in a higher incidence of bacterial vaginosis (BV) [9,10]. At present, it is not possible to know to what extent a depletion of lactobacilli *per se* is associated with the clinical exacerbation of postmenstruation, but certainly BV is one factor.

In prepubescent girls, estrogen levels are low and the vaginal epithelium is thin and lacking in glycogen. This is associated with a low abundance of lactobacilli replaced by mostly anaerobic Gram-negative rods and Gram-positive cocci. Similarly, in postmenopausal women with low estrogen levels, lactobacilli are less dominant, but become restored when hormone replacement therapy is administered [11]. The replenishment of the lactobacilli may involve an increase in

receptor sites that allow more transient binding of the organisms to epithelial cells [12].

The microbiota may vary among racial groups. Zhou *et al.* [13] observed, in a group of 144 North American caucasian and black women, that 33% of black women had communities that were not dominated by lactobacilli, while only 7% of caucasian women displayed communities that were not dominated by lactobacilli. They speculated that the lack of a protective microbiota might explain the increased susceptibility to BV in the black population. The problem can carry forward to complications associated with an aberrant vaginal microbiota, as illustrated in a study of 11,910 women enrolled at 23 - 26 weeks' gestation in which the proportion of preterm birth associated with lower genital tract infection was 21% among African Americans and 5% among whites [14]. In a study of 531 women at high risk of unplanned pregnancy, BV and sexually transmitted infection was shown to be significantly more associated with African American than white women, although race and color *per se* do not explain this finding [15].

Bacterial vaginosis

Bacterial vaginosis is a common infectious condition, affecting an estimated one third of all women at any given time and is characterized by a depletion of lactobacilli and a resulting alteration of the vaginal microbiota [16]. The reduction in lactobacilli makes for a more conducive vaginal environment for the proliferation of many anaerobic bacteria, such as *Gardnerella vaginalis, Atopobium vaginae* and *Mobiluncus curtissi.* Recent molecular techniques have allowed for a more thorough detection of bacteria than traditional culture, with the result of increased bacterial diversity associated with BV [17].

Current diagnosis of BV can be achieved by the Amsel criteria based upon the presence of a milk-like discharge, odor, a pH of > 4.5 and epithelial cells covered in Gram- negative rods. Another diagnostic test involves microscopic examination of a vaginal smear and a scoring system that is based upon the presence or absence of lactobacilli, Gram-negative rods and various bacterial morphotypes [18]. Some rapid diagnostic systems have been developed, primarily based upon elevated pH [19,20]. The symptoms and signs of BV do

significantly impact the quality of life of women [21] but are only reported in the minority of cases [22]. Irrespective of symptomatology, BV has been associated with an increased susceptibility to preterm labour perhaps owing to inflamma- tory processes [23], sexually transmitted infections including HIV infection, perhaps due to a damaged epithelial layer or altered expression of protective compounds like elafin [24,25], and pelvic inflammatory disease caused by pathogens [26]. These sequelae of BV are difficult to manage and many are not resolved with current therapeutics. This places an increased importance on developing new ways to treat and prevent this condition, with the use of probiotics being one such option.

The selection of urogenital probiotic strains

The criteria used to select bacterial strains for potential use as urogenital probiotics have evolved with time. From 1987 to 2002, the criteria included production of antimicrobial substances such as bacteriocins and hydrogen peroxide (H_2O_2), adhesiveness to epithelial cells and *in vitro* inhibitory activity against known BV pathogens [27-30]. This was based on the premise that probiotic bacteria have to adhere to or colonize epithelial cells and inhibit the adhesion and growth of pathogens in order to be beneficial. The inclusion of H_2O_2 production was based on lactobacilli isolated from healthy women often producing this substance [31]. The concept of adhesion led to the isolation of strains that were highly adherent to epithelial cells, with the expectation that they would then persist better in the host. There has only been one study correlating *in vitro* predictability with *in vivo* persistence [32], and thus it is difficult to say that this is a definitive criterion for clinical success with all probiotics. Today, the ability to persist in the vagina and produce metabolites that benefit the host are more important than adhesion scores to cells and/or mucus, and this process probably involves the ability to survive pH, cope with hormonal and modulate host responses, as well as use various compounds for growth, and somehow outcompete organisms already present. The most extensively tested probiotic combination for vaginal health, *L. rhamnosus* GR-1 and *L. reuteri* RC-14, has been detected in the vagina for up to 19 days after vaginal administration, whereas intestinal probiotic *L. rhamnosus* GG also inserted vaginally

was only detected for up to 5 days post-administration [33]. This would indicate that mining the genes of these organisms might identify factors that allow some strains to take advantage of certain biological niches. One study implanted *L. rhamnosus* GR-1 into the vagina and showed some upregulation of host defenses [34], while another showed an ability to displace BV pathogens [35].

The ability to inhibit pathogenic bacteria can be achieved through a number of different mechanisms. A study of 60 vaginal *Lactobacillus* isolates showed that the four strains exhibiting the greatest activity against BV-associated bacteria were all capable of producing H_2O_2 [29], although it was unclear that this compound was solely responsible. Clearly, it is possible to inhibit urogenital pathogens without the necessity for H_2O_2 [28,36]. In addition, while hydrogen peroxide is toxic to *G. vaginalis* at high concentrations [37], it is ineffective or irrelevant against *G. vaginalis* biofilms [38,39].

The ability of a probiotic to survive passage through the gastrointestinal tract becomes an important selection criterion when oral administration is intended. While it perhaps seems most logical to deliver probiotics by direct vaginal administration, studies have shown that oral delivery is also feasible to deliver the lactobacilli to the vagina [40-42], and the finding of indigenous lactobacilli in the rectum and vagina further supports this delivery concept [43]. Direct vaginal application may be preferable for treatment of BV; however, the ability to deliver probiotics orally could be more convenient for long-term prevention.

Regardless of the initial selection criteria used *in vitro* to identify potential probiotics, the evidence so far highlights the importance of ultimately selecting probiotic strains from clinical trials. Also, the differences in activity observed, even between strains of the same species, disproves a common misconception that all strains of *L. rhamnosus* or *L. acidophilus* are probiotic. Just because one strain (or combination of strains) has been proven as a probiotic, it does not necessarily follow that all strains of the same species are equally effective.

Probiotics for the treatment of bacterial vaginosis

The current approach to the treatment of BV is through the application of antibiotics such as metronidazole or clindamycin. Although these treatment options yield a reasonable cure rate immediately following treatment, recurrences are common within weeks and months, and side effects including drug resistance are also common [44,45]. The potential for probiotics to enhance the cure rate of antibiotics is therefore appealing. Numerous clinical trials have been undertaken with the aim of assessing the effectiveness of probiotic intervention to treat BV. A number of trials have investigated the use of probiotics or candidate probiotics to treat BV (Table 1).

A recent small trial performed by Mastromarino *et al.* [46] tested the effectiveness of three candidate probiotic strains used in combination to improve cure rate and symptoms of BV in 34 women. BV was diagnosed by Nugent score (7 - 10), Amsel criteria (3 - 4 criteria) and vaginal symptoms (discharge and/or malodor). Subjects were randomized to receive one vaginal tablet containing *L. brevis* CD2, *L. salivarius* subspecies *salicinius* FV2 and *L. plantarum* FV9 at ≥ 10^9 colony-forming units (CFU) each (18 of 34 subjects; 53%) or placebo (16 of 34 subjects; 47%) once daily for 7 days. Cure of BV was defined by Nugent score (1 - 6). Immediately following therapy, 18 of 18 subjects (100%) in the probiotic group were free of BV, with 15 (83%) and 3 (17%) subjects having a normal and intermediate vaginal microbiota, respectively. Of the placebo group, only 2 of the 16 subjects (12%) were free of BV and had an intermediate microbiota (p < 0.001). An improved BV cure rate was maintained in the treatment group 14 days after termination of therapy, with 11 of 18 subjects (61%) free of BV in the probiotics group com- pared with 3 of 16 (19%) in the placebo group (p < 0.05). Subjects taking the probiotics also experienced reduced symptoms, including discharge and malodor. This study thus substantiates the evidence for lactobacilli to treat BV, although future trials with larger sample sizes will need to be conducted.

Similarly, Hallén *et al.* [47] investigated the potential of lactobacilli to treat BV in 57 women. BV was defined by Amsel criteria (3 - 4 criteria). Subjects were randomly assigned to receive suppositories

containing 10 ^{8} - 10^9 CFU of lyophilized *L. acidophilus* (28 of 57 subjects; 49%), or placebo capsules (29 of 57 subjects; 51%), inserted intravaginally twice daily for 6 days. Immediately following therapy, 16 out of 28 women (57%) in the treatment group had normal vaginal wet smear results compared with 0 of 29 (0%) in the placebo group ($p < 0.005$), as defined as lactobacilli content of wet smears. The definition of BV cure as an improved vaginal microbiota is unclear, however, and should also include the Amsel criteria as used in the initial screening of this trial. It

should also be noted that the bacteria tested, *L. acidophilus*, was not strain-specific and thus cannot be considered a probiotic.

Rossi *et al.* [48] investigated the effectiveness of certain bacteria to normalize vaginal pH and improve symptoms associated with BV, at a longer term of therapy relative to the other trials discussed. Subjects included 40 women with BV as defined by the Amsel criteria (3 - 4 criteria). All subjects were given vaginal tablets containing *L. rhamnosus* at $> 10^6$ CFU - 1 tablet once daily for 6 days, then 1 tablet twice a week for 2 months and then 1 tablet once a week until 24 months. No placebo group was included in the trial. Following therapy, vaginal pH was < 4.5 in 24 out of 40 subjects (60%) after 12 months and in 32 out of 40 (80%) after 24 months, compared with only 4 out of 40 (10%) before treatment ($p < 0.001$ and $p < 0.02$ for 12- and 24-month follow-up, respectively). Subjects also reported a decrease in itching, vaginal discharge and burning at the 12-month and 24-month follow-up visits. Investigators of this trial did not consider differences in the subjects in terms of the length of time from treatment with antibiotics to enrolment into the trial. Also, results of this study cannot infer the effect of this treatment on BV cure because standardized tests such as the Amsel criteria or Nugent scoring were not used to determine prevalence of BV following treatment.

In the only study published so far comparing the BV cure rate of intravaginal probiotics and antibiotics, *L. rhamnosus* GR-1 and *L. reuteri* RC-14 were administered each night for 5 days versus 0.75% metronidazole gel applied vaginally twice a day to 40 women, and the probiotic proved to be more effective [49]. The subjects were randomized, but because of the treatments they were not blinded to what they received. Nevertheless, the measure of cure was not primarily based upon subject perception, but rather on Nugent

scoring and a BV-Blue test for sialidase. Whilst it is always feasible that discomfort and irritation could be swayed by subjects believing that a new therapy (i.e., probiotics) works and the old one (antibiotic) might not, the clinical presentation of odor and discharge is more definitive in combination with Nugent and BV-Blue scoring. Thus, the likelihood of bias is remote. Follow-up at days 6, 15 and 30 showed cure of BV in significantly more probiotic-treated subjects (16, 17 and 18/20, respectively) compared with metronidazole treatment (9, 9 and 11/20: $p = 0.016$ at day 6, $p = 0.002$ at day 15 and $p = 0.056$ at day 30). Confirmatory studies are needed, but some might argue that the cure rate with metronidazole in the previously mentioned study was much lower than normally expected. However, metagenomic studies are now showing that, at least in African women, metronidazole is poorly effective in curing BV and at best only useful in changing the abundance of pathogens (Hummelen *et al.*, unpublished data).

Table 1. Studies in the past two years that have examined the use of probiotics or candidate strains to improve vaginal health

Study	Design (n)	Inclusion Criteria	Intervention	Main Findings
Mastromarino et al. 2009 [46]	R, DB, PC (34)	Diagnosed with BV by Amsel criteria and presence of discharge and/or malodour	1 Lactobacillus tablet containing L. brevis CD2 + L. salivarius subsp. salicinius FV2 + L. plantarum FV9 ($\geq 10^9$ CFU each) or placebo daily for 7 days.	Higher cure rate after 7 days in probiotic group (18/18) compared to placebo group (2/16) (p <0.001) and after 21 days in probiotic group (11/18) compared to placebo group (3/16) (p < 0.05).
Rossi et al. 2010 [48]	OL (40)	Diagnosed with BV by Amsel criteria	Vaginal tablets containing Lactobacillus rhamnosus at >10^6 CFU – 1 tablet once daily for 6 days, then 1 tablet twice a week for 2 months, then 1 tablet once a week until 24 months.	Vaginal pH of <4.5 in 4/40 subjects prior to treatment compared to 24/40 subjects after 12 months and 32/40 subjects after 24 months of treatment (p < 0.001 and p < 0.02, respectively) .
Martinez et al. 2009 [51]	R, DB, PC (64)	Diagnosed with BV by the Amsel criteria and Nugent score (7-10)	Tinidazole (2 g) plus either 2 oral capsules of L. rhamnosus GR-1 and L. reuteri RC-14 (1×10^9 CFU of each strain) or 2 placebo capsules taken daily for 28 days.	Higher cure rate of BV in probiotic group (28/32) versus placebo group (16/32) (p < 0.05).
Larsson et al. 2008 [52]	R, DB, PC (100)	Diagnosed with BV by the Amsel criteria	2% vaginal clindamycin cream for 7 days followed by a vaginal lactobacilli or placebo capsule for 10 days during 3 consecutive menstrual cycles (Lactobacilli: >10^{8-9} CFU L. gasseri Lba EB01-DSM 14869 and >10^{8-9} CFU L. rhamnosus Lbp PB01-DSM 14870)	No improvement in initial cure rate in treatment group (37/48) compared to placebo group (39/50), but an increase in the avoidance of relapse after 6 months in treatment group (24/37) compared to placebo group (18/39) (p = 0.027).
Petricevic et al. 2008 [54]	R, SB (190)	Diagnosed with BV by Nugent score (7-10)	2x300 mg clindamycin for 7 days. Intervention group received vaginal Lactobacillus capsule (>10^9 CFU L. casei rhamnosus Lcr35) for 7 days following antibiotic treatment.	Higher restoration of vaginal microbiota in treatment group (69/83) versus placebo group (31/88) (p < 0.001).

Table 1. Cont. Studies in the past two years that have examined the use of probiotics or candidate strains to improve vaginal health

Study	Design (n)	Inclusion Criteria	Intervention	Main Findings
Petricevic et al. 2008 [58]	R, DB, PC (72)	Postmenopausal women aged 55-65 years with intermediate vaginal microbiota (Nugent score 4-6)	Capsules containing 2.5×10^9 CFU each of lyophilized *L. rhamnosus* GR-1 and *L. reuteri* RC-14 (intervention) or placebo once daily for 14 days	Higher improvement of restoration in probiotic group (21/35) compared to placebo group (6/37) (p = 0.0001)
Beereport et al. 2009 [58]	R, DB, PC -252	Postmenopausal women with recurrent UTIs	12-months prophylaxis with either TMP/SMX 480 mg QD or oral capsules with 10^9 cfu *L. rhamnosus* GR-1 and *L. reuteri* RC-14 BID.	The mean number of UTIs went from 7.0 to 2.8 in the TMP/SMX and 6.8 to 3.1 in the *Lactobacillus* group.
Hemmerling et al. 2009 [60]	P1, R, DB, PC (12)	Healthy women	Vaginal applicators containing *L. crispatus* CTV-05 given at one of 3 doses (5×10^8, 1×10^9, and 2×10^9 CFU/dose) or placebo once a day for 5 days	Product accepted and well-tolerated: occurrence of 45 adverse events, evenly distributed between the probiotic and placebo groups. No subjects (0%) discontinued from the study. 10/12 subjects agreed to statements regarding satisfaction of product.
Antonio et al. 2009 [61]	R, DB (90)	Healthy young females aged 14-21 years	Intravaginal capsules containing either 10^6- or 10^8-cfu *L. crispatus* CTV-05 twice daily for 3 days	Moderate colonization efficacy with 60/87 participants colonized. 36/40 subjects colonized who lacked *L. crispatus*, compared to 24/47 who initially had *L. crispatus* (p < 0.001). Decreased colonization associated with protected (p = 0.02) and unprotected (p < 0.001) sex.

R=randomized; DB=double-blind; SB= single-blind; PC= placebo controlled; P1=phase-1 trial; OL=open-label; CFU=colony-forming units *unless otherwise specified

Secondary prevention of bacterial vaginosis

Two clinical studies examined whether probiotics could treat infection and prevent recurrences. The first was a study by Anukam *et al.* [50] which showed in 125 women that probiotic *L. rhamnosus* GR-1 and *L. reuteri* RC-14 augmented cure of BV with 500 mg metronidazole therapy. The combination therapy doubled the cure rate of BV (88 vs 40%; p < 0.001) and did not lead to recurrent infection, albeit the follow- up was short. A second independent study by Martinez *et al.* [51], showed similar results (87.5 vs 50% cure; p < 0.05) using the same probiotic lactobacilli orally (2 capsules/day of 1 X 10^9 CFU for each strain) plus 2 g tinidazole to treat 64 women diagnosed with BV. In addition, at 5-week follow-up, 75% of women in the probiotic group had a normal vaginal microbiota compared with only 34.4% in the placebo group.

A larger trial (n = 100) was undertaken by Larsson *et al.* [52] which also tested two strains, *L. gasseri* Lba EB01-DSM 14869 and *L. rhamnosus* Lbp PB01-DSM 14870, each at > 10^{8-9} CFU/capsule administered once daily for 10 days during three consecutive menstrual cycles, as a means to reduce recurrences of BV following 2% clindamycin cream therapy for 7 days. The initial cure rate in each group as determined by a score of 1 in the Hay/Ison scoring system was 37 of 48 women (77%) in those who subsequently received lactobacilli and 39 of 50 (78%) in those randomized to then receive placebo. Women who remained positive for BV after the first menstrual cycle were treated with antibiotics and discontinued from the study. At the 6-month follow-up, 24 of 37 subjects (64.9%) in the lactobacilli group remained BV-free compared with 18 of 39 subjects (46.2%) in the placebo group (p = 0.027). The investigators also isolated lactobacilli from subjects in the treatment group and verified that these matched the strains that were administered. This study therefore demonstrates the potential for these strains to help reduce the occurrence of BV, although more investigation is warranted.

A large trial (n = 255) conducted by Eriksson *et al.* [53] investigated the use of lactobacilli-containing tampons (*L. gasseri, L. casei* var. *rhamnosus* and *L. fermentum,* total of 10^8 bacteria) following 3 days of clindamycin ovule administration for BV. Following treatment, there was no improvement in cure rate of BV (51 of 91 =

56% with lactobacilli and 59 of 96 = 62% with placebo). The failure indicates that only certain strains of lactobacilli have the ability to augment antibiotics and reduce the risk of BV recurrences. The use of vaginally applied *L. casei* var. *rhamnosus* Lcr35 daily for 7 days, following treatment with 2 X 300 mg oral clindamycin for 7 days [54] in 190 women resulted in 83% showing a marked reduction in Nugent score by ≥ 5 grades (mean difference of 6.61), compared with only 35% in the control group (mean difference of 4.13; p < 0.001). It is important to note that this trial was observer-blind only, and so subjects from the control group were aware of the contents of their capsules and thus cannot be considered placebo. Nevertheless, this study demonstrated a beneficial effect of lactobacilli administered with clindamycin and raises the question of whether this remedy worked because of the strain or the route of administration, compared with the study using tampons [52]. A genomic comparison study might prove useful to compare the *L. rhamnosus* Lcr35 used here versus the *L. casei* var. *rhamnosus* used in the tampon study. In addition, it is feasible that the strains contained in the tampon countered each other through either bacteriocin production or by one strain negating a beneficial effect of another on host immunity.

A study by Shalev *et al.* [55], albeit with groups too small to be clinically significant, investigated the effect of yogurt enriched with bacteria to prevent recurrent BV in 46 women who have had ≥ 4 documented episodes in the past year. BV was defined by pH > 4.5, positive amine test and presence of clue cells in microscope analysis of vaginal secretions. Subjects were randomly assigned to two groups. In group 1, 23 of 46 subjects (50%) consumed 150 ml yogurt enriched with *L. acidophilus* (> 10^8 CFU/ml) daily for 2 months, followed by no yogurt for 2 months, and then 150 ml pasteurized yogurt daily. In group 2, 23 of 46 subjects (50%) consumed pasteurized yogurt for 2 months, followed by no yogurt for 2 months, and then consumed yogurt enriched with *L. acidophilus.* A significant reduction in the episodes of BV was seen in the first group, from 60% before treatment to 25% after the first month, while group 2 saw a decrease in episodes from 70 to 50% (p = 0.004). An increase of *L. acidophilus* in both the vagina and rectum was also seen in the first group compared with the second group. There was a considerable dropout rate in both groups for reasons unclear, which made it

difficult to specify the number of participants treated successfully at each follow-up.

A study of 84 BV patients randomized to receive either oral metronidazole 500 mg twice a day for 7 days, or one vaginal tablet containing freeze-dried *L. rhamnosus* once a week at bedtime for 2 months starting 1 week after the last antibiotic administration, showed a significant improvement long-term effect (up to 90 days) with the lactobacilli prophylaxis (p = 0.05) [56].

In the first study of its kind of postmenopausal women, 21 of 35 (60%) who received daily oral *L. rhamnosus* GR-1 and *L. reuteri* RC-14 for 14 days and only 6 of 37 (16%) receiving placebo showed a reduction in the Nugent score by at least two grades (p = 0.0001) [57]. Although this does not prove cure of BV, the clear displacement of BV organisms in favor of lactobacilli shows that probiotic lactobacilli treatment can impact vaginal health even when estrogen levels are low after menopause. The fact that the microbiota was improved within such a short timeframe argues well for adding a new option to the management of postmenopausal women at risk of BV. Another study with the same probiotic strains in postmenopausal women susceptible to urinary tract infections (UTIs), showed that 1 year of daily therapy was as effective as daily antibiotic in preventing recurrences [58]. The mean number of reported symptomatic UTIs the year before the study was 7.0 in the trimethoprim-sulfamethoxazole (TMP/SMX) and 6.8 in the probiotic group. After 12 months of prophylaxis these fell to 2.8 and 3.1, respectively. After 1 month on TMP/SMX, resistance to TMP/SMX, trimethoprim and amoxicillin increased from about 20 - 40% to about 80 - 95% in fecal and asymptomatic bacteriuria *E. coli.* This study not only showed that long-term safe and effective appli- cation of probiotics to women whose vaginal microbiota is the source of uropathogens and infection, but also showed that this approach significantly reduced antibiotic resistance amongst uropathogens.

The ability of probiotic lactobacilli to restore and main- tain a normal vaginal microbiota is important, given the high prevalence of BV [59]. In a study of 64 women, oral treatment with *L. rhamnosus* GR-1 and *L. fermentum (reuteri)* RC-14 once daily for 60 days resulted in the vaginal microbiota being restored from an asymptomatic BV state to a healthy state in 37% of subjects compared with 13% receiving placebo (p = 0.02). Six of the 25

subjects (24%) in the placebo group developed BV at day 35 and 4 (16%) at day 56 compared with 0 of the 23 (0%) in the treatment group (p < 0.05) [42]. This study, like that of Petricevic *et al.* [54] highlights the benefit of the everyday use of these lactobacilli strains as they help restore the vaginal microbiota and help prevent asymptomatic BV.

The safety record of probiotic lactobacilli for vaginal application is excellent. Hemmerling *et al.* [60] performed a Phase I dose-ranging safety trial for *L. crispatus* CTV-05, in 12 healthy women. Safety was assessed by recording the incidence of adverse events, while tolerability was assessed by recording the number of subjects who prematurely discontinued themselves from consuming the product because of an adverse event. Acceptability was evaluated through the use of a self-administered questionnaire. Subjects were instructed to insert vaginal applicators containing *L. crispatus* CTV-05 given at one of three doses (5×10^8, 1×10^9, and 2×10^9 CFU/dose; 3/12 subjects [25%] for each dose) or placebo (3/12 subjects; 25%) once a day for 5 days. Overall, 45 adverse events were found to occur evenly across the three doses and placebo group. Most events (91%) were mild in nature, while the other 9% were unrelated to product use, indicating that the product was safe. None of the subjects discontinued themselves and most (83%) agreed with statements regarding the satisfaction and comfort of the product, which indicated tolerability and acceptability of the product. The subjects in this group were healthy, unlike one of the target populations of BV women for this probiotic product. In addition, the sample size of 12 limits the reproducibility of findings from this study. Thus, more investigation will be required to test the safety and tolerability of this product in BV women.

Another strain that shows promise as a vaginal probiotic is *L. crispatus.* A company study that was not published in peer-reviewed literature showed that vaginal application of *L. crispatus* CTV-05 in BV patients did not statistically improve clinical cure rates at 30 days. The strain only 'colonized' 70% patients, but when it did there was better resolution of BV compared with women who received placebo. Although this led to the company terminating commercialization, studies on the strain continued. Antonio *et al.* [61] investigated the ability of *L. crispatus* CTV-05 to persist in 90 healthy young females aged 14 -21 years. Quantitative culturing methods were used to

measure the abundance of *Lactobacillus* species, while molecular techniques detected the abundance of the CTV-05 strain. Two groups of 45 subjects (50%) were randomly assigned to receive intravaginal capsules containing either 10^6- or 10^8 -CFU *L. crispatus* CTV-05 twice daily for 3 days. The CTV-05 strain showed moderate ability to persist in 60 of the 87 participants (69%). The strain was found in 36 of 40 subjects (90%) who originally lacked *L. crispatus*, compared with 24 of 47 subjects (51%) who had been colonized with indigenous *L. crispatus* (p < 0.001), indicating that the indigenous strains outcompeted the candidate probiotic. A decrease in colonization of the administered strain was linked to protected (p = 0.02) and unprotected (p < 0.001) sex.

Given the lack of advances in managing BV during the past 50 years, it is perhaps not surprising that women are receptive to the idea of using probiotics [62]. One study assessed the satisfaction of a product containing *L. crispatus* CTV-05 in 424 women who suffer from BV [63]. Satisfaction was assessed by having the subjects complete a standardized questionnaire after randomization to receive either an intravaginal capsule containing 1 X 10^8 CFU *L. crispatus* or placebo, applied twice daily for 3 days, monthly for 3 months. Only 232 of the 424 subjects (54.7%) completed the three follow-up visits for reasons not stated. A total of 175 of 232 subjects (75.4%) stated that they were satisfied with the capsule, which was found to be associated with improved Nugent score. No significant differences were found between the treatment and placebo group, although these numbers were not provided.

Expert opinion

There is clearly a significant need for new therapeutic and prophylactic approaches to the management of urogenital infections. Since the clinical observation of Bruce *et al.* in 1973 [64], efforts have been made to understand better not only the composition of the healthy vaginal microbiota but also the properties of lactobacilli that seem to protect the host from infection.

Metagenomic and other such techniques are uncovering the microbes that can colonize or transiently survive in the vagina. The methods used to select candidate probiotic strains are not so advanced and still in many cases consist of *in vitro* assays on

adhesion and production of antimicrobial agents, plus species commonly found in the vagina. This is somewhat 'hit and miss', as noted in studies that improve the health of the host, and others that seem to make no or little impact. Genomic sequencing of strains will help to determine some characteristics that may be beneficial *in vivo*, but the absence of an animal model, and the unsubstantiated tightening by regulatory agencies such as the FDA in allowing probiotic studies to be done in humans, mean that the development of new efficacious vaginal probiotics are some way off, at least in the USA. Nevertheless, a number of groups around the world are attempting to develop and test lactobacilli strains for vaginal use. In doing so, if they decide to use multiple strains, they need to do studies that show the benefits and absence of counteractive effects of these combinations. At the very least, competitive adhesion, bacteriocin and immunological assays should be done for this purpose.

Probiotics to date

Of the clinical studies carried out in an attempt to administer lactobacilli to the vagina, very few have had appropriate sample size and trial design, or strains selected with appropriate properties. Even though essentially only a handful of strains have been tested so far, the data strongly indicate the potential to improve vaginal health with probiotic lactobacilli. Studies have now shown that *L. rhamnosus* GR-1 and *L. reuteri* RC-14 can provide a positive impact on the vaginal microbiota in pre- and postmenopausal women. These or other strains will not colonize the host, nor always actually reach the vagina after oral intake, or dominate over time. Rather, the ultimate goal is for them to reduce the pathogens that ascend from the rectum and counter these and others that enter through sexual contact, in such a way as to lower the risk of disease. In addition, the therapy should provide a functional milieu that maintains microbial and host-immune homeostasis in a manner that optimizes the health of the host. This may mean allowing recovery of the woman's own beneficial microbiota, or repriming her natural defenses.

For a vaginally applied probiotic, current regulations characterize them as drugs or medical devices, with the former making the development process expensive and the latter difficult to be approved with suitable claims. This has delayed (and will continue to delay) the

availability of products for use by women, which is unfortunate as intravaginal application is needed to treat BV and to displace aberrant and recalcitrant microbiota.

Future studies

In the future, many new products are envisaged based upon an ability to manipulate the vaginal microbiome. Some will comprise probiotic strains, either singly or in combinations, others will be prebiotic nutrients designed to improve the abundance of nonpathogens, and others may target immunomodulatory effects. Interventions will probably include the creation of recombinant strains that deliver specific anti-infective compounds [65-67] particularly against viruses such as herpes simplex and HIV. Challenges for such applications will be substantial, including containment, lack of adverse effects and an ability to persist in sufficient numbers to neutralize the viruses when they enter, especially if they are protected by sperm and seminal fluid, and if host cells are present for their binding and invasion.

Challenges also come from scaling up strains from in vitro, animal and pilot human studies to a product that is suitable for development. These include being able to grow strains that can be fastidious, dry and prepare them in a form that survives storage and suitable shelf-time, and that delivers appropriate live numbers to the target site. In some cases, such as if L. iners were to be a developed as a probiotic, these problems are insurmountable at present. Companies with the expertise in mass culture of bacteria often do not have the time, expertise and financial incentive to do the R&D work required for new product development. In addition, most companies in the probiotic arena are in the food and supplements industry and either not willing or able to develop vaginal drugs or devices. Partnerships with financiers and biotech or pharmaceutical companies may be required to overcome these issues. The extent to which women suffer from urogenital infections and complications stemming from them is simply too great not to make major efforts to develop new products.

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9.

Lactobacillus rhamnosus GR-1 and *L. reuteri* RC-14 to prevent or cure bacterial vaginosis among women with HIV

Ruben Hummelen
John Changalucha
Nicodemus L Butamanya
Adrian Cook
J Dik F Habbema
Gregor Reid

Abstract

Objective: Bacterial vaginosis (BV) among women with HIV is highly prevalent, difficult to cure and significantly associated with HIV transmission. We aimed to enhance cure and prevention of BV among women living with HIV with long term oral *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14 supplementation.

Methods: We conducted a randomized, double blind, placebo controlled among 65 women living with HIV with an aberrant microbiota (Nugent score 4-10) who were randomized to receive daily probiotics or placebo for 6 months. Those having BV (Nugent score 7-10) received additionally metronidazole for 10 days (400 mg twice daily).

Results: We did not find an enhanced cure rate of adjuvant probiotics to metronidazole treatment of BV. Among those with an intermediate vaginal flora, probiotics tended to increase the probability of a normal vaginal flora (odds ratio = 2.4, p = 0.1) and significantly increased the probability of a beneficial vaginal pH (odds ratio = 3.8, p = 0.02) at follow-up.

Conclusion: We conclude that supplementation of probiotic *L. rhamnosus* GR-1 and *L. reuteri* RC-14 did not enhance the cure of bacterial vaginosis among women living with HIV, but may prevent the condition among this population.

Introduction

The vaginal flora is normally dominated by lactobacilli and can gradually shift towards a complex mix of anaerobic bacteria, *Gardnerella vaginalis* and *Mycoplasma hominis,* a condition called bacterial vaginosis (BV) [1]. Various observational studies have shown that BV is highly prevalent [2] and facilitates the transmission of HIV [3-5]. Efforts to reduce the prevalence of BV have been unsuccessful [6, 7], and innovative treatment strategies are desperately needed. Especially for women living with HIV among whom the cure rate of BV appears to be lower and the prevalence higher [6].

Lactobacillus rhamnosus GR-1 and *L. reuteri* RC-14 have been shown to improve the vaginal flora after oral administration [8-11], and enhance the cure rate of BV among women treated with metronidazole [12]. The apparent safety of probiotics in people living with HIV [13-15] led to the design of the current study. Our primary aim was to determine whether long term *L. rhamnosus* GR-1 and *L. reuteri* RC-14 supplementation could enhance the cure of BV among women living with HIV treated with metronidazole. In addition, we aimed to assess whether long term supplementation could prevent women with an intermediate vaginal flora from developing BV.

Methods

A randomized, double blind, placebo controlled trial with a follow-up of 25 weeks was conducted. Primary outcome was the cure rate of BV at two weeks follow-up. Secondary outcomes were; recurrence rate of BV, the presence of a beneficial vaginal pH (<4.7), symptoms (itching, odor, discharge) and prevention of BV among those with an intermediate vaginal flora at baseline.

Participants were randomized to receive either daily capsules of freeze dried *L. rhamnosus* GR-1 and *L. reuteri* RC-14 (2×10^9 viable organisms) or identical looking placebo capsules, twice daily for 25 weeks (provided by Chr Hansen, Horsholm, Denmark). Additionally, participants diagnosed with BV by Nugent score (7-10) received metronidazole for 10 days (Aventis, twice daily 400 mg orally). A block randomised list was generated by a computer and a statistician

not involved in the data collection prepared a tube set with either placebo or probiotics for each participant accordingly.

The Medical Ethical Review Committee of Erasmus Medical Centre, The Netherlands, and the Medical Research Coordinating Committee of the National Institute for Medical Research, Tanzania, approved the study design and protocol. Participants were informed of the purpose of the trial and gave their signed or thumb printed informed consent before participation. This trial was registered at clinicaltrial.gov NCT00536848.

Women who attended the HIV care and treatment clinic at Sekou-Toure regional hospital, Mwanza, Tanzania, were invited to participate. Participants were consecutively enrolled between October 2007 and February 2008 and the follow-up continued until July 2008. Inclusion criteria were, confirmed HIV infection, not eligible to be treated with antiretroviral medication, abnormal vaginal flora (Nugent score 4 – 10) and being 18 - 45 years old. Exclusion criteria were pregnancy, lactation, menstruation at time of screening, hypersensitive to metronidazole and presence of cervicitis, genital ulcers or *Trichomoniasis* during gynaecologic examination.

During screening, a gynaecological examination was performed to diagnose cervicitis (mucopurulent endocervical exudate on visual inspection) and measure the pH using a dipstick (range: 2.0 – 9.0, Merck, Germany). Two swabs from the vaginal wall were taken: one sample was used to diagnose *Trichomoniasis* (presence of motile flagellates on wet mount), the other sample was used to make a Gram stained smear to evaluate *Candida* colonization (budding yeast) and grade the vaginal flora according to the Nugent score at the National Institute for Medical Research, Mwanza, Tanzania. For quality control of the Nugent scoring all slides at baseline, 2 weeks and 25 weeks were re-evaluated by the microbiological laboratory of Weill-Bugando University college of Health Sciences, Tanzania. Agreement between the laboratories was substantial (kappa = 0.67, 95% CI; 0.57 – 0.76). The gynaecologic examination described was repeated at follow-up visits at 2, 5, 15 and 25 weeks. At a baseline visit information on demographics, gynaecologic history and current genital symptoms was collected using a structured questionnaire. During follow-up visits at 2, 5, 10, 15, 20, 25 weeks participants were interviewed about vaginal symptoms, adverse events and remaining capsules were counted to assess compliance. When symptoms and

signs of BV were present during follow-up, participants were (re-) treated with metronidazole from the follow-up visit at week 5 onwards.

To detect a difference in cure rate of BV at two weeks follow-up between 80% in the probiotics group and 40% in the placebo group the calculated sample size was 46 patients (23 in each group, two sided test with $\alpha = 0.05$ and $\beta = 0.80$). While sample size was based on a cross-sectional comparison, power was increased in some analyses by the use of longitudinal methods for repeated measurements.

We defined cure of BV as a BV Nugent score at baseline (7-10) with a normal Nugent score (1-3) during follow-up. Recurrence was defined as a BV Nugent score at baseline, a normal Nugent score during follow-up (first visit with normal score) and then a BV Nugent score again after initial cure (first visit with BV score).

The prevalence of a normal vaginal flora (Nugent 1-3) was compared cross-sectionally between the probiotic and placebo groups. Differences were tested using a chi-square test and with a Fisher's exact test when less then 5 participants per sub-group were present. To take into account the correlation between measurements of the same subject we used generalized estimating equations with a binominal outcome, a logit link and an exchangeable correlation structure to compare the overall probability of a normal vaginal flora and a beneficial vaginal pH during follow-up.

Compliance with the study regimen was calculated as the number of capsules that had been taken divided by the total number of capsules that should have been taken, and tested using a T-test. Participants contributed to analyses until participation ended due to loss to follow-up or initiation of anti-retroviral medication. Data was stored in an access database and analyzed using SPSS version 15.0.

Results

Of 229 screened patients, a total of 65 participants were eligible. After randomization, 4 participants were excluded from analyses as 2 were HIV-negative during a re-check, 1 had a normal baseline vaginal flora and 1 did not return for follow-up after the baseline visit. Of those remaining, 29 received probiotics (20 BV and 9 Intermediate) of whom 23 completed the study, and 32 received placebo (23 BV

and 9 Intermediate) of whom 28 completed the study (Figure 1). Loss to follow-up was similar (p = 0.3) between the probiotic [4 of 29 (14%)] and placebo group [2 of 32 (6%)], there was also a similar (p = 1.0) loss due to anti-retro viral treatment initiation among the probiotic [2 of 29 (7%)] and the placebo group [2 of 32 (6%)] (Figure 1). No clinically relevant differences were detected between groups (Table 1).
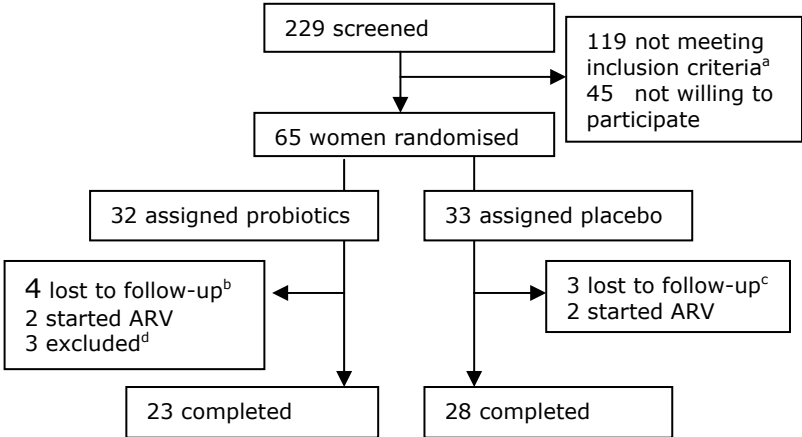
Figure1. Study profile

ARV: Anti retro-viral treatment
[a] 86 Negative Nugent score, 15 sexual transmitted infection and 18 eligible for ARV.
[b] 2 losses due to travelling, and 2 for unknown reasons.
[c] 2 losses due to travelling, and 1 for unknown reasons.
[d] 2 HIV negative, 1 Nugent score 1-3 at baseline

After two weeks follow-up of those having BV at baseline 8 of 20 (40%) participants were cured among the probiotics group compared to 12 of 22 (54%) among the placebo group (p = 0.3) (table 2). Cross sectional comparison of the prevalence of a normal vaginal flora did not reveal any significant differences (Table 2). At 15 weeks a trend was seen (p = 0.08) for a higher prevalence of a normal flora among the probiotics group [9 of 17 (53%)] than the placebo group [5 of 20 (25%)]. The overall probability of a normal vaginal flora during follow-up did not differ between the groups (odds ratio 1.06 for probiotic group, p = 0.9). No difference (p = 1.0) was detected between the recurrence rates of BV among the probiotics group [7 of 12 (58%)] and the placebo group [9 of 15 (60%)]. The probability of

a beneficial pH (pH < 4.7) (table 3) and the occurrence of vaginal symptoms during follow-up were similar between groups (data not shown).

Among those with an intermediate vaginal flora at baseline the prevalence of a normal vaginal flora was consistently higher among the probiotics group, but did not reach significance with cross-sectional comparison (table 2). Longitudinal comparison showed a trend of a higher probability among the probiotics group of a normal vaginal flora during follow up (odds ratio 2.4, p = 0.1). Among those receiving probiotics the prevalence of a vaginal pH of < 4.7 was consistently higher with cross-sectional comparison, being significant (p = 0.04) at 5 weeks of follow-up (Table 3). Longitudinal comparison showed that participants receiving probiotics had a significantly higher probability of a pH < 4.7 (odds ratio = 3.8, p = 0.02), this association remained after adjusting for baseline pH. No differences in vaginal symptoms during follow-up were detected.

Overall, no difference in cure rate was detected between those with a CD4 count of < 350 cells/μl [14 of 26 (54%)] and those with a CD4 count of > 351 cells/μl [6 of 16 (38%)] (p = 0.3). The probability of cervicitis appeared to be lower among the probiotics group [2 of 29 (7%)] than the placebo group [7 of 32 (22%)] (p = 0.1). There was no difference in *Candida* colonization between the probiotic group [13 of 29 (47%)] and the placebo group [9 of 32 (28%)] (p = 0.2).

Among those receiving probiotics, 7 participants reported an adverse event (6 gastro-intestinal, 1 vaginal) compared to 10 participants receiving placebo (5 gastro-intestinal, 2 vaginal, 2 itching skin and 1 dizziness). A physician rated all events mild or moderate, no severe events occurred and treatment was not interrupted on any occasion. There was no difference (p = 0.2) in compliance between the probiotic group (92% of days compliant) and the placebo group (89% of days compliant).

Table 1. Baseline characteristics

Characteristic		Placebo (n=9) n(%)	Probiotics (n=9) n(%)	p	Placebo and metronidazole (n=23) n (%)	Probiotics and metronidazole (n=20) n (%)	p
Age (years)	< 30	3 (33%)	3 (33%)	1.0	10 (44%)	8 (40%)	0.8
	≥ 31	6 (67%)	6 (67%)		13 (57%)	12 (60%)	
Education	No schooling	1 (11%)	1 (11%)	1.0	1 (4%)	1 (5%)	1.0
	Form 1-7	8 (89%)	7 (78%)		22 (96%)	18 (90%)	
	Grade 1-8	0	1 (11%)		0	1 (5%)	
CD4 (cells/µl)	< 350	5 (63%)	7 (88%)	0.6	14 (61%)	13 (65%)	0.8
	≥ 351	3 (38%)	1 (13%)		9 (39%)	7 (35%)	
Married		3 (33%)	3 (33%)	1.0	6 (26%)	10 (50%)	0.1
Number of sexual partners past 3 months	0	4 (44%)	7 (78%)	0.3	8 (35%)	10 (50%)	0.3
	1	4 (44%)	2 (22%)		15 (65%)	10 (50%)	
	≥ 2	1 (11%)	0		0	0	
Parity	0	1 (11%)	1 (11%)	1.0	1 (4%)	3 (15%)	0.3
	≥ 1	8 (89%)	8 (89%)		22 (96%)	17 (85%)	
Birth control	None	6 (67%)	7 (78%)	1.0	14 (61%)	16 (80%)	0.2
	Condom	1 (11%)	0		6 (26%)	3 (15%)	
	Hormonal	2 (22%)	2 (22%)		3 (13%)	1 (5%)	
History vaginal symptoms, treatment last episode if history.	No history	2 (22%)	3 (33%)	1.0	7 (30%)	5 (26%)	0.8
	No treatment	4 (44%)	3 (33%)		10 (44%)	10 (53%)	
	Antibiotics	1 (11%)	1 (11%)		5 (21%)	1 (5%)	
	Antifungal	2 (22%)	2 (22%)		1 (4%)	3 (15%)	

Table 2. Nugent score during follow-up.

	Intermediate group (Nugent 4-6)			Bacterial vaginosis group (Nugent 7 – 10)		
Week	Placebo Normal[a] n (%)	Probiotics Normal[a] n (%)	P	Placebo and metronidazole Normal[a] n (%)	Probiotics and Metronidazole Normal[a] n (%)	P
2	3/9 (33%)	7/9 (78%)	0.2	12/22 (55%)	8/20 (40%)	0.3
5	4/9 (44%)	6/8 (75%)	0.3	6/20 (30%)	5/20 (25%)	0.7
15	2/6 (33%)	3/8 (38%)	1.0	5/20 (25%)	9/17 (53%)	0.08
25	3/8 (38%)	5/8 (63%)	0.6	9/20 (45%)	8/17 (47%)	0.9

Table 3. Vaginal pH during follow-up

	Intermediate group (Nugent 4-6)			Bacterial vaginosis group (Nugent 7 – 10)		
Week	Placebo pH < 4.7 n(%)	Probiotics pH < 4.7n(%)	P	Placebo and Metronidazole pH < 4.7 n(%)	Probiotics and Metronidazole pH < 4.7n(%)	P
Baseline	1/8 (13%)	4/9 (44%)	0.3	0/22 (0%)	0/16 (0%)	-
2	3/7 (43%)	6/8 (75%)	0.3	10/19 (53%)	5/16 (31%)	0.2
5	2/8 (25%)	6/7 (86%)	0.04	7/20 (35%)	4/19 (21%)	0.3
15	1/5 (20%)	2/7 (29%)	1.0	5/17 (29%)	6/17 (35%)	0.7
25	1/7 (14%)	2/7 (29%)	1.0	8/17 (47%)	5/15 (33%)	0.4

Discussion

This study showed that the adjuvant use of *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14 did not improve the cure of BV in women with HIV. Treating BV is difficult among women living with HIV. This is evidenced by the finding that of the total BV group only 11 of 40 women (25%) were cured after metronidazole treatment at five weeks. This rate is profoundly lower than among non-HIV infected African woman (40%) and non-HIV infected Caucasian women (78 - 81%) after 30 days [12, 16].

A trend was found for a higher probability for a normal vaginal flora among those having an intermediate vaginal microbiota when taking probiotics and a significantly higher probability of a beneficial vaginal pH (< 4.7) at follow-up among the probiotics group. This suggests that, as in non-HIV infected subjects, the orally administered lactobacilli can reach the vagina or at least influence the environment to increase the count of indigenous lactobacilli [9, 17]. However, the failure of the antibiotics to eradicate the BV pathogens, perhaps as they are invariably in dense biofilms [18], or because a fully functional immune system is conjointly required, meant that a normal lactobacilli flora was not easily restored. The intransigence of BV organisms in HIV infected women needs further investigation as the continued presence of these bacteria increases the risk of transmission of HIV to a newborn child or partner.

In summary, this study showed that curing BV in women infected with HIV is extremely difficult, and while probiotics did not improve this cure, further studies are warranted to explore their use in preventing BV among this population.

Acknowledgements

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10.

General discussion

I. What is the long-term impact of probiotic use on the immune function of HIV patients?

Answer: It is not yet proven that use of probiotics, and specifically *Lactobacillus rhamnosus* GR-1, has a long-term impact on the immune function of HIV patients.

Comment: Two randomized controlled trials had previously suggested a benefit in immune function to HIV patients of probiotic consumption of up to two months [1,2] . We examined the impact of yogurt supplemented with *L. rhamnosus* GR-1 by monitoring the development of the immune function among a cohort of consumers and non-consumers with HIV for up to three years (chapter 4). We found that those consuming probiotic yogurt had a modest increase in CD4 count compared to the control group. This study therefore confirmed previous findings of beneficial effects of probiotics on the immune function of HIV patients. An important strength of this study was the long period of follow-up, an asset that would have been difficult to achieve using a prospective study design. However, due to the observational nature of the study, the results observed could have been due to selection bias of the intervention and control groups. Despite this limitation the similarities between the groups indicated that this bias was likely limited.

To further explore these findings, we conducted a randomized, placebo controlled, trial to assess whether an effect on immune function would be detectable after 25 weeks of probiotic use, this time using *L. rhamnosus* GR-1 and *L. reuteri* RC-14 in dried capsule form. In contrast to the observational study, no impact on the immune function was detected. This leads to three potential explanations. Firstly, because the intervention in the observational study included both a probiotic strain and yogurt the effect might have been caused by an enhanced nutritional status, resulting from consuming yogurt. Secondly, the effect observed in the observational study could have been due to the fact that the population studied was in a more advanced stage of HIV disease. This sub-population could have responded better to the intervention as a reduced gut barrier and the translocation of microbial products can become more profound at a later stage of disease [3]. Thirdly, the limited sample

size in the randomized trial may have prevented detection of effects with CD4 count as primary outcome (95% confidence interval -158 to 210 cells/µl). Trials with a sufficiently long follow-up and an adequate sample size are needed to further investigate this concept.

Following the study of Kaiser and colleagues [4] that showed a substantial increase in CD4 counts in HIV subjects given high doses of micronutrients, a similar, more physiologically based formula that was edible in yogurt was developed and tested in two human studies (Hummelen, Hemsworth & Reid, manuscript in preparation). In addition, with a view to sustainability and practical translation at the local sub-Saharan level, a yogurt formulation was recently developed that combines a locally grown vegetable (Moringa Oleifera), exceptionally rich in vitamins and minerals, with a yogurt supplemented with *L. rhamnosus* GR-1 (van Tienen, Hullegie, Hummelen & Reid, manuscript in preparation). The potential for such an intervention to provide further support for people with HIV and for whom access to ARTs is limited, is worthy of testing both from a clinical point of view and in terms of practical implementation at community kitchen sites being created in Tanzania, Kenya, Rwanda and potentially beyond.

II. Which micro-organisms constitute the vaginal microbiota among women with HIV?

Answer: *Lactobacillus iners* and *Gardnerella vaginalis* were found to be present in all subjects. In a BV state, the vaginal microbiota was dominated by *Lachnospiricieae*, *Prevotella bivia* or *Gardnerella vaginalis*. When BV is absent the vaginal microbiota was dominated by *L. iners* or *L. crispatus*.

Comment: In chapter 6, we describe the development and use of a novel technique to identify micro-organisms of the vaginal microbiota from a large cohort of Tanzanian women infected with HIV. By the use of this technique we acquired an extraordinary insight in the vaginal microbiota. Our investigations disproved the long held assumption that BV was associated with absence of lactobacilli, while pathogens were absent in health. Rather, the presence of *Lactobacillus iners* and *Gardnerella vaginalis* was detected in every

sample. This finding raises the question of whether these two species represent a "core vaginal microbiota" in all women. To address this question, we are currently analyzing vaginal samples from a large Brazilian cohort and from three Canadian studies, totaling more than 500 samples. The early results from a cohort in Vancouver suggest that depending on how deep the sequence abundance is explored, it is possible that these two species can be found in the majority of women. Cumulatively, these studies will enable us to compare the vaginal microbiota of non-African women with the population studied (African women with HIV) using the same technique. Whilst this does not allow assessment of how HIV infection impacts the vaginal microbiota by direct comparison of HIV+ and HIV- women from the same population, we aim to explore this in due course.

Another assumption previously held was that distinct *Lactobacillus* species (i.e*. L. iners*, *L. crispatus*) have a similar function in the vaginal tract, for example in maintaining a low vaginal pH. In our study, *L. iners* was found to only exert a modest influence on the vaginal pH, in contrast to *L. crispatus,* which appears to have a more profound impact. Interestingly, *L. iners* was found in an environment heavily colonized with pathogens and during antibiotic treatment, while *L. crispatus* appeared to be more easily displaced and killed. This suggests that the two *Lactobacillus* species fulfill different ecological functions in the vaginal tract, and have different abilities to survive. The recent genomic sequencing of *L. iners* has identified some of the factors that appear to allow this organism to persist so well [5].

The deep sequencing showed that no single pathogen is associated with BV. Rather, four different dominating micro-organisms are aligned with the current clinical diagnoses. This suggests that either the clinical diagnoses are sub-optimal, or the condition is caused by certain metabolic functions that can be induced by a range of pathogenic organisms. Investigations into these metabolic functions of the vaginal microbiota are now underway (Jean Macklaim, personal communication).

It is also possible that BV is caused by, or causes, immunological and host defense alterations that pave the way for the displacement of lactobacilli and propagation of pathogens. To investigate this, *in vitro* studies were performed to assess whether differences exist in the response of vaginal epithelial cells to various lactobacilli and *G.*

vaginalis. Preliminary results indicate that *L. iners* and *G. vaginalis* both provoke a very modest host response, in contrast to *L. crispatus* which induces the secretion of pro-inflammatory cytokines (IL-6 and IL-8) (Bisanz, Hummelen *et al.* manuscript in preparation). These findings suggest that *L. crispatus* may stimulate a strong host response that inhibits pathogen growth and colonization. Further studies are being carried out to assess the role of the host response. For example, the gene-expression patterns of vaginal epithelial cells obtained from women with various compositions of the vaginal microbiota are currently being analyzed (Hummelen, Bisanz *et al.* manuscript in preparation).

This evolving understanding of vaginal health has been made possible by the recent development of methods that facilitate the large scale sequencing of nucleotides. This has enabled the high-throughput analyses of genomic information, termed metagenomics, present in various human and environmental samples [6]. DNA sequencing by syntheses (Roche 454) is currently routinely used for metagenomic analyses, as it can sequence relatively long strands of DNA. However, it is imprecise as many insertions and deletions occur compared to Illumina, which sequences shorter strands of DNA [7]. For single genome sequencing, this is not a problem with Roche 454, but for micro-organism identification it means that analyses can only be done to the genus (e.g. *Lactobacillus*), and not to the species level (e.g. *Lactobacillus iners*). We have been the first to develop and use the Illumina sequencing method to identify micro-organisms. We describe in chapter 7 its capabilities and demonstrate that hundreds of samples can be analyzed at a sufficient depth to sample all diversity, at a fraction of the costs of conventional 454 analyses.

III. Can probiotics prevent or improve the cure rate of BV among women with HIV?

Answer: Additional use of *L. rhamnosus* GR-1 and *L. reuteri* RC-14 with metronidazole treatment does not improve the cure rate of BV in HIV patients, but it may help to prevent the condition.

Comment: In contrast to studies on HIV negative women [8,9], the probiotic strains did not improve the cure rate of BV among women with HIV. One factor that might explain this finding is the

apparent difference in the vaginal microbiota between the studied African cohort and non-Africans [10,11,12,13]. The latter showed high presence of hydrogen peroxide producing *L. crispatus* and *L. jensenii*, organisms associated with reduced occurrence of BV [12]. The low prevalence of *L. crispatus* and the virtual absence of *L. jensenii* among our population (chapter 6) could have contributed to the inefficacy of the probiotic strains and the rapid recurrence of BV.

Furthermore, he altered intestinal microbiota of people living with HIV, with high numbers of pathogens [14,15], could have prevented the probiotic strains from surviving the gut passage. To test this theory, the probiotic strains could be instilled directly into the vagina, which was shown to be feasible in HIV negative women [9], or different probiotics such as *L. jensenii* or *L. crispatus* could be tested to see if they survive gut passage and transfer to the vagina.

An additional explanation may be that a functional immune system is essential for lactobacilli to help cure BV. This could work via up-regulation of host-defences in the vagina including of the secretion of sIgA which then help dislodge the BV biofilms and allow better eradication by the antibiotics [16].

The finding that probiotic supplementation increased the likelihood of having a low vaginal pH during follow-up and a reduced probability of acquiring BV, suggests that probiotics could still have a role to play for women with HIV. Currently there is an urgent need to develop interventions to counter BV in this population [17]. This thesis emphasizes that new interventions need to be tested HIV among women with HIV specifically, as the findings from non-infected women are not necessarily transferable to this population.

Further considerations

Assessment of interventions that target the intestinal microbiota in HIV patients

Drawing from the results and experience obtained in conducting the studies described in this thesis, the following recommendations can be made. Firstly, before testing the efficacy of interventions on clinical outcomes, it would be valuable to identify biological markers that could provide insight in the potential of the intervention. Table 1 summarizes markers that could potentially be used for initial

screening of interventions targeting the intestinal microbiota of HIV patients.

Table 1. Biological markers for assessment of interventions that target the intestinal microbiota in HIV patients.

Measurement	Outcome	References
Plasma LPS	Microbial translocation	[18,19,20,21,22]
Plasma Total 16s RNA	Microbial translocation	[18]
Plasma secreted CD14	Microbial translocation	[18,22]
Urine Lactoferrin:Mannitol ratio	Intestinal permeability	[23,24]
Urine Xylose	Intestinal absorption	[22,24]
Fecal microbiota	Intestinal microbiota	[15,25]
Fecal calprotectin/ lactoferrin	Intestinal inflammation	[15]
Plasma CD4/CD25 cells	Immune activation	[26]
Natural Killer cells	Innate immunity response	[26]

Secondly, an intervention targeting the intestinal microbiota may have a more favorable outcome among those whose disease progression is characterized by leakage of microbial products from the intestinal tract. These sequela appear to become more profound later during the course of HIV infection [3,18,24]. Although ART reduces leakage of microbial products, still 20% of patients have persistent inflammation associated with poor CD4 count recovery [27]. Among children, ART fails altogether to reduce levels of leakage of microbial products [28]. Those characteristic may define the outcome of an intervention targeting the intestinal microbiota. For example, a Brazillian study on probiotics and HIV had selected a subpopulation of children and specifically those not responding to ART [2]. This may have selected those whom are likely to have a more favorable response to probiotic intervention and therefore, only limited inferences can be made for other sub-populations.

Thirdly, changes to Natural Killer cell cytotoxicity and immune activation markers (CD4/CD25) have been observed with a 12 week follow-up period and with a limited sample size (n = 57) [25,26]. This suggests that immune markers, other than CD4 count, could indicate the potential of an intervention to preserve immune function. One

criticism of biological markers to screen intervention clinical use, is that they have only limited predictive value for clinical outcomes. Therefore, when a potential benefit of an intervention is detected, this needs to be verified with a trial that has a sufficiently large sample size and length of follow-up, that can measure the impact of the intervention on defined clinical outcomes (e.g. mortality) or valid surrogate outcomes (e.g. CD4 count) [29]. The assessment of these outcomes likely requires a longer follow-up and a larger sample size.

Conclusions

- It is not yet proven that probiotic use, and specifically *L. rhamnosus* GR-1, has a long-term impact on the immune function of HIV patients
- *L. iners* and *G. vaginalis* are always present in the vaginal microbiota of HIV positive women living in East-Africa.
- When BV is present, the vaginal microbiota is dominated by *Lachnospiricieae*, *G. vaginalis* or *Prevotella bivia*.
- *L. crispatus* is strongly associated with a low vaginal pH (<4.5), but if *L. crispatus* is not present, a large fraction of *L. iners* is required to produce a low pH.
- The use of Illumina sequencing to profile the human microbiota is an effective and efficient method to increase the depth of microbial analyses.
- *L. rhamnosus* GR-1 and *L. reuteri* RC-14 do not improve antibiotic cure BV in women with HIV, but they may reduce the risk of developing the condition.

Recommendations

The following recommendations are made with respect to future directions:
- We recommend the conduct of a long term (>1 year), sufficiently sized, randomized trial to assess the long-term impact probiotics on the immune function of people living with HIV.
- We recommend the development of new interventions to restore and maintain vaginal health in women living with HIV.
- Illumina sequencing with combinatorial barcoding is recommended as standard for analyzing the human microbiota.

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Summary

The composition of the intestinal and vaginal microbiota has a profound impact on the health of the host. With an aberrant intestinal microbiota, and a frequent occurrence of a disturbed vaginal microbiota, HIV patients are at particular risk to experience adverse events. This thesis uncovers the vaginal microbiota of HIV infected women and explores whether probiotics can have an impact on health. Part I (chapter 2-5) describes whether probiotics can be used to target the intestinal microbiota to impact the progression of HIV. Part II (chapter 6 -7) describes an assessment of the vaginal microbiota of women with HIV. And part III (chapter 8 – 9) provides an assessment of probiotics to impact a disturbed vaginal microbiota among women with HIV.

I. What is the long-term impact of probiotic use on the immune function of HIV patients?

Chapter 2 provides a critical assessment of studies conducted of nutritional supplements to delay the progression of HIV. It concludes that vitamin B, C, E and folic acid can delay the progression of HIV. In contrast, vitamin A, iron and zinc have been associated with adverse events and caution is warranted for their use. Supplementation with selenium, *N*-acetyl cysteine, prebiotics and probiotics has considerable potential but evidence needs to be further substantiated. In Chapter 3 a theoretical framework is provided for the application of probiotics to ameliorate the impact of HIV on the epithelial barrier and the intestinal microbiota. It concludes that specific probiotic strains have the potential to counter these pathological mechanisms and may help to preserve the immune function of HIV patients.

An observational study is described in chapter 4 on the impact of probiotic *L. rhamnosus* GR-1 in yogurt on immune function of HIV patients. A total of 150 HIV patients were followed retrospectively for up to three years and their immune function was monitored. An increase in immune function was observed in the group who consumed the yogurt with *Lactobacillus rhamnosus* GR-1 compared to those who did not. Furthermore, those consuming yogurt had a smaller increase in CD4 count before the start of yogurt consumption

than after commencing consumption. Hence, the results suggest that probiotic yogurt use is associated with a preservation of immune function among HIV patients. This study is followed by a randomized, placebo controlled, trial, described in chapter 5, on the impact of daily use of encapsulated *L. rhamnosus* GR-1 and *L. reuteri* RC-14 for 25 weeks on the immune function of HIV patients. The effects observed in the observational study could not be confirmed in this trial. While *L. rhamnosus* GR-1 and *L. reuteri* RC-14 can be safely consumed at $2x10^9$ CFU/day, the treatment did not universally preserve the immune function of HIV patients.

II. Which micro-organisms constitute the vaginal microbiota among women with HIV?

Chapter 6 provides an in depth investigation of the vaginal microbiota in HIV positive women in Tanzania using a novel metagenomic technique. This study reveals a core microbiota consisting of *Garnerella vaginalis* and *Lactobacillus iners*, and suggests that *L. iners* and *L. crispatus* have two different functional roles. Also it describes that antibiotic treatment of BV perturbs the vaginal microbiota but rarely results in the establishment of a lactobacilli dominant state. Chapter 7 explores the attributes of the technique developed to identify the bacteria of the vaginal microbiota. It demonstrates that the technique, based on combinatorial barcoding and Illumina sequencing, greatly increases the depth and phylogenetic resolution of bacterial identification compared to methods currently in use, such as denaturing gradient gel electrophoresis or 454 Roche sequencing. This technique identified around 200 species present in the vaginal microbiota in 272 samples processed with one sequencing run. This also greatly reduced the costs and time involved in microbiota analyses.

III. Can probiotics prevent or improve the cure rate of BV among women with HIV?

Chapter 8 reviews mechanistic and clinical studies that have been performed to assess whether there is a potential for probiotics to have an impact on the vaginal microbiota. The review concludes that *L. rhamnosus* GR-1 and *L. reuteri* RC-14 have been shown to improve

the cure rate of BV and positively impact the vaginal microbiota among HIV negative women. To test whether probiotics are also effective in improving the vaginal microbiota among women with HIV, a randomized placebo controlled trial is described in chapter 9. The study assessed whether *L. rhamnosus* GR-1 and *L. reuteri* RC-14 in capsule form administered orally improve the cure rate of metronidazole, or reduced the risk of developing BV among women with HIV. It concludes that the probiotics did not improve the cure rate of BV but may reduce the risk of developing the condition.

Conclusions

- It is not yet proven that probiotic use, and specifically *L. rhamnosus* GR-1, has a long-term impact on the immune function of HIV patients
- *L. iners* and *G. vaginalis* are always present in the vaginal microbiota of HIV positive women living in East-Africa.
- The use of Illumina sequencing to profile the human microbiota is an effective and efficient method to increase the depth of microbial analyses.
- *L. rhamnosus* GR-1 and *L. reuteri* RC-14 do not improve antibiotic cure of BV in women with HIV, but they may reduce the risk of developing the condition.

Samenvatting

De samenstelling van de bacteriële flora heeft een groot effect op de gezondheid van de gastheer. Met een afwijkende darm flora en het frequent voorkomen van een verstoorde vaginale flora hebben HIV patiënten een verhoogd risico om de nadelige effecten hiervan te ervaren. Dit proefschrift beschrijft de vaginale flora bij vrouwen met HIV en onderzoekt of probiotica een effect kunnen hebben op de gezondheid van HIV patiënten. Deel I (hoofdstuk 2-5) beschrijft of probiotica ingezet kunnen worden om de darm flora te beïnvloeden en hiermee de verslechtering door HIV te vertragen. Deel II (hoofdstuk 6-7) geeft een omschrijving van de vaginale flora bij vrouwen met HIV. En deel III (hoofdstuk 8-9) bestudeerd of probiotica ingezet kunnen worden om een verstoorde vaginale flora bij vrouwen met HIV te herstellen.

I. Wat is het lange termijn effect van probiotica op de immuunfunctie van HIV patiënten?

Hoofdstuk 2 beschrijft studies die hebben geëvalueerd of voedingssupplementen de verslechtering van het immuunsysteem door HIV kunnen vertragen. Dit hoofdstuk concludeert dat vitamine B, C, E en foliumzuur de achteruitgang van het immuunsysteem door HIV kunnen vertragen. Vitamine A, ijzer en zink hebben hier geen effect op en zijn kunnen bijwerkingen veroorzaken. Hierdoor moet kan standaard gebruik van deze supplementen door HIV patiënten worden afgeraden. Het gebruik van selenium, *N*-acetylcysteïne, prebiotica en probiotica is veelbelovend maar wetenschappelijk bewijs schiet tekort om deze supplementen nu al aan te raden.

In hoofdstuk 3 wordt uiteengezet hoe probiotica mogelijk een rol kunnen spelen het verminderen van de effecten van HIV op de epitheliale darmbarrière en de darmflora. Dit hoofdstuk concludeert dat specifiek geselecteerde probiotica stammen mogelijk van invloed kunnen zijn bij deze pathologische mechanismen en mogelijk een rol kunnen spelen bij het langer behouden van een goede immuunfunctie bij HIV patiënten.

In hoofdstuk 4 wordt een observationele studie beschreven naar het gebruik van yogurt met *L. rhamnosus* GR-1 onder HIV patiënten en effecten van deze interventie op hun immuunfunctie. De

immuunfunctie van 150 HIV patiënten (probiotica en controle groep) werd tot 3 jaar retrospectief gevolgd. De studie concludeert dat onder deelnemers die de probiotica yogurt consumeerden een verbetering werd waargenomen ten opzichte van de deelnemers die geen probiotica yogurt consumeerden.

Een gerandomiseerde, placebo gecontroleerde studie wordt beschreven in hoofdstuk 5 waarbij HIV patiënten *L. rhamnosus* GR-1 en *L. reuteri* RC-14 in capsule vorm gebruikten of capsules voor 25 weken. Hoewel de studie liet zien dat het gebruik van probiotica niet geassocieerd was met bijwerkingen konden de effecten van probiotica op de immuunfunctie van HIV patiënten niet bevestigd worden.

II. Welke bacteriën maken deel uit van de vaginale flora bij vrouwen met HIV?

Hoofdstuk 6 beschrijft een onderzoek naar de vaginale flora bij vrouwen met HIV in Tanzania waarvoor een nieuwe techniek werd ontwikkeld en toegepast. De studie laat zien dat *Gardnerella vaginalis* en *Lactobacillus iners* bij alle Afrikaanse vrouwen deel uitmaken van de vaginale flora. Daarnaast laat de studie zien dat *Lactobacillus iners* en *Lactobacillus crispatus* een verschillende functionele rol vervullen als deel van de vaginale flora. Met het in kaart brengen van de vaginale flora voor en na behandeling van een verstoorde vaginale flora, ook bacteriële vaginose (BV) genoemd, met antibiotica werd aangetoond dat de flora wel drastisch veranderde maar dat dit zelden leidde tot een *Lactobacillus* dominante flora.

Hoofdstuk 7 onderzoekt de karakteristieken van de techniek die ontwikkeld werd om de vaginale flora in kaart te brengen. Dit hoofdstuk laat zien dat de techniek, die gebaseerd is op het combineren van barcodes met de analyse van stukjes bacterieel DNA (Illumina sequencing), alle diversiteit in de samples kon waarnemen met een hele goede identificatie van bacterie soorten. In vergelijking de gangbare techniek (denaturerende gradiëntgelelektroforese (DGGE) werd een grotere diversiteit aangetoond en in vergelijking met de analyse van langere stukjes DNA (454 sequencing) konden bacteriën beter geïdentificeerd worden. De ontwikkelde techniek reduceerde ook sterk de kosten en snelheid waarmee flora analyse gedaan kunnen worden.

III. Kunnen probiotica een bacteriële vaginose onder vrouwen met HIV voorkomen of de genezinggraad verbeteren?

In hoofdstuk 8 worden de klinische en mechanistische studies beschreven die onderzoeken of probiotica de vaginal flora kunnen beïnvloeden. Het hoofdstuk concludeert dat Lactobacillus rhamnosus GR-1 en Lactobacillus reuteri RC-14 de genezingsgraad van van bacteriële vaginose kunnen verbeteren en de vaginale flora positief kunnen beïnvloedden.   Om de vraag te beantwoorden of probiotica ook onder HIV-positieve vrouwen de vaginale flora positief kunnen beïnvloeden is een gerandomiseerde, placebo gecontroleerde trial beschreven in hoofdstuk 9. Vrouwen met HIV en bacteriele vaginose werden behandeld met antibiotica voor 7 dagen en gebruikten daarnaast dagelijks orale capsules met Lactobacillus rhamnosus GR-1 en Lactobacillus reuteri RC-14 of placebo voor 25 weken. Een andere groep vrouwen met HIV met een minder ernstig verstoorde vaginale flora kregen alleen probiotica of placebo voor 25 weken. Het onderzoek concludeert dat het gebruik van probiotica niet de genezingsgraad van bacteriële vaginose verbeterde maar mogelijk het risico op het ontwikkelen van bacteriële vaginose verlaagd.

Conclusies

- Het is nog niet bewezen dat het gebruik van probiotica, en specifiek *Lactobacillus rhamnosus* GR-1, een lange termijn invloed hebben op de immuunfunctie van HIV patiënten.
- *Lactobacillus iners* en *Gardnerella vaginalis* zijn altijd aanwezig in de vaginale flora van HIV-positieve vrouwen in Oost-Afrika.
- Het gebruik van het analyseren van korte stukjes DNA (Illumina sequencing) om de humane flora te analyseren is een effectieve en efficiënte methode om de diepte van microbiële analyse te verbeteren.
- *Lactobacillus rhamnosus* GR-1 en *Lactobacillus reuteri* RC-14 verbeteren niet de genezingsgraad van antibiotica voor bacteriële vaginose van vrouwen met HIV, maar verminderen mogelijk het risico op de ontwikkeling van bacteriële vaginose.

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I could not have imagined that an interest in bugs would ultimately lead me to a journey on three continents, allow me to collaborate with an international team of scientists, and involve hundreds of enthusiastic participants. A number of people have been key to guiding and accompanying me on this journey, and finally, in this section, I get to acknowledge them.

This journey started off with a conference in Gent, Belgium. Prof.dr. Gregor Reid gave a talk on the human microbiota and HIV, which immediately sparked my interest. The talk closed with, "turn a different corner and we would never have met". Fortunately, we did meet, and an exciting cascade of opportunities has followed since then. Gregor, it was only two years ago that we sat down in Amsterdam and thought of a plan to further develop this work into a PhD thesis. This is just one of the many examples of your passion for seeing students succeed. I deeply value your mentorship over the past several years, the opportunities that you have created, and great times in various pubs on the globe. This is not even to mention the upcoming wedding for which you deserve most of the credit (though we did a little work too).

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With a project idea, an e-mail address, a tent and a sleeping bag, I arrived at Mwanza Airport, Tanzania in July 2006. Not knowing what would come of the new adventure, I was introduced to dr. John Changalucha, director of the National Institute for Medical Research, Mwanza. Dr. Changalucha, your leadership, patience and endless support have resulted in solid and interesting projects that we could work on together. I admire how altruistically you give your time, energy, and advice to those you are working with and many others.

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mention. Arja and Yolanda, you have completed a nice project in Mwanza at an incredible pace, thank you for our collaboration.

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I arrived in Canada with some boxes of tediously collected swabs, hoping to do a small study. This study evolved into a fascinating experiment that has changed the way we analyze and understand the composition of the human microbiota. This shift can be largely credited to prof.dr. Greg Gloor. Greg, thank you so much for turning a simple clinician into a simple clinician who at least appreciates the trouble of laboratory work and who can do some PCR. You are an excellent teacher.

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A special note to Lesley, you have made me feel welcome right from the start. I will never forget you cheering for me at my race, and our laughs together. Dr. Wayne Miller, I am thankful for all your editing work, your strong enthusiasm and great input for the projects I was working on (even if it was investigating the health effects of Tim Horton's French Cappuccinos.)

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questions. Amy, thank you for your amazing input to many aspects of the projects we were working on. Rod, among many other things, thank you for the manuscript we wrote together; Marc for the innumerable slides and great laughs; Sarah, too many practical issues to mention; and Kate, for your introduction to the Reid lab. Many thanks to the administrative support team at the Lawson Health Research Institute and members of the Students Association that have made me feel at home. To our Wageningen collaborators: Drs. Paul Vos, Belinda van't Land and Klaske van Norren, I am very thankful, and feel indebted to your great input in writing our manuscript that is featured in this thesis. It was an absolute privilege and pleasure to collaborate with you.

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I want to especially mention Greg and Susan Hemsworth; thank you for welcoming me into your family and for the many good times in Canada. My brothers and sisters, Rob, Jos, Klaske and Ryan, Anne and Ruth, Arjen and Froukje, thanks for inspiring me and our friendship.

Dad and Mum, I am deeply thankful for the great examples you have been and the safe haven you always have provided. It's extraordinarily how two people teaming up can have such an impact on my life and the lives of many others. Mum, you are a remarkable woman. Your encouragement and fine intuition have shaped and inspired me to become the person I am. Dad, you are a great example and mentor. Thank you for sparking of an interest in a number of random things including growing vegetables, adventure and yes, human health.

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About the author

Ruben Hummelen (1984) was born in Ngqeleni, South-Africa, and grew up in Ede, the Netherlands. He graduated from secondary school (Ichthus College, Veenendaal) in 2003 after which he enrolled in Medical School at the Erasmus University Rotterdam. In 2004 he participated in a research project on the gut microbiota and inflammatory bowel disease (department of immunology, prof.dr. H. Drexhage) which sparked of his interest in the human microbiota. In 2005 he was selected for a research master in Clinical Epidemiology at the Netherlands Institute of Health Sciences (Nihes). The studies incepted during this master were developed further and are presented in this thesis (promoters: prof.dr. G. Reid and prof.dr. J.D.F. Habbema). Studies were conducted from 2007 - 2008 at the National Institute for Medical Research, Mwanza, (director: dr. J. Changalucha) and Sekou-Toure regional hospital, Mwanza, Tanzania (supervision: dr. N. Butamanya). Projects were completed and analyzed at the department of Public Health, Erasmus University Rotterdam, the Netherlands. Further research was conducted in Canada from 2009 – 2010 at the University of Western Ontario, London, Canada. Ruben is founding member and past program director of the Students and Fellows Association of the International Scientific Association of Probiotics and Prebiotics. He currently serves on the editorial board of Gastroenterology Research and World Journal of Gastrointestinal Pathophysiology. In 2009 he obtained his Masters degree in Clinical Epidemiology and is currently completing his clinical internships in Rotterdam. Ruben is actively involved in social work in the Tarwewijk, Rotterdam, and acts as chair of the foundation friends of House of Hope.

International Publications

1. Irvine SL, **Hummelen R**, Hekmat S, Looman CW, Habbema JDF, Reid G. Probiotic yogurt consumption is associated with an increase of CD4 Count among people living with HIV/AIDS. Journal of Clinical Gastroenterology. 2010; 44: 201-5

2. **Hummelen R**, Hemsworth J, Reid G. Micronutrients, N-Acetyl Cysteine, Probiotics and Prebiotics, a Review of Effectiveness in Reducing HIV Progression. Nutrients 2010;2:626-51.

3. **Hummelen R**, Fernandes AD, Macklaim JM, Dickson RJ, Changalucha J, Gloor GB, Reid G. Deep Sequencing of the Vaginal Microbiota of Women with HIV. PloS one 2010; 5(8): e12078

4. Gloor G, **Hummelen R**, Macklaim JM, Fernandes AD, Reid G. Microbiome profiling by combinatorial barcoding with Illumina sequencing. PloS one 2010; 5(10): e15406

5. **Hummelen R**, Changalucha J, Butamanya NL, Cook A, Habbema JDF, Reid G. *Lactobacillus rhamnosus* GR-1 and *L. reuteri* RC-14 to prevent or cure bacterial vaginosis among women with HIV. International Journal of Gynaecology and Obstetrics 2010: 111(3):245-8

6. **Hummelen R**, Vos AP, van't Land B, van Norren K, Reid G. Altered host-microbe interaction in HIV: a target for intervention with pro- and prebiotics. International Reviews in Immunology 2010; 29: 485-513.

7. MacPhee R, **Hummelen R**, Bisanz JE, Miller WL, Reid G. Probiotic strategies for the treatment and prevention of bacterial vaginosis. Expert Opinion in Pharmacotherapy 2010; 11(18):2985-95.

8. Shane AL, Cabana MD, Vidry S, Merenstein D, **Hummelen R**, Lynch S, Merenstein DJ, Sanders ME, Tancredi DJ, Vidry S. Guide to designing, conducting, publishing and communicating results of clinical studies involving probiotic applications in human participants. Gut Microbes 2010; 1: 243 - 53.

9. **Hummelen R**, Changalucha J, Koyama TEK, Butamanya N, Habbema JDF, Reid G. Effect of 25 weeks probiotic supplementation on immune function of HIV patients. Submitted

10. **Hummelen R**, Hemsworth J, Changalucha J, Butamanya N, Hekmat S, Reid G. Trial of micronutrient supplemented yogurt with or without probiotic *Lactobacillus rhamnosus* GR-1 among anti-retroviral naïve HIV patients. Submitted

11. Monachese M, Cunningham-Rundles S, Diaz MA, Ermond E, Guerrant R, **Hummelen R**, Kemperman R, Kerac M, Kort R, Merenstein D, Panigrahi R, Ramakrishna B, Safdar N, Shane A, Trois L, Reid G. Probiotics and prebiotics to combat enteric diarrheal diseases and HIV in the developing world: a consensus report. Submitted

12. Dols JAM, Boon JA, Bontekoe R, Monachese M, Changalucha J, Butamanya N, Varriano S, Vihant O, Hullegie Y, van Tienen A, **Hummelen R**, Reid G. The challenges of assessing the impact of probiotic yogurt on the well-being of HIV positive women in Tanzania. Submitted

13. **Hummelen R**, Bisanz JE, Macklaim J, MacPhee R, Hammond J, Gloor GB, Reid G. The vaginal microbiome of post-menopausal women and correlation with inflammatory and atrophic changes. Submitted

Erasmus MC
Universitair Medisch Centrum Rotterdam

PhD Portfolio Summary

Summary of PhD training and teaching activities

Name PhD student: Ruben Hummelen Erasmus MC Department: Public Health Research School: Nihes		PhD period: 2007-2010 Promotor(s): Prof.dr. G. Reid, Prof.dr. J.D.F. Habbema	
1. PhD training		**Year**	**Load**
Research skills			
- Master of Clinical Epidemiology, Netherlands Institute for Health Sciences (Nihes), Rotterdam, The Netherlands		2006-'9	30 ECTS
In-depth courses			
- Summer course Epidemiology and Biostatistics, Johns Hopkins University, USA		2007	120 hours
- Course on protection of human research subjects and Good Clinical Practices (GCP), London School of Hygiene and Tropical Medicine, Mwanza site, Tanzania.		2008	40 hours
Presentations and international conferences			
- European Conference on Probiotics, Krakow, Poland*		2008	32 hours
- 6th Meeting of the International Scientific Association for Probiotics and Prebiotics (ISAPP), London, Canada*		2008	36 hours
- International scientific conference on functional foods, Zilina, Slovakia*		2009	30 hours
- 5th International Yakult Symposium, Amsterdam, The Netherlands#		2009	36 hours
- Sackler symposium of the National Academy of Sciences, California, USA		2010	20 hours
- 7th Meeting of ISAPP, California, USA*		2010	24 hours
- TNO beneficial microbes conference, Noordwijkerhout, the Netherlands*		2010	50 hours
- 8th Meeting of ISAPP, Barcelona, Spain#		2010	32 hours
- Human Microbiome Research Conference, National Institutes of Health, St. Louis, Missouri, USA$		2010	1 hour
- Bill & Melinda Gates Foundation exploratory meeting Enteric and diarrheal diseases, January 5-7, London, UK#		2010	5 hours
- 6th international Symposium on Probiotics, Montreal, Canada$		2010	1 hour
*Presentation, #Poster presentation, $Abstract			
Awards and grants			
- Grant in Aid Danone Institute Canada		2007	
- Best presentation European Conference on Probiotics, Krakow, Poland		2008	

	Year	Load
- Nominated Young Investigators Award, International Scientific Conference on Scientific Foods, Zilina, Slovakia	2008	
- VSB Scholarship 2009 for research	2009	
- Travel Grant for 8th meeting ISAPP	2010	
2. Teaching activities	**Year**	**Load**
- Guest lecture, Scientific meeting National Institute for Medical Research, Tanzania	2008	6 hours
- Lecture, Symposium Stola, Erasmus University Rotterdam, the Netherlands	2009	4 hours
- Lecture, Lawson Health Research Institute, London, Canada.	2009	2 hours
- Lecture, Meeting Canadian Research and Development Centre for Probiotics, London, Canada	2010	2 hours
- Guest lecture, Department of Nutrition, Fanshawe college, London, Canada	2010	4 hours
- Guest lecture, Meeting department of Biochemistry, University of Western Ontario, London, Canada.	2010	2 hours
- Lecture, Staff Conference, University of Western Ontario, London, Canada.	2010	4 hours
Supervising theses		
- Bachelor thesis (Nutrition)	2008-'9	80 hours
- Bachelor thesis (Microbiology)	2010	24 hours
- Master thesis (Medicine)	2010	24 hours
- Master thesis (Public Health)	2008-'9	90 hours
3. Other activities		
- Program director Students and Fellows Association (SFA) of ISAPP	2009-'10	30 hours
- Organiser and chair session at 8th meeting of ISAPP 2010.	2010	14 hours