From the DEPARTMENT OF LABORATORY MEDICINE
Karolinska Institutet, Stockholm, Sweden

LACTOBACILLUS BASED TREATMENT OF VAGINAL INFECTIONS

SONAL PENDHARKAR

Stockholm 2013
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Published by Karolinska Institutet. Printed by Karolinska University Press, US-AB

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ISBN 978-91-7549-281-0
Dedicated to Aai-Baba (mom-dad)
ABSTRACT

The vaginal microbiota (VMB) is a complex and delicate balance between different bacterial species and is normally dominated by Lactobacillus species. Under the effect of hormonal changes, behavioral or sexual activity VMB could lose this balance resulting in diseases. Bacterial vaginosis (BV) is one of the most common vaginitis affecting women and is defined as an increase in proportion of anaerobic bacteria and a reduction or absence of lactobacilli. BV has been linked with increased risk of acquiring sexually transmitted diseases (STDs) including infection by human immunodeficiency virus type-1 (HIV-1). Vulvovaginal candidiasis (VVC) is the second most common cause of vaginitis after BV, which is recognized by an overgrowth of yeast mainly belonging to the genus Candida and albicans species. The standard clinical treatment for these conditions prescribes antibiotics (clindamycin and/or metronidazole) and antifungal (fluconazole). This thesis focuses on investigating clinical interventions for the treatment of BV and VVC and attempting to express neutralizing antibody fragments in Lactobacillus against HIV-1 for future applications.

An extended antibiotic treatment with clindamycin and metronidazole together with adjuvant lactobacilli was tested in order to achieve long lasting cure and reduce relapse of BV (paper I). Five different combinations of Lactobacillus strains were tested which included newly characterized strains (paper I) and the commercial EcoVag® capsules (a mixture of L. gasseri DSM 14869 and L. rhamnosus DSM 14870). The cure rate was 74.6% after six months and 65.1% after 12 months. No significant difference was observed in cure rates depending on whether the women were colonized by any of the given strains. However, change of sexual partner was significantly associated with relapse of BV. The results were further confirmed in another clinical trial (paper II, trial I).

We subsequently assessed the efficacy of a prolonged treatment with EcoVag® lactobacilli in combination with similar antibiotic treatment in BV patients (paper II, trial II). The cure rate was 66.7% and 62.5% after six months and 12 months respectively and was comparable to that in paper I. Prolonged treatment with lactobacilli did not significantly improve the colonization by EcoVag® strains. Overall colonization by any lactobacilli and by EcoVag strains was associated with cure of BV. Once again change of sexual partner was associated with relapse of BV. We also tested for the first time, a combination of EcoVag® and anti-fungal for treatment of recurrent VVC and evaluated if lactobacilli can colonize women when the microbiota is not disturbed by antibiotics. All the women were cured of VVC after six months and 87.5% remained cured after 12 months. EcoVag® strains colonized more in the presence of prior antibiotic treatment in BV patients and in women with VVC who were not colonized by lactobacilli in the beginning of the study.

The VMB has been extensively studied in European and American populations albeit less so in the African population. Therefore we have identified the vaginal Lactobacillus species in a group of 40 women in South Africa (paper III). We found that the vaginal Lactobacillus species identified were similar to those in other populations, suggesting that the strategies utilising probiotic and engineered lactobacilli could be applied in Africa. Finally llama-derived single domain antibody fragment (VHH) against HIV-1 glycoprotein gp140 was expressed in L. paracasei BL23 (our model strain) (paper IV). The VHH was expressed in secreted and cell surface anchored form, which were able to bind to recombinant HIV-1 glycoproteins. The VHH is currently being tested for in vitro neutralization of various HIV-1 viral strains. These results provide the basis for the use of lactobacilli and genetically modified lactobacilli for treatment of BV, yeast infection and HIV-1 in both developed and developing world.
LIST OF PUBLICATIONS

I. Extended antimicrobial treatment of bacterial vaginosis combined with human lactobacilli to find the best treatment and minimize the risk of relapses.

II. Vaginal colonisation by probiotic lactobacilli and clinical outcome in women conventionally treated for bacterial vaginosis and yeast infection.
    Pendharkar S, Brandsborg E, Hammarström L, Marcotte H and Larsson P-G. *Manuscript*

III. Identification and characterisation of vaginal lactobacilli from South African women.

IV. *In vitro* neutralization of HIV-1 by *Lactobacillus* produced VHH antibodies.
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LIST OF ABBREVIATIONS

AIDS  Acquired immunodeficiency syndrome
AP   Alkaline phosphatase
APF  Aggregation promoting factor (from \textit{Lactobacillus crispatus})
ATCC American Type Culture Collection
BCA  Bicinchoninic acid
BHCG β-subunit of human chorionic gonadotropin
BSA  Bovine serum albumin
BV   Bacterial vaginosis
CFU  Colony forming units
DMEM Dulbecco's Modified Eagle Medium
DSM  Deutsche Sammlung von Mikroorganismen
ELISA Enzyme-linked Immunosorbent assay
ENV  Envelope
FITC Fluorescein isothiocyanate
g Grams
GMP  Good manufacturing practice
gp Glycoprotein
H$_2$O$_2$ Hydrogen peroxide
HIV-1 Human immunodeficiency virus-1
HPV  Human papilloma virus
HSV-2 Herpes simplex virus-2
IC50 Half maximum inhibitory concentration
ID50 Median infective dose
ITT  Intent to treat
IUD  Intra uterine device
MRS  Lactobacilli media developed by de Man, Rogosa and Sharpe
NNRTI Non-nucleoside reverse transcriptase inhibitor
NRTI Nucleoside reverse transcriptase inhibitor
PBS-T Phosphate buffer saline-Tween$^\text{®} 20$
RLUs Relative light units
SDS  Sodium dodecyl sulphate
TMB  3,3’, 5,5’-tetramethylbenzidine
RT  Room temperature
R-VVC Recurrent Vulvovaginal candidiasis
VHH Variable domain of heavy chain from \textit{Camelidae}
VMB  Vaginal microbiota
VVC  Vulvovaginal candidiasis
1 INTRODUCTION

1.1 Vaginal microbiota (VMB)

The human body is long known to coexist with microbial communities specific to different mucosal sites such as gastrointestinal tract, oral cavity and vagina. The number of bacteria that closely exist with humans outnumber human cells by a factor of 10 (1). A bacillus for the first time was isolated from vagina a century ago in 1892 by Doderlein and was named after him which was later renamed as *Lactobacillus* (2). VMB is a complex balance of different bacterial species present in the vagina. In a healthy woman, VMB is dominated by *Lactobacillus* species and the most commonly identified species are *L. crispatus, L. jensenii, L. gasseri, L. iners* followed by *L. rhamnosus, L. reuteri, L. vaginalis, L. acidophilus, L. casei, L. fermentum, L. plantarum* and *L. salivarius* (3–7). Other than the lactobacilli, *Prevotella, Peptostreptococcus* and *Porphyromonas* are the species also normally present in the vagina but in very low numbers (8). In some of the studies, *Gardnerella vaginalis* has also been reported to be present in women with *Lactobacillus* dominated microbiota (9,10). More than 50 vaginal microbial species have been identified (11–13) which coexist with each other. Studies on vaginal microbiota of different populations have resulted in a mixed opinion on dominant colonizing lactobacilli in women from different ethnicity. Some studies performed with white, black, Hispanic and Asian women have suggested that the composition of VMB vary between these ethnic groups and was not dominated by lactobacilli in black women (14,15). However, the studies from Nigeria and South Africa suggest that the colonizing lactobacilli in these cohorts are similar to those identified in European and American populations (5,6). An internal factor that affects the composition of VMB is menstrual cycle. During the menstrual cycle, the proportion of colonizing lactobacilli increases as the cycle progress post menses whereas species like *Prevotella* and *Bacteroides* increase significantly before menses (16). A *Lactobacillus* dominated microbiota confers protection against sexually transmitted diseases (STDs) including infection by HIV-1, *Neisseria gonorrhoeae*, herpes simplex virus-2 (HSV-2) and human papillomavirus by preventing pathogenic organisms from surviving and infecting the host cell (17–21).

1.2 *Lactobacillus*

*Lactobacillus* is a genus of Gram-positive facultative anaerobic or microaerophilic rod-shaped bacteria. *Lactobacillus*, along with *Lactococcus, Streptococcus, Leuconostoc, Enterococcus* to name a few, is the member of lactic acid bacteria (LAB) group. These
are recognized to produce lactic acid as a fermentation product and maintain a lower pH (< 4.5) which is protective against pathogenic microbes (22). *Lactobacillus* is a major part of the vaginal and gastrointestinal microbiota. In the vagina, *Lactobacillus* provide a protective environment by maintaining a low pH, by producing bacteriocin and through competitive exclusion (23–26). During the perinatal period and from puberty to menopause, high estrogen levels stimulate the deposition of glycogen on the vaginal epithelium. This glycogen is metabolized anaerobically by lactobacilli to produce acetic and lactic acid and lowers the vaginal pH (27). Among the identified vaginal colonizers from the *Lactobacillus* genus *L. crispatus* has been shown to stabilize the VMB and is less affected by the changes during the menstrual cycle (28,29), whereas *L. iners* is suggested to be a dominant part of the VMB when the microbiota is in a transitional stage between abnormal and normal (28,30).

The presence of lactobacilli in general and their impact on microenvironment contribute to the homeostasis of VMB. Many of the *Lactobacillus* species including *L. acidophilus* and *L. salivarius* were tested for production and activity of bacteriocin against BV associated bacteria (31–33) and were found to inhibit their growth. H$_2$O$_2$ and its effect have been studied by several groups and have resulted in contradictory results. Many of the studies did establish an inverse association between the presence of H$_2$O$_2$ producing bacteria and BV and have also shown an inhibitory effect of H$_2$O$_2$ against pathogens like *Neisseria gonorrhoeae*, HIV and diverse anaerobes (19,23,25,34,35). However, in contrast, a study by O’Hanlon *et al.* showed that H$_2$O$_2$ present in the vaginal fluid could not suppress the growth of BV associated bacteria (36). Factors such as adherence to epithelial cells, colonization resistance and other antimicrobial agents also contribute to the vaginal colonization and subsequent health status.

1.3 **Imbalance of the vaginal microbiota**
The vaginal microbiota represents a delicate balance between beneficial lactobacilli and other anaerobes, *Mycoplasma* and *G. vaginalis*, which in a normal microbiota are below detectable levels. This delicate balance is affected by several participating factors like hormonal levels, vaginal practices, sexual practices, bacterial interactions, host defense and very recently studied dietary indices (37–39) which could lead to dramatic changes in VMB and clinical conditions like BV and VVC.
1.3.1 **Bacterial vaginosis (BV)**

Menge and Kronig at the end of 19th century first described the isolation of an anaerobic organism in addition to *Lactobacillus* from vagina (40). Using culture based techniques, Curtis in 1923, associated “white-discharge” syndrome with black-pigmented anaerobes, curved anaerobic motile rods, anaerobic cocci and Gram-variable rods (41). He also noted depletion of vaginal *Lactobacillus* in women with this syndrome. It was thus known that the symptomatic “vaginal discharge syndrome” was associated with significant changes in VMB. Later on, a Gram-variable coccobacillus was identified to be associated with the white discharge syndrome and named as *Gardenerella vaginalis* by Gardiner and Dukes in 1955 (41). For a long time this syndrome was associated only with *G. vaginalis* until 1980, when Spiegel *et al.* established an association between non-specific vaginitis and dramatic variations in VMB (42–44). Thereafter this syndrome was named as bacterial vaginosis. Further on the markers for diagnosing BV were identified by Amsel *et al.* as: 1- vaginal discharge with a pH level above 4.5; 2- malodourous discharge on addition of 10% potassium hydroxide (KOH); 3- presence of at least 20% of epithelial cells coated with bacteria named as “clue cells” and 4- a typically white thin homogenous vaginal discharge (45). Nugent *et al.* in 1991 standardized a method to interpret gram stained vaginal smears to diagnose BV (46), which since then has been widely accepted in clinics for the diagnosis of BV and classifying the VMB. In brief, a score of zero to four is given for the decreasing number of lactobacilli, a score of zero to four for increasing number of *Gardnerella/Bacteroides* morphotypes and a score of zero to two is given for increasing number of curved Gram variable rods. The sum of all these scores is a representative of the status of VMB as normal (0-3), intermediate (4-6) and BV (7-10).

In addition to Nugent scoring, Hay/Ison also defined a way of grading the vaginal smears with two additional categories. According to Hay/Ison scoring a vaginal smear is given grade I (normal) with presence of only lactobacilli morphotypes, grade II (intermediate) with reduced lactobacilli and other morphotypes, grade III (BV) with mixed morphotypes and few or no lactobacilli, grade 0 with epithelial cells with no bacteria seen and grade IV with only Gram positive cocci (47,48). BV is defined as an increase in proportion of BV associated bacteria like *G. vaginalis, Bacteroides (Prevotella), Mycoplasma hominis, Mobiluncus* species, *Atopobium vaginae* and reduction or absence of lactobacilli (49,50). The prevalence of BV is highest in sub-Saharan Africa (6.4-58.3%) whereas, it is comparatively less prevalent in other regions (Fig. 1) (51).
1.3.3 Vulvovaginal candidiasis (VVC)

After BV, VVC is the most common vaginitis affecting women. VVC is caused by overgrowth of yeast belonging to the genus Candida and Candida albicans is one of the most common causative species and is a commensal of skin, gastrointestinal and reproductive tracts. Non-albicans species which have recently been associated with VVC are Candida glabrata, Candida tropicalis, Candida parapsilosis and Candida krusei (52,53). Seventy to 75% of women experience VVC at least once in their lifetime (54,55), where 40 to 50% of women suffer from 1 or more episodes after treatment for first episode (56). Candida species that have been associated with recurrence are non-albicans species (57) and C. glabrata is the most common species (58). Symptoms of VVC include thick vaginal discharge with a pH less than 4.5 and the presence of alkali resistant blastospores or hyphae of C. albicans in vaginal smears. Unlike in BV, lactobacilli are not depleted in VVC and coexist with Candida. A similar observation was made in paper II presented in this thesis.

1.3.3 Risks associated with disturbed vaginal microbiota

The disturbance in VMB with loss of lactobacilli increases the risk of acquiring vaginal infections including infection by HIV-1, Neisseria gonorrhoeae and favors the growth of G. vaginalis. BV associated bacteria have been shown to induce HIV-1 replication and shedding in the genital tract (59,60), which may increase infectivity of HIV-1 for
women with BV (61). H₂O₂ producing lactobacilli have been shown in vitro to inhibit G. vaginalis, other anaerobes, N. gonorrhoeae and HIV-1 (19,23,25,35,62). Other exhibited properties of lactobacilli which may protect the vaginal ecosystem include competition for attaching to epithelial cells and stimulation of the local immune system (11,63). Women harbouring H₂O₂ producing lactobacilli have been shown to be less likely than women without these lactobacilli to have BV as well as STD pathogens such as N. gonorrhoeae, Chlamydia trachomatis, and Trichomonas vaginalis (34,62,64). Moreover, abnormal VMB is shown to be associated with HSV-2 and HPV infections (20,21). BV associated VMB is also considered to be a risk factor for premature delivery, a leading cause of prenatal mortality (65,66).

Hence, a VMB with no or a lower proportion of lactobacilli creates a permissive environment with a higher vaginal pH, lack of H₂O₂, bacteriocins and no competition with pathogens for binding to epithelial cells providing an opportunity for BV associated bacteria to colonize and STD pathogens to cause infection. In addition, BV-associated bacteria produce proteases like sialidases and prolidases, adversely affect vaginal mucosal cell exfoliation and degradation of mucus, glycoproteins, which are thought to be major components of the first line of defense against infection (67).

1.4 Treatment of vaginal infections
The current treatment strategies for vaginal infections include use of antibiotics, antifungal drugs alone and in combination with probiotic lactobacilli. In order to improve the cure rate and reduce recurrence rate, investigators are currently assessing the possible combination of antibacterial and antifungal drug together with probiotics.

1.4.1 Current treatment regimens for BV and VVC
According to the current treatment regimen women with BV are given clindamycin and/or metronidazole in the form of vaginal cream or administered orally (68). Treatment of BV prescribes either 2% clindamycin vaginal cream daily (5 grams (g)) for 7 days or 0.75% metronidazole vaginal cream (5 g) daily for 7 days or 500 milligrams (mg) of oral metronidazole twice daily for 7 days. However, resistance for clindamycin in anaerobic bacteria has been observed after the treatment (69). Side effects for metronidazole has also been registered including nausea, metallic taste, transient neutropenia and gastrointestinal side effects (especially when administered orally) to name a few (70).
In the case of VVC, the current treatment strategy prescribes single dose of 150 mg fluconazole which has been shown to be inhibiting 90% of Candida species (71). Previously used oral ketoconazole which was effective in treating recurrent infection, use of vaginal boric acid or flucytosine to control C. glabrata are associated with severe side effects or are not safe for long term use (72–74). Hence, fluconazole is favoured over other antifungal drugs.

1.4.2 Combination therapy with lactobacilli

Use of lactobacilli with probiotic effect as a combination treatment with antimicrobial drugs has been suggested and investigated widely because of failure of antimicrobials to prevent recurrences. Treatment with antibiotics hamper the colonization of native vaginal microbial species post treatment mainly lactobacilli and in several cases leads to recolonization by pathogenic microbes, thereby increasing the recurrence rate (75,76). The properties that are considered ideal for probiotic lactobacilli include adherence to vaginal epithelial cells, co-aggregation, exclusion of pathogen adhesion and production of bacteriocin. The route of administration of lactobacilli is also important. In most of the studies conducted till date, lactobacilli have been delivered intra-vaginally. Specific strains of lactobacilli have been tested for vaginal colonization and treatment of yeast infection or BV, when administered intra-vaginally or orally (77–80). The Lactobacillus strains tested, including L. rhamnosus GR-1, L. reuteri RC-14, L. crispatus CTV-05, L. gasseri DSM 14869, L. rhamnosus DSM 14870, L. fermentum LF10 and L. acidophilus LA02, were shown to improve the cure rate of BV and VVC (77,79,81–84). However, more studies are needed to establish the observed effect in general population.

In paper I and II presented in this thesis, we were able to improve the long term cure rates for BV and VVC using EcoVag® capsules containing the above mentioned L. gasseri DSM 14869 and L. rhamnosus DSM 14870. These strains were originally isolated from Scandinavian women and were proven to be most effective in adhesion to vaginal epithelial cells, growth, acid and H₂O₂ production. The capsule contains 10⁸ - 10⁹ colony forming units (CFU) of each Lactobacillus strain per capsule.
1.5 HIV-1 infection

In 1982, the term AIDS was introduced by U.S. Centers for Disease control and prevention, to name the disease responsible for ongoing epidemic. Thereafter in 1983, HIV was for the first time isolated by Luc Montagnier and his team in France (85), which was shown by Robert Gallo and his team in the USA to be the cause of AIDS (86). HIV-1, the first isolated virus, has caused infection worldwide. The virus establishes infection by interacting primarily with the host CD4 T-lymphocyte (87), macrophages and dendritic cells that express CD4 and CCR5/CXCR4 cell receptors.

The transmission of HIV-1 occurs by direct blood-to-blood contact or contact with contaminated blood products or syringes. However, unprotected heterosexual intercourse has remained the major cause of HIV-1 transmission and the region with the highest HIV prevalence is Sub-Saharan Africa. According to a global report in 2011 by UNAIDS, sub-Saharan Africa represents 69% of all HIV infections worldwide (Fig. 2) (88).

![Global HIV Prevalence 2009](image)

*Fig 2. Global HIV prevalence 2009. Source: UNAIDS Global report 2012*

In the case of sexual transmission of HIV-1, the mucosa is the site of infection for the virus. In the vagina, the mucous membrane of the female genital tract, which is mainly composed of multilayers of stratified epithelia, offers a good barrier to the virus. The mucus with pH around 4 immobilizes virus-like particles and prevent them from causing infection (89). Lactobacilli colonizing the vagina environment with acid production and exhibit other protective properties. In the absence of these beneficial lactobacilli, women who suffer from BV become vulnerable to vaginal infections and
infection by HIV-1. Vaginal epithelia abraded by bacterial vaginosis, genital infections (eg. HSV-2 and syphilis), use of intravaginal preparations for “dry sex” and sexual trauma can critically increase the rate of HIV transmission (90). It has been shown that the lack of commensal lactobacilli in the vagina leads to increased risk of acquiring HIV infection in women and transmission to their male partners (91,92). These conditions highly favor the occurrence and transmission of HIV-1.

1.5.1 Strategies against HIV-1 transmission

Zidovudine was the first antiretroviral drug to be introduced in 1987 followed by didanosine and zalcitabine. To overcome the risk of developing drug resistance and to minimize viral replication, two or more drugs are used together in combination which is of high clinical relevance and benefit (93). This therapy is named as highly active antiretroviral therapy (HAART) and the drugs prescribed belong to different classes. There are several different antiretroviral drugs available today for clinical use based on different stages of HIV life cycle. They are of five different classes; entry inhibitors (eg. maraviroc and enfuvirtide), nucleoside analog reverse transcriptase inhibitors (NRTI) (eg. deoxythymidine, zidovudine, stavudine, tenofovir), non-nucleoside analog reverse transcriptase inhibitors (NNRTI) (eg. nevirapine, delavirdine, efavirenz, and rilpivirine), integrase inhibitors (eg. raltegravir) and protease inhibitors (PI) (eg. atazanavir, darunavir, ritonavir, indinavir, lopinavir, nelfinavir) all with different modes of action. Antiretroviral therapy was successful in rapidly reducing the viral load and reviving the host CD4 T-cell count (94,95). However, lack of complete normalization of T-cell levels upon treatment (96), complexity related with the use of antiretroviral drugs and side effects (97) are still major challenges.

Another branch of emerging anti-HIV drugs are microbicides. Microbicides are compounds that can be applied in vagina or rectum to protect against HIV-1 and other sexually transmitted infections. To prevent new cases of HIV-1 infection concept of “ABC” is being highly promoted which according to UNAIDS can be defined as abstinence from sex, being faithful to your sexual partner and condom use. However in male dominated societies this might not function and hence a female controlled microbicide formulation active against HIV-1 could prove effective.
1.5.2 Microbicides

Microbicides can act in different ways and also have varying degrees of specificity (98,99). The first generation of microbicides was broadly reactive, but non-specific in its activity. Nonspecific microbicides consist of buffering agents, detergents or surfactants that include nonionic, anionic or cationic compounds. For example, formulations like cellulose sulphate, Carraguard® (a synthetic naphthalene sulfonate polymer), and PRO2000® (containing the polyanion carrageenan) provide a physical barrier that prevents pathogens from interacting with host cells. So far mostly non-specific microbicides, such as surfactants and polyanions, have completed phase III clinical testing and none has demonstrated clear statistical evidence of protection. These failures could be conquered by developing microbicides with increasing specificity and well-defined mechanisms of action that might be less prone to unwarranted adverse effects on the integrity of the natural barriers to transmission. The life cycle of HIV provides a number of points at which a microbicide could theoretically prevent infection. Each step of the virus entry pathway and replication is a potential target for novel inhibitors. The CD4 binding site (CD4bs) and the co-receptor binding site (CoRbs) of gp120 as well as gp41, which mediate fusion, are therefore attractive targets for various kinds of microbicides (100,101). In a recent clinical trial in South Africa, tenofovir an NRTI in a microbicide gel formulation was found to lower the HIV-1 incidence rate in treatment group (102). The development of antibodies that target the conserved envelop epitopes (gp120) also clearly represent a desirable approach to block viral transmission. A few human monoclonal antibodies (mAbs) against gp120 (such as b12 or VRC01) that broadly neutralize diverse HIV-1 strains have been isolated from HIV-1–infected patients (103,104). Vaginally administered b12 and VRC01 mAbs have also been shown to protect against mucosal SHIV challenge in animal models, suggesting the use of antibodies as potential vaginal microbicides (105,106).

1.6 VHH

VHH are the variable domain of llama heavy chain only antibody naturally found in the members of Camelidae family (Fig. 3). It consists of a single immunoglobulin domain and is the smallest naturally occurring antigen-binding molecule known to date (107). VHHs exhibit several advantages over conventional antibodies, as they are markedly more acid and heat resistant and, being formed by a single polypeptide, are easier to express in a functional recombinant form. These properties make VHH suitable for
therapy at mucosal sites such as the vagina where the acidic environment can limit the functionality of conventional antibodies. In addition, owing to their naturally longer CDR2 and CDR3 regions, llama VHH antibody fragments are superior at accessing clefts such as active sites of enzymes and canyons on virus capsids. VHH have low immunogenicity and they are unlikely to exhibit untoward side effects during chronic application (108–110).

![Diagram of antibody structures](image)

**Fig 3.** (A) Conventional IgG, (B) *Canis lupus* heavy chain antibody and (C) VHH domain

### 1.6.1 VHH as neutralizing antibody molecules against pathogens
In 2006, Baral *et al.* and van der Vaart *et al.* generated broadly cross-reactive and protective VHH fragments targeting a number of trypanosome isolates and globally prevalent strains of rotavirus (111,112). VHH, being one-tenth the size of conventional antibodies, are expected to diffuse more readily inside the crevices at the interface between a virus and its target cells, thus blocking infection.

Thereafter, Forsman *et al.* in 2008 isolated and characterized VHH fragments reacting with the CD4 binding sites of gp120, which were broadly neutralizing HIV-1 subtypes (113). This study showed that the VHH antibodies could be generated against HIV-1 in llamas, which are specific against CD4bs on gp120 but the VHH generated against HIV-1 displayed limited neutralization. However, the same group was able to select a VHH “J3”, which neutralized 96 out of 100 tested HIV-1 strains belonging to subtypes A, B, C, D, BC, AE, AG, AC, ACD, CD, and G with low IC50 values (median) ranging between 0.32 to 4.42 µg/ml (112). In comparison with the monoclonal antibody VRCo1, that neutralized 88.4% of the 69 viral strains tested (104), VHH J3 was more potent neutralizing 94.2% of the same set of 69 viruses. Thus, VHH represents a novel tool for prophylaxis and therapy against HIV-1 (114).
1.7 **Lactobacillus** mediated delivery of VHH antibody fragments against HIV-1

A major impediment to passive administration of specific microbicides is to provide a practical and continuous delivery of the product. To date the largest number of intravaginal drug delivery systems for microbicides are in the form of creams, gels, suppositories or intravaginal rings (115). Although commonly used for topical intravaginal delivery of microbicides, gels or creams must be applied immediately before sex. Vaginal rings also have disadvantages as insertion is unpleasant for some women and it may cause vaginal irritation and discharge. Since some exogenously administered lactobacilli were shown to colonize the vaginal tract and restore a normal VMB, an attractive idea is to use lactobacilli as vectors for *in situ* delivery of antibody fragments against HIV in the vaginal tract. Once administered, the lactobacilli would persist for days or weeks in the vaginal tract allowing production of microbicides *in situ*. This approach requires the use of lactobacilli that can persist in the vaginal tract, which, also explains why we selected lactobacilli that can colonize the vagina and attempted to improve the colonization in paper I and II.

Efforts have been made to design a *Lactobacillus* strain (*L. jensenii* 1153) to secrete the first two extracellular domains of human CD4 (2D CD4), RANTES (a natural ligand of CCR5) and cyanovirin-N (a carbohydrate binding protein) isolated from cyanobacteria (116,117). Thereafter a vaginal isolate *L. reuteri* RC-14 was also modified to produce the HIV entry and fusion inhibitors 2D CD4, MIP-1β, and T-1249 (118). The microbicides produced by lactobacilli were shown to neutralize HIV-1 *in vitro*. Lactobacilli have previously been engineered and used for delivery of functional VHH antibody fragments against rotavirus and *Lactococcus* phages (119,120). These *in vitro* studies prove the concept of expressing functional VHH antibody fragments that can neutralize pathogens. These antibodies were expressed using our model strain *L. paracasei* BL23. Similarly, vaginal lactobacilli eliciting probiotic properties could be engineered to produce VHH antibody fragments as a prophylaxis against HIV-1. A better understanding of the species composition and ecology of bacterial ecosystems is required for developing engineered probiotic lactobacilli and *Lactobacillus* based prophylaxis against BV and HIV in developing countries. Therefore, we have identified the cultivable vaginal *Lactobacillus* species in South African women (paper III).
2 AIMS

The aims of this thesis were to assess the clinical interventions (antimicrobials in combination with lactobacilli) for long-term cure of vaginal infections such as bacterial vaginosis (BV) and vulvovaginal candidiasis (VVC), and to successfully produce a functional VHH antibody fragment against HIV in lactobacilli for future applications. The specific aims were:

- To characterize human vaginal lactobacilli and to evaluate an extended antibiotic treatment regimen together with adjuvant lactobacilli for improving the cure rate of BV.
- To investigate the colonization by lactobacilli and clinical outcome in women receiving prolonged treatment with EcoVag® lactobacilli, in combination with antibiotic or anti-fungal treatment.
- To identify the cultivable vaginal *Lactobacillus* species in South African women and to compare that with the *Lactobacillus* microbiota identified in European population.
- To express a neutralizing VHH antibody fragment against HIV-1 in *L. paracasei* BL23 (a model strain).
3 MATERIALS AND METHODS

3.1 Lactobacilli used for treatment (Paper I and II)

In paper I, *Lactobacillus* strains isolated from healthy Swedish women (80) were characterised and nine were selected for the clinical trial. The lactobacilli were fermented, lyophilised, and dispensed as a mixture of three different strains (10^9/capsule) in gelatin capsules according to GMP standard (Gruppo Clerici Sacco SS.r.l., Cadorago Italy together with Bifodan A/S, Hundested, Denmark). Other strains from commercial products, currently used for treatment of BV, were also tested. These strains include *L. gasseri* (Lba EB01-DSM 14869) and *L. rhamnosus* (Lbp PB01-DSM 14870) contained in the commercially available EcoVag® vaginal capsules (10^8 CFU/capsules) (Bifodan A/S, Denmark). Capsules containing a mixture of EcoVag® strains plus *L. gasseri* DSM 15527, were also tested. These *Lactobacillus* strains were initially isolated from healthy women in Norway and characterised by Bifodan. The second probiotic preparation used was LaciBios® (oral capsules containing *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14 (around 10^9/capsule)). These *Lactobacillus* strains have been described in the studies by Reid and collaborators (81,121). In paper II, only the EcoVag® vaginal capsules from Bifodan were used for the treatment.

3.2 Study population (Paper I-III)

In Paper I, the study was conducted at an outpatient private gynaecological clinic in Drammen, Norway from January 2007 until January 2011. A total of 76 patients were included in the study. In paper II, the first clinical trial including patient with BV was conducted in an outpatient private gynecological clinic in Drammen, Norway from July 2009 until January 2011 and the second trial including both patients with BV and yeast infection was carried out in Skövde, Sweden. A total of 10 women in the first and 26 women in second clinical trial have been included. In both studies, women included were regularly menstruating women, 18 years or older, with normal gynaecological status, not pregnant or breast-feeding and without signs of other genital tract infections. Exclusion criteria were patients with hormonal IUD without regular menstruation; women infected with *Chlamydia trachomatis*, or *Trichomonas vaginalis*, or with a clinical *Candida* infection.
In paper III, forty premenopausal and HIV uninfected black women aged 18–44 years with or without BV were recruited for the study. Study participants were randomly selected among women who visited the Perinatal HIV Research Unit (PHRU) in Chris Hani Baragwanath Hospital Inclusion criteria were their willingness to provide the informed consent, passing the assessment of understanding, BHCG negative status (non-pregnant) and belonging to a low risk group for HIV acquisition. The women were non-menstruating and not taking antibiotics at the time of sample collection.

3.3 Clinical method (Paper I and II)

In paper I and II, women had a routine gynecological examination with a non-lubricated speculum and a vaginal ultrasound at inclusion. A sample of vaginal secretion was analysed for vaginal pH using special pH strips (range 3.8-5.0). The diagnosis of BV was based on Amsel criteria (45), i.e. fulfilling at least 3 of 4 criteria; thin homogenous discharge, vaginal pH above 4.5, positive amine test, and presence of clue cell during microscopical investigation using a phase contrast microscope. A vaginal sample was also taken and air-dried for Hay/ISON scoring.

In paper II, yeast infection was diagnosed on the basis of clinical examination showing thick vaginal discharge with a pH of less than 4.5 and a wet smear & KOH smear confirming the presence of alkali resistant blastospores or hyphae of Candida albicans using a phase contrast microscope seen on magnification of 400 times. At inclusion, vaginal samples were also tested for Chlamydia trachomatis infection using strand-displacement amplification (CT amplified DNA assay; Becton-Dickinson) according to the local laboratory routine. Samples for Neisseria gonorrhoeae culture were only taken when deemed clinically motivated. In paper III, BV was diagnosed on the basis on Nugent score.

3.4 Treatment of BV and yeast infection (paper I and II)

In paper I, women in the BV group were given a seven days course of daily 2% vaginal clindamycin cream (Dalacin vaginal cream 2%, Pfizer Norway Ltd) together with oral clindamycin 300 mg BID for 7 days (Dalacin 300 mg, Pfizer Norway Ltd) and a 5 days course of vaginal metronidazole (Zidoval gel 75 g, Meda AS, Norway) was given after first and second menstruation. Immediately after clindamycin and the first metronidazole treatment, vaginal Lactobacillus capsules were given for 5 days (Fig 4A). Oral clindamycin treatment was also given to the patient's sexual partner (122).
In paper II, the first clinical trial was designed with one group of 10 women diagnosed with BV. The treatment with antibiotic and lactobacilli was similar but women were given only one course of metronidazole instead of two (after the first menstruation). In the second trial (trial II), 10 women were recruited in each of the three different groups which are as follows: 1- women with BV receiving antibiotics and lactobacilli (Fig 4B), 2- women with yeast infection receiving anti-fungal drug and lactobacilli (Fig 4C) and 3- women with yeast infection receiving only the anti-fungal drug. Women with BV received similar antibiotic treatment as in the first trial and a prolonged treatment with vaginal lactobacilli. After each antibiotic course, EcoVag® capsules were given for 10 days (instead of 5) and after the second menstruation once every week for the next four months.
Women with *Candida* infection were given a 28 day course of Fluconazole 50 mg every day followed by vaginal EcoVag® capsules for 10 days. After the first menstruation, EcoVag® capsules were given again for 10 days along with a weekly course of 200 mg Fluconazole for two months. This was followed by a third course of Fluconazole, where women were given 200 mg of the drug once every two weeks for next six months along with lactobacilli once every week for four months. A group of women with *Candida* infection are being treated only with Fluconazole for controlling the effect of lactobacilli in the *Candida* treatment protocol.

### 3.5 Sample collection (Paper I-III)

In paper I and II (trial I), after every menstruation the patients took a self-swabbed vaginal culture whereas in paper II (trial II) vaginal swab sample was taken twice every month (on day 7 and day 21). In paper III, swab samples were taken at a single time point. In all the studies, vaginal swabs were collected in Amies agar gel medium with charcoal (Copan Venturi Transsystem® Copan Diagnostics, Italy). A glass smear was also taken which was air dried and sent in a sealed envelope along with the swab to our laboratory in Karolinska Institutet. The smear was gram stained for BV scoring.

### 3.6 Isolation of *Lactobacillus* from swab samples (Paper I-III)

Upon arrival, swabs were streaked onto Rogosa agar, blood agar plates and de Man-Rogosa-Sharpe (MRS) agar plates. Plates were incubated for 48 hours at 37°C in an incubator in anaerobic condition using BD GasPack™ EZ gaz generating systems (Becton, Dickinson and Company, Sparks, MD) and the colonies with Gram positive rods were grown in MRS broth medium for preparing the frozen stocks (15%) and genomic DNA isolation. Four to eight colonies were picked per sample.
3.7 Identification and genotyping of the isolated species (Paper I-III)

*Lactobacillus* species were identified by amplifying and sequencing the complete 16S rRNA gene (1.5 kb). The obtained sequences were then subjected to nucleotide-nucleotide BLAST using BLASTN (http://www.ncbi.nlm.nih.gov/) and subsequently compared to the 16SrRNA sequences of typed strains to validate the results (papers I to III). Genotyping of the isolated *Lactobacillus* species in paper I and II was performed initially by using PCR amplification of the bacterial repetitive extragenic palindromic DNA sequences (REP-PCR) and then distinguishing the typed isolates further by performing RAPD (Rapid Amplified Polymorphic DNA)-PCR.

3.8 Hydrogen peroxide (H₂O₂) production test

*Lactobacillus* isolates were streaked onto a 20 ml MRS agar plate containing 0.25 mg/ml 3,3’, 5,5’-tetramethylbenzidine (TMB) and 0.01 mg/ml of horseradish peroxidase (HRP) (Sigma-Aldrich). Plates were incubated in anaerobic condition using the BD Gas Pack™ anaerobic container system for 72 hours and were exposed to air for 30 minutes before scoring them for blue coloration. HRP generates O₂ from any H₂O₂ produced by the lactobacilli, which in turn oxidizes the TMB substrate to form a blue pigment. On the basis of the blue coloration, isolates were scored for H₂O₂ production as 0 for no blue coloration, 1 for light blue, 2 for moderate and 3 for dark blue coloration.

3.9 Statistical analysis

In paper I, "Time from treatment to relapse" has been calculated in months and was used to explore differences in time to relapse between groups by survival analysis with Kaplan Meier procedure. A logistic regression analysis was performed and an Odds ratio (OR) with 95% confidence interval (CI) was calculated. All comparisons both between and within groups was performed two-tailed with a significance level of 5%. All the analyses in this study were performed in PASW (SPSS) v.18 and in accordance with the principal of intention to treat (ITT). In paper II, the difference in frequency of isolation of any lactobacilli or *L. rhamnosus* DSM 14870 and *L. gasseri* DSM 14869 was compared by Fisher exact test. Two-tailed P values less than 0.05 were considered statistically significant. Odds ratio to analyze association between change of partner and relapse of BV was calculated at 95% confidence interval using contingency table analysis.
In paper III, Fisher’s exact test was performed to test the relation between BV status (normal microbiota vs. BV) and the presence of any lactobacilli, specific *Lactobacillus* species and high/low H$_2$O$_2$-producing lactobacilli. Two-tailed P values less than 0.05 were considered statistically significant. All the comparisons in Paper II and III were performed with GraphPad Prism 4 software (GraphPad Software, Inc., La Jolla, Ca).

**Methods applied in Paper IV**

3.10 Bacterial strains, plasmids and growth conditions

*E. coli* DH5α (Invitrogen, Carlsbad, CA) was grown on Luria-Bertani (LB) plates at 37°C or in LB broth with 220 rpm orbital shaking at 37°C. *L. paracasei* BL23 (previously known as *L. casei* or *L. zeae* ATCC 393 pLZ15) (123) was grown in Lactobacilli MRS agar (Difco, Becton Dickinson, Sparks, MD) plates at 37°C anaerobically using BD GasPack™ EZ gaz generating systems (Becton, Dickinson and Company) or in MRS broth without shaking. VHH fragments were expressed in *Lactobacillus* under the high activity APF promoter in the expression vector pAF100 and pAF900 (124). When appropriate, the concentrations of antibiotics used were 100 μg/ml ampicillin or 300 μg/ml erythromycin for *E. coli* transformants and 5 μg/ml erythromycin for *L. paracasei* transformants.

3.11 VHH J3

A llama was immunized with a mixture of gp140 trimers derived from a subtype BC HIV-1 strain CN54 (gp140 CN54) and a subtype A strain 92UG037 (gp140 UG37) and VHH J3 was selected directly from the phage library for their ability to neutralize HIV-1 pseudovirus as described by McCoy *et al* (114). VHH J3 was the best candidate neutralizing 96% of the 100 tested HIV strains and was hence chosen for expression in lactobacilli.

3.12 Expression of VHH in *L. paracasei*

The gene encoding J3 was cloned in two different expression cassettes, pAF100 which allows the protein to be secreted out from lactobacilli in the culture supernatant (secreted version) and pAF900 which mediate display of the protein on cell surface (anchored version) (Fig. 5) (124). The J3 VHH was fused to an E-tag at C-terminal before the stop codon in the secreted version and between the VHH gene and prtP
anchor sequence in anchored version for detection and purification of the protein. We modified our original expression cassette by removing the extra N-terminal sequence present in between the signal peptide and cloned VHH. The plasmids were transformed into L. paracasei BL23 (123) by electroporation as described previously generating L. paracasei mJ3-pAF100 and L. paracasei mJ3-pAF900.

3.13 Western blotting

The L. paracasei BL23 transformants were grown in MRS broth with 5 μg/ml erythromycin until an OD600 of 1.0. The cultures (1 ml) were centrifuged at 8000 rpm for 5 minutes (min) to separate the cell pellet and supernatant. The supernatant was filter sterilized, pH adjusted to 7.0, mixed with Laemmli buffer in 1:1 ratio and boiled for 5 min. The cell pellet was washed twice with PBS, re-suspended in 100 μl Laemmli buffer and boiled for 5 min. The cell extract was centrifuged at 13,000 rpm for 5 min to remove cell debris and the supernatant containing the soluble proteins was recovered. Supernatant and cell extract were run on SDS–polyacrylamide gel at 170 volts and the proteins were transferred onto a nitrocellulose membrane (Hybond-ECL, GE Healthcare, Little Chalfont, Buckinghamshire, UK). The membrane was blocked with PBS-T (PBS with 0.05% (v/v) Tween 20 + 5% (w/v) milk powder) and successively incubated with mouse anti E-tag antibody (1μg/ml, Phadia AB, Sweden) at room temperature (RT) for 2 hours (h) and HRP (horse radish peroxidase) labeled goat anti-mouse antibody (DAKO A/S, Glostrup Denmark) at RT for 1 h. The signal was detected by chemiluminescence using ECL Plus™ Western Blotting detection system (GE Healthcare).

3.14 Enzyme linked immunosorbent assay (ELISA)

96 well microtiter plates were coated with 50 μl Bal.26 gp120 (NIBSC, Hertfordshire, UK) at 2 μg/ml in PBS overnight (o/n) at 4°C. Plates were subsequently blocked with 1% BSA (in PBS containing 0.05% Tween 20, PBS-T) for 4–6 h at RT. After washing
the plates with PBS-T, dilutions of *Lactobacillus* culture supernatants containing secreted VHH and *Lactobacillus* purified VHH were added and the plates were incubated at 4°C o/n. *E. coli* purified VHH J3 (114) was used as positive control for binding. Supernatant from *L. paracasei* pAF100-ARP1 producing secreted VHH against rotavirus was used as a negative control (124). Plates were subsequently washed three times with PBS-T and mouse anti E-tag antibody was added at 1 µg/ml in blocking solution followed by incubation at RT for 2h. Plates were then washed three times with PBS-T and incubated with alkaline phosphatase (AP) conjugated rabbit anti-mouse antibody at 1/1000 (Dako A/S, Glostrup Denmark) in blocking solution. Following additional 1 h incubation at RT, the plates were washed thrice with PBS-T and once with PBS. After the washes, 100 µl of diethanolamine buffer (1M, pH 10.0) containing 1mg/ml p-nitrophenyl phosphate (Sigma-Aldrich, St. Louis, MO) was added and the absorbance was read after 10-30 min at 405 nm in a Varioskan Flash (Thermo Scientific, Waltham, MA).

### 3.15 Purification of the VHH and quantitation

Purification of the secreted VHH was performed by using a HiTrap™ anti E-tag Column (GE-Healthcare) according to the manufacturer’s instructions. Eluate was concentrated on Amicon ultra-3K centrifugal filter (Millipore, Carrigtwohill, County Cork, Ireland).

### 3.16 BCA assay for protein quantitation

The concentration of purified VHH was determined using the Micro BCA™ Protein Assay Kit (Pierce, Rockford, IL) with bovine serum albumin (BSA) as a standard.

### 3.17 Flow cytometry

Flow cytometry was used for detection of anchored VHH on *Lactobacillus* surface and for its binding to HIV-1 envelope glycoproteins. For detection of J3 on the surface of lactobacilli 50 µl of the *Lactobacillus* cultures of J3-pAF900 construct grown to an OD600 of 1.0 in MRS were harvested by centrifugation (8000 rpm, 3 min) and washed three times with 500 µl PBS. Bacteria were re-suspended in 50 µl PBS with 1% BSA (PBS-BSA) and incubated on ice for 30 min sequentially with 50 µl anti E-tag antibody (10 µg/ml) and 100 µl fluorescein isothiocyanate (FITC) conjugated anti-mouse immunoglobulins (diluted 1/200) (Jackson Immunoresearch Laboratories, West Growe,
PA), all diluted in PBS-BSA. Bacteria were washed with 500 µl PBS between all three incubations.

To test binding of surface anchored J3 to HIV-1 glycoproteins, 50 µl of *L. paracasei*-mJ3-pAF900 construct were harvested and washed three times in PBS. Bacteria were incubated on ice for 30 min with biotinylated Bal.26 gp120 (NIBSC, Hertfordshire, UK) and CN54 gp140 (Polymun Scientific, Klosternevburg, Austria) at concentration of 10 µg/ml followed by incubation with streptavidin PE (diluted 1/200) for 30 min on ice. Glycoproteins were biotinylated using EZ-LinkTM Sulfo-NHS-LC-Biotinylation kit (Thermo Scientific) as per the manufacturer’s protocol. Non-transformed *L. paracasei* BL23 and *L. paracasei* pAF900-ARP1 producing surface anchored VHH against rotavirus were used as a negative control (124).

### 3.18 *In vitro* neutralization assay

*Lactobacillus* produced VHH was tested for neutralizing activity *in vitro* using the TZM-bl luciferase assay against a range of HIV-1 pseudoviruses. For the *Lactobacillus* secreting VHH, VHH purified from the supernatant was tested. For the surface anchored lactobacilli, the bacteria were used for pre-absorption of the virus before inoculation of the target cells. The TZM-bl cells were cultured in DMEM (Gibco BRL Life Technologies) containing 10% heat-inactivated FBS µg/ml gentamicin (Sigma). Cell monolayers were disrupted at confluency by treatment with 0.25% trypsin in 1mM EDTA. Envelop (Env) pseudotyped viruses were obtained by co-transfecting the Env plasmid with pSG3ΔEnv (125) using FuGENE transfection reagent (Roche) as previously described (126). Neutralization was measured by a reduction in luciferase gene expression after single-round of infection of TZM-bl cells with Env-pseudotyped viruses (126). VHH IC50 titers were calculated as the reciprocal viral dilution (ID50) causing 50% reduction of relative light units (RLUs).
4 RESULTS

4.1 Paper I

Most women experience BV and many of them suffer from relapses. In order to reduce the frequency of relapses and to find out if an extended antibiotic treatment against bacterial vaginosis (BV) together with adjuvant lactobacilli treatment could cure BV. In this study 63 women diagnosed with BV were included and were offered a much more aggressive treatment than given in current clinical practice with repeated clindamycin and metronidazole treatment and vaginal Lactobacillus administration. Both newly characterized and commercially available Lactobacillus strains were used for vaginal administration.

The women receiving EcoVag® were colonized by L. rhamnosus 14870 in 6 out of 8 cases (75%). The strain persisted more than 2 months in three patients, i.e. about 2 weeks after stopping its administration and until month 3 and 5 in two other patients (one and three months after stopping the treatment respectively). Of the women receiving the characterised lactobacilli isolated from Swedish women (Group 1 to 3), L. crispatus strains (4R5, 8R6 and 23B33) were the ones showing the best colonization. Although colonizing a lower proportion of women (around 33%), L. crispatus 4R5 (Group 1), L. crispatus 23B33 (Group 2), and L. crispatus 8R6 (Group 3) persisted until month 6 in 7 out of 9 women colonized by one of these strains i.e. 4 months after stopping the treatment. However, no significant difference was observed in the cure rate depending on which Lactobacillus strain was given and whether the women were colonized by lactobacilli. The patients were followed as long as possible or until the relapse. The cure rates obtained after month 6, 12 and 24 were 74.6%, 65.1% and 55.6%. One of the most noticeable observations in the study was occurrence of relapse of BV in women after having a new sexual partner (Odds ratio of 9.3). To conclude, aggressive treatment with antibiotics combined with vaginal administration of lactobacilli gave a long term cure.

4.2 Paper II

Paper II represents two trials which were conducted in order to confirm results of paper I and to improve Lactobacillus colonization and BV treatment. We also tested for the first time, a combination of EcoVag® and anti-fungal for treatment of recurrent yeast
infection and evaluated if lactobacilli can colonize women when the microbiota is not disturbed by antibiotics.

In trial I, 10 women diagnosed with BV were recruited and given the combination treatment with antibiotics and lactobacilli as mentioned before (methods, section 3.3.). In trial II, both women with BV and yeast infection were recruited. A similar treatment with clindamycin and metronidazole was given for BV but the Lactobacillus dose was increased. EcoVag® vaginal capsules were given for 10 days after every antibiotic treatment and after the second menstruation once every week for four months. Women with yeast infection also received a similar Lactobacillus treatment along with fluconazole. Another modification in the protocol was that the swab samples were collected twice every month on day 7 and 21 as the level of lactobacilli may vary during the menstruation cycle.

In trial I, nine of the 10 women treated for BV got colonized by either of the EcoVag® Lactobacillus strains during the study. In eight women, EcoVag® strains persisted for at least two weeks after stopping the treatment. In three women, either of the strains was identified at month four (two months after the treatment was stopped) and persisted until month five (three months after the treatment) in two of them. L. gasseri DSM 14869 was more frequently isolated than L. rhamnosus DSM 14870 (17 vs 9 out of 42 samples) but the difference was not significant (P=0.098). The cure rate after month 6 was 50 % with four women cured and four with a relapse of BV. Two women did not complete the sixth month follow up.

In trial II, either of the EcoVag® strains was identified until month five in 5 of the 10 women (71%). L. gasseri was the dominant colonizer in this group colonizing seven women at some stage during the study and was regularly isolated in two women out of seven until month six. L. rhamnosus was isolated from five women but more sporadically. In three women, both the EcoVag® strains were found to colonize individually at different sampling points. Some of the interesting observations were: - 1- EcoVag® strains colonized women but other Lactobacillus species were seen colonizing throughout the study; 2- EcoVag® strains were more frequently isolated from women that did not have lactobacilli at the start of the study compared to women who had lactobacilli at the start of the treatment; 3- No significant difference was observed in the frequency of isolation of EcoVag® strains or other lactobacilli between
sample 1 (day 7) and sample 2 (day 21). 4- A comparison of both trial I and II shows that colonization by EcoVag® lactobacilli in women with BV did not improve significantly upon increasing the dose. The cure rate for BV group was 66.7% and 62.5% after month 6 and 12. Three women had a relapse after having a new sexual partner.

The frequency of isolation of any Lactobacillus species during the course of the study was associated with cure of BV in trial I and II whereas, the frequency of isolation of EcoVag® strains was significantly associated with the cure of BV in trial II only. As previously observed, a change in sexual partner was associated with relapse of BV with an Odds ratio of 77 (95% CI: 2.665 to 2225).

In the second group of women with Candida infection, EcoVag® strains were isolated from 8 out of 9 (89%) women at some time point during the study but the Lactobacillus microbiota was dominated by non EcoVag® Lactobacillus strains. L. rhamnosus DSM 14870 was isolated from six women and L. gasseri DSM 14869 from five women. L. rhamnosus DSM 14870 could be isolated up to month 5 in three women. Both strains could be isolated at some point in three women. Overall L. rhamnosus DSM 14870 and L. gasseri DSM 14869 were isolated in 16 (19%) and 10 (12%) out of 86 samples respectively. A comparison of both the trials showed that EcoVag® lactobacilli could be isolated more frequently in BV patients pretreated with antibiotics (41 out of 86 samples, 48%) than from Candida infected patients pretreated with anti-fungal drugs (24 out of 86 samples, 28%) (P<0.05). The cure rate for VVC was 100% after 6 months and 87.5% after 12 months.

4.3 Paper III
The vaginal microbiota has been extensively studied in populations in western countries but less so in African population. Therefore, we conducted a project in South Africa to study the vaginal microbiota in a group of 40 women from Soweto. Vaginal swab samples were collected and analyzed for the presence and identification of Lactobacillus species, and the VMB was assessed by Nugent scoring using the vaginal glass smears. Lactobacilli were identified in 19 out of 21 women harbouring a normal vaginal microbiota, in three out of five women with intermediate microbiota and in eight out of 14 women with BV. The most commonly identified Lactobacillus species were L. crispatus, L. iners, L. gasseri, L. vaginalis and L. jensenii isolated from 10
(33%), 8 (27%), 7 (23%), 5 (17%) and 5 (17%) respectively. Importantly, *L. crispatus* was isolated only from women with a normal vaginal microbiota whereas the other species were isolated from women both with and without BV. Microscopical examination of the smears revealed that in this cohort, clue cells were only observed in smears with high Nugent scores, thus confirming as association between the presence of clue cells and BV.

Presence of any lactobacilli and in particular *L. crispatus* was significantly associated with normal vaginal microbiota (Fisher test, normal vs. BV microbiota, \( P = 0.0386 \) and \( P = 0.024 \) respectively) but not the colonization by other *Lactobacillus* species. The isolated *Lactobacillus* species were characterized for their production of hydrogen peroxide (\( \text{H}_2\text{O}_2 \)). Production of \( \text{H}_2\text{O}_2 \) was observed in the majority of the *L. crispatus* (90%), *L. jensenii* (86%) and *L. vaginalis* (80%) isolates and in a lower proportion of the *L. gasseri* isolates (30%). Though the association was not significant, isolates producing high levels of \( \text{H}_2\text{O}_2 \) were more frequently isolated from women having low Nugent scores compared to women with high scores.

### 4.4 Paper IV

The variable domain of heavy chain only antibody (VHH) from *Camelidae* family is the smallest known antibody derivative to neutralize pathogens. The VHH J3 eliciting a potent and broad neutralizing activity against HIV-1 was expressed in *L. paracasei* BL23 in secreted and anchored form. Expression of VHH J3 was analyzed by western blot and bands of the expected size were detected for secreted (15.24 KDa) and surface anchored (39.83 KDa) J3. Flow cytometry was performed to confirm the surface display and binding of J3 to Bal.26 gp120. VHH J3 expressing cells gave a strong signal for both display and binding to the glycoprotein, whereas the non-expresser *L. paracasei* BL23 and *L. paracasei* producing VHH against rotavirus (ARP1) did not bind to gp120. The binding of secreted J3 to HIV-1 gp120 was shown by ELISA. The *Lactobacillus* purified J3, *E. coli* produced J3 and *Lactobacillus* expressing surface anchored J3 are currently being tested for *in vitro* neutralization against various HIV-1 strains.
5 DISCUSSION

Ever since HIV-1 was identified as the causative pathogen of the AIDS epidemic, trials till date are continued in order to come up with a vaccine or treatment, which can prevent or cure HIV/AIDS. Despite of the success of HAART, HIV-1 is of a major concern because of the genetic variability of the virus and associated side effects of anti-HIV drugs. Moreover, education, social environment, sexual behavior, availability of medical resources and one’s own health affect the rate of HIV incidences majorly. Therefore, a combination of different strategies is required to battle HIV-1. The prevalence of HIV is highest in Sub-Saharan Africa and it is striking to see that the same is also true for BV (51) which points towards a possible link between these two diseases. Research findings in this field has established a link between BV and HIV-1 infection according to which in the absence of lactobacilli and overgrowth of anaerobic bacteria, a condition identified in BV, increases the risk of acquiring HIV-1 infection and its transmission (17,20,91). BV is one of the most common vaginitis followed by VVC that affects women in their reproductive age and these should be addressed with high importance.

A novel approach towards improving vaginal health and preventing HIV-1 from causing infection would be to develop designer probiotic, which is capable of colonizing the vagina and programmed for in situ production of microbicide. For this purpose VHH antibody fragment is our choice of microbicide. Among sub-Saharan African countries, South Africa is still the country most severely affected by HIV/AIDS and therefore we aim in the future to test our designer probiotic approach in South Africa. A stepwise development of all the projects and important research findings are discussed in this section.

Clinical trials in Scandinavia

5.1 Treatment of BV and Lactobacillus colonization

According to a Meta-analysis BV can be treated with metronidazole and clindamycin with an expected one month cure rate of 70-80% and 82% respectively (127) which is rarely observed in clinical practice. Despite of the success of treatment with antibiotics, high recurrence rate is still a problem. Therefore, in order to achieve a long-term cure, we designed a study accessing the impact of an aggressive antibiotic treatment with
administration of both clindamycin and metronidazole combined with vaginal administration of *Lactobacillus* capsules (paper I).

In paper I, five different *Lactobacillus* formulations were tested in women: EcoVag® capsules containing *L. gasseri* DSM 14869 and *L. rhamnosus* DSM 14870, capsules containing EcoVag® strains plus *L. gasseri* DSM15527 and capsules containing three different mixtures of three newly characterized strains (paper I). Each mixture of newly characterized lactobacilli contains one strain of each of the following species: *L. crispatus*, *L. gasseri* and *L. jensenii*. The antibiotic treatment consisted of one course of clindamycin treatment (oral and vaginal) followed by two courses of metronidazole vaginal gel. *Lactobacillus* capsules were given for 5 days after both the clindamycin treatment and the first treatment with metronidazole. The obtained cure rate was 74.6% and 65.1% after 6 and 12 months respectively, which is higher than in most of the other studies (paper I). A similar cure rate was obtained with different preparations of lactobacilli administered. Among the strains tested, only three *L. crispatus* strains and *L. rhamnosus* DSM 14870, could be isolated from the vaginal samples. *L. rhamnosus* DSM 14870 colonized a higher proportion of women than *L. crispatus* strains (75% vs 30%) but the later colonized for a longer period (up to four months after the treatment was stopped). However, no correlation was found between colonization and cure of BV. In comparison to other well-studied probiotic strains, these results present longer colonization with given lactobacilli. Previous studies, where vaginal colonization was assessed for *L. crispatus* CTV-05, *L. rhamnosus* GR-1 and *L. fermentum* RC-14, 20 to 40% of the women not treated with antibiotic were colonized for 4-weeks post intra-vaginal administration (82,128,129). In another study, vaginal capsules containing a mixture of *L. gasseri* LN40, *L. fermentum* LN99, *L. casei* subsp. *rhamnosus* LN113 and *Pediococcus acidilactici* LN23 were administered for five days to women after conventional treatment with clindamycin (130). Following the first menstruation after *Lactobacillus* administration, 53% of the women were colonized by any of the five strains but only 26% after the second menstruation. In accordance with our results, no correlation was found between colonization and the clinical outcome for BV. The difference in colonization by the probiotic lactobacilli might depend on factors such as the microbiota at enrollment and vaginal intercourse (82). Thus it was clear to us that in order to achieve long-term cure and reduce the relapses, an aggressive antibiotic treatment combined with lactobacilli provides an opportunity for the VMB to stabilize for a long duration.
5.2 Modification of the protocol and treatment of BV

Considering the prospects of a vaginal probiotic, especially a microbicide expressing lactobacilli for prophylaxis against HIV-1, long-term colonization of the administered lactobacilli is of utmost importance. As EcoVag® colonized a higher proportion of women, we further designed experiments in order to improve the persistence of EcoVag® strains and treatment of BV. It was previously shown that lactobacilli are resistant to metronidazole but may be sensitive to high concentrations (131). Therefore, in the next trial performed with EcoVag® lactobacilli in BV patients (paper II, trial-1), we reduced the metronidazole treatment to one course. This time either of the two EcoVag® strains or both colonized 90% of women and persisted up to 3 months after the treatment was stopped in 33% of the treated women. In contrast to the paper I, in this trial, L. gasseri DSM 14869 was more frequently isolated than L. rhamnosus DSM 14870, although not to a significant degree (17 vs 9 out of 42 samples, P=0.098). These results are slightly better than in our previous study where EcoVag® strains were isolated in 75% of the women and in 13% of them, three month after stopping the treatment.

Later on, Eriksson et al. in 2011 showed that clindamycin could be present in the vagina in low concentrations 5 days after the treatment is ceased (132). Since EcoVag® was given for five days immediately after the clindamycin treatment; the presence of residual clindamycin might have affected Lactobacillus colonization. Therefore, the treatment protocol was further modified (paper II, trial II). We modified the protocol by increasing the EcoVag® lactobacilli dose from 5 to 10 days after both the antibiotic courses and prolonged the treatment for next four months by giving lactobacilli once weekly (Paper II, trial II). We were also interested to note any changes in vaginal colonization by EcoVag® lactobacilli during the menstrual cycle and therefore decided to collect vaginal samples twice a month. A swab sample and a glass smear were collected every month at day 7 and day 21 after menstruation. It has been observed that the composition of vaginal microbiota changes during menstrual cycle. After four months, 75% of women were identified with EcoVag® in trial II compared to 50% of women colonized by EcoVag® in trial I (paper II). Hence the colonization and persistence of EcoVag® lactobacilli slightly improved by increasing the EcoVag® dose but not significantly. Furthermore, the frequency of isolation of EcoVag® strains throughout the study was similar in both the trials. However, a significant increase in colonization of other Lactobacillus strains was observed in the second trial. We
speculated that the administered lactobacilli (EcoVag® capsules) may favor colonization by other Lactobacillus strains or species by improving the vaginal environment although, it might also be because a higher proportion of women harbored other lactobacilli at the time of inclusion in trial II (44%) than in trial I (0%). The probable colonization resistance offered by the endogenous lactobacilli to the newly administered lactobacilli following an antibiotic treatment could explain this. A similar finding was described by Antonio et al. where, the presence of endogenous lactobacilli decreased colonization by probiotic L. crispatus CTV-05 (82). It has previously been shown that the proportion of lactobacilli increases after menstruation and the number of other non-Lactobacillus species increases before menstruation (16,133). No difference was observed between isolation of EcoVag® strains or other lactobacilli over the cycle. However, in our study, EcoVag® strains were provided weekly.

The cure rate at month 6 was 50% for trial I and 66.7% in trial II. This cure rate was better than in most published studies but lower than in Paper I (74.6%). This might be because only one course of metronidazole was given in the present study instead of two (80). The cure rate at month 12 in trial II (62.5%) was however comparable to the one previously reported (65.1%). In Paper I, we observed no correlation between isolation of EcoVag® strains and cure of BV but we did not consider long-term persistence of lactobacilli. In trial II of paper 2, we observed that the frequency of isolation of EcoVag® over the course of the study was associated with cure. Furthermore, in both trial I and II, the frequency of isolation of any lactobacilli (EcoVag® strains or other Lactobacillus strains) during the treatment period resulted in a better clinical outcome.

Overall, a factor which was strongly linked with relapse of BV in paper I and II was the change of sexual partner. Whether it is because of increased sexual activity after change of partner or because BV may act as a STD associated condition, we can only speculate that a stable sexual relationship and use condom might reduce the risk of relapse.

5.3 Colonization of EcoVag® lactobacilli in the presence of endogenous lactobacilli and cure of VVC

It was not certain whether the administered lactobacilli would colonize in the presence of endogenous lactobacilli and for how long it will persist in the vagina. This is also of importance from the microbicide point of view that the engineered lactobacilli are able to colonize and persist in women who harbor endogenous lactobacilli. To investigate
this, we included a group of women in trial II who were diagnosed with VVC, a condition arising from overgrowth of yeast but where lactobacilli are still present. These women were treated with fluconazole, which is an anti-fungal drug and not expected to kill lactobacilli. Women included in the study had recurrent episodes of VVC and were offered an aggressive fluconazole treatment with intent to treat together with lactobacilli as described in trial II (paper II).

We observed a difference in colonization in BV and VVC treated patients. EcoVag® strains colonized better in women treated with antibiotics for BV than in women who were treated with fluconazole for VVC. This could be because of: 1- inefficient colonization by EcoVag® lactobacilli in the absence of prior antibiotic treatment and; 2- in the presence of certain lactobacilli, which may affect colonization with probiotic lactobacilli (134). This is also reflected in our observation that among the women treated for VVC, EcoVag® strains were more frequently isolated from women who did not have lactobacilli at the start of the study. All the women were cured from of VVC at 6 months follow up and 87.5% of these women were still cured at the second follow up (from 12-18 months after the initial treatment). Although a few clinical studies have presented conflicting results on the use of lactobacilli to reduce recurrence of VVC, most of the women in our group remained cured for 12-18 months. Since the fluconazole dose given in our study is very high, we question the possible role of given lactobacilli in long-term cure of VVC. In order to investigate the efficacy of EcoVag® lactobacilli for vaginal colonization and treatment of VVC, we included a group of women with R-VVC who are receiving a similar fluconazole treatment without adjuvant EcoVag® lactobacilli. We are still awaiting the results of this control group.

5.4 Which *Lactobacillus* species colonize South African women?

**Will EcoVag® strains colonize women in Africa?**

The VMB has been studied extensively in Western populations albeit less so in African populations. Studies performed in North America, which compared the composition of VMB in different ethnic groups, reported the dominance of non-*Lactobacillus* species in black women (14,15). However, studies performed in Africa suggest that the vaginal *Lactobacillus* species colonizing African women are in fact similar to those identified in western populations (5,6). It was thus important for us to conduct a study in Soweto, South Africa to identify the colonizing vaginal *Lactobacillus* species and determine whether the *Lactobacillus* microbiota in these women is similar to that identified in
western population. If the *Lactobacillus* species isolated from African women are similar to those in America and Europe where probiotic studies have been performed, similar strategies utilizing probiotic and engineered lactobacilli could be applied in Africa. Furthermore, if needed, the isolated strains could be characterized and tested for colonization. The pre-dominant *Lactobacillus* species identified in our cohort were *L. crispatus*, *L. iners*, *L. gasseri*, *L. vaginalis* and *L. jensenii* similar to European and American women (3,4,135). We also observed that the presence of lactobacilli and in particular *L. crispatus* was associated with normal VMB. None of the women colonized by *L. crispatus* had BV or intermediate microbiota as per the Nugent scores. The role of *L. crispatus* in stabilizing the VMB has been presented by Verstraelen *et al.* in a longitudinal analysis of VMB in 2009 (28). Other *Lactobacillus* species were isolated from women irrespective to the Nugent scores.

Various studies have shown an association between colonization by H$_2$O$_2$ producing lactobacilli and normal VMB, where lack of such lactobacilli increased the risk of developing BV (19,23,25,35). On the contrary, a study by O Hanlon *et al.* reported that the physiological concentrations of H$_2$O$_2$ below 100 millimolar did not kill BV associated bacteria (36). In order to find out any association in our cohort we tested the isolated lactobacilli for the production of H$_2$O$_2$. In accordance with previous studies (136,137), the majority of *L. crispatus*, *L. jensenii* and *L. vaginalis* isolates were H$_2$O$_2$ producers. The presence of H$_2$O$_2$ producing lactobacilli was higher in women with normal VMB, although not to a significant degree which could be due to the small sample size and the single sampling point. Moreover, at the time of sample collection, behavioral factors that might affect the VMB and the phase of menstrual cycle were not noted (except that the women were non-menstruating). Therefore a longitudinal study with bigger cohort and by considering all the factors might give more information on the association between H$_2$O$_2$ producing lactobacilli and the VMB.

### 5.5 Lactobacilli expressing VHH antibody fragments

Due to their smaller size and single domain nature, VHH are easy to express as functional recombinant proteins in bacteria or yeast. As previously reported by our group, *Lactobacillus* produced VHH against rotavirus (ARP1) elicited effective binding to and neutralization of rotavirus (112,119). Lactobacilli expressing surface anchored ARP1 were able to reduce the viral load and alleviate diarrhea symptoms in a mouse pup model. Similarly, a *Lactobacillus* colonizing the vaginal tract and producing VHH
antibody fragment could be used for neutralization of HIV-1. In paper IV, we have expressed a VHH against HIV-1 gp140 (VHH J3) in secreted and surface anchored form in a model Lactobacillus strain (L. paracasei BL23). The Lactobacillus produced VHH were shown to bind to Bal.26 gp120 and are currently being tested for in vitro neutralization of HIV-1 strains. An estimated amount of VHH produced by lactobacilli is \( \approx 3 \mu g/ml \), which might not be enough for in vivo protection against the virus. On the contrary, the advantage of having lactobacilli expressing surface anchored VHH is that it offers multiple VHH molecules attached on cell surface thus, increasing the avidity. Moreover, the virus once captured by VHH on the Lactobacillus surface would form an aggregate, thereby reducing the chance of dissociation of the virus-antibody complex. Once aggregated the virus particles would lose their infectivity, reducing the infectivity of virus. Compared to the secreted VHH, anchored VHH is expected to be more potent taking advantage form the anchored VHH and by being attached on the epithelial cells.

The initial phase of infection offers several targets for neutralizing antibodies, which in the case of HIV-1 are gp120 and gp41 on the virus whereas CD4 and CCR5/CXCR4 receptors on the host cell are also potential targets. An advanced approach would be to have a polyclonal antibody mix like preparation of lactobacilli expressing VHH with different targets thus increasing its avidity. The success of VHH dimers expressing lactobacilli at binding to rotavirus and in preventing the development of diarrhea in mouse model proved that it is possible to express functional VHH with different specificities in dimer form (138). No loss in the expression levels of VHH was noticed. Similarly VHH J3, the best candidate, could be coupled with another VHH with a different target. The concept would allow us to have custom made lactobacilli expressing our key molecules to target various antigens or pathogens (like HSV-2, HPV).
6 CONCLUSIONS

The ultimate aim of these and the future projects is to build an efficacious, economical, comfortable and protective strategy for prophylaxis against HIV-1 infections and maintain vaginal health. The following conclusions could be drawn from the four projects:

Paper I and II
- An aggressive antibiotic treatment combined with administration of EcoVag® can provide a long lasting cure against BV and VVC.
- A prolonged treatment with EcoVag® lactobacilli together with antimicrobials could improve long-term cure against BV and VVC and reduced the rate of relapse.
- The change of sexual partner is associated with relapse of BV.

Paper III
- The vaginal lactobacilli colonizing South African women is similar to those identified in European and American populations.
- The presence of lactobacilli, specifically *L. crispatus*, is associated with a normal vaginal microbiota.

Paper IV
VHH antibody fragments can be functionally expressed in lactobacilli in both secreted and surface anchored forms.
7 FUTURE PERSPECTIVES

*Lactobacillus* plays an important role in maintaining the vaginal microbiota and has been successfully used for improving the vaginal health and for the delivery of therapeutic molecules on mucosal sites. Taking into account the recent advances in the field of host-microbe interactions, it is irrefutable to accept the importance of a healthy host microbiota. Considering these emerging facts, it is of primary importance to develop therapeutic strategies targeted to improve vaginal health, which may further have a deeper impact on prevention or treatment of sexually transmitted diseases such as infection by HIV-1, HSV-2 etc. One of the major thrust areas, which can be benefited by the virtue of the same ideology, is the mother-child health. The first microbial population, which colonizes the human body, is derived from mother’s vaginal microbiota or skin during the process of birth. This clearly indicates the absolute importance of a balanced vaginal microbiota since it contributes to the normal delivery and, later on governs the metabolic fate of an individual. This in turn opens up a wide scope for developing new strategies to improve mother’s vaginal microbiota during the pregnancy period in order to improve child health and prevent premature birth. One of the strategies could be the use of probiotic vaginal *Lactobacillus* strains during the pregnancy. The probiotic lactobacilli could be provided in formulations like yoghurt, drink or a vaginal capsule containing freeze-dried lactobacilli.

Addressing BV and VVC in Scandinavia by modifying clinical interventions and use of lactobacilli, we were able to improve cure rates. A long-term vaginal colonization by administered lactobacilli is of primary importance if we want to target African population where the prevalence of both BV and HIV-1 is the highest. Till date no *Lactobacillus* colonization study has been performed in South Africa and this is what we aim for taking a step forward in this direction. EcoVag® lactobacilli will be assessed for colonization in South African women which would in turn have an impact on future studies. Having recently obtained the genome sequence of EcoVag® lactobacilli, it will also be possible to perform quantitative analysis by real-time PCR and to follow the colonization of these strains over the menstruation cycle. With the emerging high throughput sequencing techniques, the vaginal microbiome and the shift in microbial populations could be studied both in women with normal microbiota and with BV before and after administration of EcoVag®. The possible gene interactions or the resulting pre and/or probiotic effects could also be studied using the gene microarray.
technology benefiting the field of vaginal probiotics. If the EcoVag® strains appear not to colonise women in South Africa, Lactobacillus strains isolated from South African women could be further characterized and tested for colonization.

To overcome the hurdles related to the use of current microbicide formulations against HIV-1 infection, a novel strategy would be to genetically modify the probiotic lactobacilli to deliver therapeutic molecules on the mucosal surface in the vagina. The best colonizing Lactobacillus strains from our study in South Africa could be engineered for production of VHH and, these recombinant designer probiotic lactobacilli could colonize the vagina and produce VHH in situ. To stabilize the expression of VHH, the VHH encoding gene will be inserted in the Lactobacillus genome and the gene encoding thymidine will be knocked down in order to ensure that the genetically modified lactobacilli will not survive outside the human body. However, before performing a clinical trial with the genetically modified lactobacilli, the concept will have to be tested in a non-human primate model, in order to ensure that the modified lactobacilli are protective against mucosal SHIV challenge. An added benefit with the VHH conferred by its small size is that, they could be expressed as homo/hetero dimers increasing the avidity with similar specificity. This would also overcome the problem of relative lower expression of the recombinant VHH in lactobacilli.

The modified lactobacilli could be freeze dried and incorporated in capsules or tampons, allowing the stockpiling of microbicide without any need of strict storage conditions. Once administered, the lactobacilli would colonize the vagina and persist while producing the microbicide in situ. This would bypass the need of repeated and frequent administration. To conclude, Lactobacillus based strategies for the delivery of microbicides against HIV-1 or other sexually transmitted pathogen, would function as a bi-headed arrow, where one arm would target for an improvement of the host health and another arm to prevent pathogen from causing infection. The strategy thus offers a discrete, affordable, easy to use and coitally independent HIV prevention, which can be applied against various pathogens and could be reciprocated in a bigger population with no known side effects.
8 POPULAR SCIENCE SUMMARY

The female body is a complex and delicate system that plays a major role in flourishing the human society but, this also exposes them to various health complications. Disturbance of the balance of beneficial and non-beneficial bacteria residing in the vagina is one such example where, our own body, physical/sexual activity and environmental factors influence this balance. Lactobacilli in the vagina are such beneficial bacteria, which maintain the vaginal environment by producing acid and compounds active against other microbes. The disturbed balance of vaginal bacteria causes health issues like bacterial vaginosis (BV) and yeast infection (vulvovaginal candidiasis (VVC)), which in turn amplifies the risk of getting infected by other pathogens including HIV-1. Theoretically, in such a condition one has to deal with two different health complications at the same time and therefore, an approach towards these issues would be to bring back the dominance of beneficial bacteria in vagina and to equip these bacteria to produce anti-microbials that will kill pathogens. Thus, the basis of the thesis work can be very simply read through the conversation below:

Patient: Hello Dr., I have vaginal discharge and foul smell and itching too®. Is that a sign of an infection?

Dr.: Yes, these are the signs of a vaginal infection. It happens when you lose the beneficial bacteria and other non-beneficial ones overgrow. But don’t worry, I will give you some probiotic bacteria that will improve your symptoms and will also protect you from other infections.

But Dr., how does this work?
**Dr.:** Hmm, well, imagine the society we live in. We have all kinds of people living together, but there are some who can cause trouble to others. Therefore, we have police, the ones who are chosen from our own society because they are strong, brave and show characteristics of a soldier. They are selected and trained to combat bad elements in our society. They learn tactics for fighting and to use a weapon when needed for protecting the society. In this way, they maintain law and order, and if we are attacked by some highly trained enemies then, our police uses equally or even better combat skills to bring them down and save the society from destruction.

**So, who’s who Doctor??**

**Dr.:** Our body is like a society, where all different good and bad bacteria live together. When the bad bacteria start to grow more than the good ones, they cause trouble to our body. Therefore, we need the good bacteria, which can reside in our body and fight with the bad ones. Thus, the good bacteria are chosen from our own body after a lot of research and are designed to produce compounds that will stop a pathogen from causing infection.

**Wow, that sounds exciting and safe. Thank you!**
9 ACKNOWLEDGEMENTS

The compilation of this PhD thesis marks the final phase of my doctoral program. This has been an extraordinary journey, giving me a vast experience in different facets of life and research during these years. This would not have been possible without the presence and contribution of numerous people directly or indirectly related to my work. I take this opportunity to convey my deepest gratitude to all those who have contributed in making this possible.

My deepest and sincere appreciation to my principal supervisor and mentor, Dr. Harold Marcotte. Thank you Harold, for your contribution in making my PhD fruitful. I greatly appreciate your scientific insights, all the scientific discussions we have had together and valuable advices on scientific writing. You have been a fantastic supervisor, supporter and mentor.

I convey my sincere gratitude to Professor Lennart Hammarström, my co-supervisor and the group leader, for allowing me to pursue my doctoral studies in his group and under his able guidance. I thank you sincerely for everything that you have done for me. In these five years, I have gained strength and self-confidence, which would help me throughout my life and yes, “Sky is the limit”. Thank you!

Ammi (Anne-Marie Duan), I sincerely thank you for making the start of my journey in Stockholm as smooth as possible. I am also grateful to Pernilla Klyve, for her ever ready state of mind to help us deal with official matters in no time. Thank you for all those wonderful chats we have had and I wish you all good time ahead. My deepest gratitude to Naradja Wissmar, you are a fantastic lady. I thank you for everything, your energetic and lovely presence, enthusiasm, cheerfulness and kindness. I thank you for giving me a great friend and for all those moments when you have cheered me up. I wish you all good health and happiness in life. Renee Engqvist, Ingegerd Löfving and Kerstin Bergman, three lovely ladies in our group. I am grateful to all three of you for your help with any kind of work in lab. I specially thank Renee and Ingegard, for sharing all those wonderful stories from the time, when I was probably not even born. Thank you for being there.

I take this opportunity to thank all the former and current members of my group, with whom I have spent most of my time in lab or in office. Ashwin Kotnis, Neha Pant, Beatriz Alvarez-Gonzalez, Kasper Krogh-Andersen, Lily, Yoko, Alberto Cagigi, Cornelia Rosner, Giuseppe Cappellano, Noel de Miranda, Georgia Kokarakí, Annika Lindkvist, Kyriaki Liadaki, Chenglin Wu, Ryan Ramanujam, Magdalena Janzi, Likun Du, Stephan Borte, Ning Wang, Andrea Bjorkman, Konstantinos Georgiou, Omar Alkhairy, Yin Lin, Che Kang Lin, Gokce Gunaydin and Margarita Bartish, thank you all for making the place lively with your presence, sweet and spicy conversations, jokes, scientific discussions and for bringing all different colors and flavors to the Hammarstrom groups. My best wishes to the new members of Hammarstrom groups: Dr. Jinqiao Sun, Mia Olsson & Dr. Hasan.

During the years of my doctoral program, I have got support directly or indirectly from senior researchers and professors at the Division of Microbiology and Virology through their valuable comments and questions during the joint seminars. I thank you all for your support and promoting scientific talks and conversations between the divisions and also in the department. Alenka Jejcic, Marjan Amiri, Sophie Curbo,
Soni-Priya, Xiaoshan Zhou, Joao Paredes, Marcus Buggert, Samir Abdurahman, Jessica Nyström, Piotr Novak, Gustaf, Lars, Selina, Sepideh, Fredrik, Marcus, Anette and Antony Chen, I am grateful to all of you for all the interesting and fun full of conversations at work and at the lunch table. Fredrik and Thore, you have been data/life saviors to almost all of us. Besides that, you are fantastic guys and I want to thank you for your help and for short & funny chats.

I sincerely thank my Ph.D. half time committee members Prof. Kristina Broliden, Prof. Eva-Sverremark Ekstrom and Prof. Vinod Divan, for their very encouraging response and guidance. My deepest gratitude to Dr. Manpal Sridhar, principal scientist at the National Institute of Animal Nutrition and Physiology, Bangalore, India, for her positivity and support during my Master’s dissertation project. Mam, you have been an inspiration to me ☺ I also extend my sincere regards to the faculty members of Rungta College of Science and Technology, Bhilai and MATS University, Raipur, India (especially Mrs. Deepa Pillai, Dr. Divya Tiwari and Mr. V. P. Roy) for sowing the seeds of science in me.

I heartily thank all my friends from Graduate Student’s Association board, for the very enthusiastic participations and fantastic-long-fruitful meetings. I have enjoyed working with all of you.

At work and away from work, I feel lucky to have found jems like Marcel Frankowiack, Shuba Krishnan, Kathrin Reiser, Jenny Svard, Halime Ekici, Babilonia Barqasho, Kajsa Noyan and Simin Zhang. You guys are fantastic and without your precious presence I would have not imagined my stay here. Your presence, continuous support, cheerfulness and inspiring talks (especially from Halime) have made my life happier and easier. I will miss you all very much.

My friends from outside lab and from India; Erwin Brenndörfer & Xiaoli Linda Hu, Christopher & Milica Udhe, Esra Karaca, Ruchi Bansal, Agata Wasik, Annie Pettersson, Deepika, Kalai, Arivind, Shwetha & Subbu, Senthil Vasan & Sujareetha, Vivek Sunkari & Saranya, Balaji, Varsha, Sravya, Aishwarya, Vidhu, Poonam and Avanti, I thank you all from the bottom of heart for being a part of my journey and making it easy and memorable. I would cherish the memories I have with you. My heartiest thanks and gratitude to Saritha, Rakesh, Robby, Sini, little Julia and Devesh & Marika for showering immense love and care. You have given me my own family outside India. Thank you!

At last but not the least; I would like to extend my deepest regards and humble gratitude to my family for being extremely supportive and believing in me and my abilities. My parents Mr. Suhas Pendharkar and Mrs. Swati Pendharkar and my brother Salil Pendharkar (“Pillu”), there are not enough words with which I can thank you for your unconditional love and support. Aai-Baba (mom-dad) you have been the true inspiration behind whatever I have achieved in my life. I thank you for giving me this beautiful life and for being the best guide and critic in my life. I am proud to be your daughter. My deepest gratitude and love to my best friend - my husband Sameer Kulkarni. You have been the true partner of my life. Since the last eight years, that I have known you, despite of all physical boundaries you have always been next to me for cheering me and inspiring me to leave behind the hurdles and proceed in my life happily. Your support has been truly extraordinary and I look forward to gather and cherish many more precious and sweet memories with you. Love you!
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