UROGENITAL PROBIOTICS
Potential role of *Lactobacillus* in the prevention of urogenital infections in women

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Till mina nära
The secret of being a bore is to tell everything.
- Voltaire
Abstract

The human vaginal ecosystem is dominated by *Lactobacillus* species. An altered vaginal flora can result in symptomatic conditions such as bacterial vaginosis and vulvo-vaginal candidiasis, and urogenital colonisation by uropathogenic bacteria can cause urinary tract infection. The protective role of lactobacilli is gradually being accepted and clinical studies have been carried out in order to evaluate the use of promising probiotic bacteria, which are defined as “live microorganisms which when administered in adequate amounts confer a health benefit on the host”.

This thesis includes an investigation into the ecological role of lactobacilli in the genital tract in healthy women, with respect to the relationship to other species and vaginal pH. Furthermore, in order to find different probiotic strains with promising probiotic qualities, *Lactobacillus* strains were screened in two diverse screening processes. The selected strains were further evaluated in clinical trials.

The prevalence of group B streptococci (GBS) and yeast was significantly dependent on the number of vaginal lactobacilli among healthy women. GBS were less frequently found in women with high numbers of vaginal lactobacilli than in women with low numbers and the prevalence of yeast was significantly higher in women with 3-6.99 log_{10} lactobacilli sample^{-1} than in women with less than 3 or ≥7 log_{10} lactobacilli sample^{-1}. Furthermore, the first screening made on 511 strains isolated from the female genital tract resulted in the final selection of a *Lactobacillus plantarum*, designated LB931. The screening showed that LB931 had a strong technical growth, survived through freeze-thawing, produced substances bactericidal to uropathogenic bacteria and was a rapid and strong producer of hydrogen peroxide. Further characterisation showed that LB931 possessed the properties required for probiotics with the capability to prevent urogenital infections. LB931 could be supplied to the genital tract through the usage of panty liners impregnated with the strain. In the second screening, *Lactobacillus fermentum*, designated Ess-1, was the only one out of 126 *Lactobacillus* strains with strong capacity to inhibit *Candida albicans* and *Candida glabrata*. Additional characterisation showed that *L. fermentum* Ess-1 had the properties that are needed to prevent over-growth of *Candida* in the vulvo-vaginal tract. The result of the case study showed that a high and frequent dosage of Ess-1 is needed and that improved vulvo-vaginal candidiasis specific diagnostic criteria are required.

In conclusion, *L. plantarum* LB931 and *L. fermentum* Ess-1 are promising probiotic strains to be used in the prevention of recurrent urogenital infections in women and to enhance the normal flora in healthy women.

Keywords: *Lactobacillus*, normal flora, probiotics, urogenital infections, *Candida*
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Abbreviations

UTI  urinary tract infection
BV  bacterial vaginosis
VVC  vulvo-vaginal candidiasis
RVVC  recurrent vulvo-vaginal candidiasis
spp.  species
VEC  vaginal epithelial cell
H₂O₂  hydrogen peroxide
PAMP  pathogen associated molecular pattern
OTC  over-the-counter
BLS  bacteriocin-like substance
IL  interleukin
TNF  tumour necrosis factor
CFU  colony forming unit
ITS  interference test strain
LB931  Lactobacillus plantarum LB931
MRS  deMann, Rogosa and Sharpe
PFGE  pulsed field gel electrophoresis
Ess-1  Lactobacillus fermentum Ess-1
SP  study period
GBS  group B streptococci
Papers in this thesis

This thesis is based on the following papers and manuscript:

I. **Daniel Rönnqvist**, Ulla Forsgren-Brusk & Eva Grahn Håkansson. Lactobacilli in the female genital tract in relation to other genital microbes and vaginal pH. *Acta Obstetricia et Gynecologica*, 2006; 85; 726-735

II. **Daniel Rönnqvist**, Helena Ström, Ulla Forsgren-Brusk & Eva Grahn Håkansson. Selection and characterization of a *Lactobacillus plantarum* strain promising as a urogenital probiotic. *Microbial Ecology in Health and Disease*, 2005; 17; 75-82


IV. **Daniel Rönnqvist**, Ulla Forsgren-Brusk, Malte Brännström & Eva Grahn Håkansson. *Lactobacillus fermentum* E ss-1 administered to women with vulvo-vaginal candidiasis: report of six cases. (manuscript)

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1.0 Introduction

1.1 Microbial ecology in the lower genital tract in females

1.1.1 Introduction
The vaginal flora was first reported by Albert Döderlein, as early as 1892. Döderlein found that the microflora was homogenously colonised with Gram-positive rods, which were designated the name “Döderlein’s bacilli”. Over the years, these bacilli have been identified as \textit{Lactobacillus} spp. and are still believed to be the dominant components of the genital microflora; however, periodically in combination of other anaerobic and aerobic bacteria species and yeast, among other microbes\textsuperscript{1,2}. The ecological role of lactobacilli in the female genital tract has been thoroughly studied and there is a strong global belief that lactobacilli are part of the defence against pathogenic species. Several means of action have been suggested, but the specific mechanisms and the interaction between these defence mechanisms are not fully understood. The last decade’s increased global interest in women’s health has led to reports concerning the microbial ecology of the female genital area. Furthermore, urogenital infections, such as urinary tract infection (UTI), bacterial vaginosis (BV) and vulvo-vaginal candidiasis (VVC), most often include treatment with antibiotics and antimycotics, which have led to a raised concern regarding drug resistance among pathogenic species. In search for alternative treatments, therapies with cranberry juice, cranberry extract and vitamin A have been suggested for prevention of recurrent UTIs\textsuperscript{3-5}, carbophytic gel has been suggested in treatment of BV\textsuperscript{6} and boric acid has shown good effect on women with VVC\textsuperscript{7}. Additionally, it has been suggested that the improvement of the genital lactobacilli flora can play a key role in the prevention of these conditions\textsuperscript{8}. As a consequence of these alternative treatments, the expansion of antibiotic and antymycotic resistance could be reduced\textsuperscript{9-11}.

1.1.2 The normal microflora
The microbiological flora in the lower genital tract in women is very complex and unique for every woman\textsuperscript{12}. For that reason, there is no clear definition of what constitutes a normal genital flora. A majority of the studies that concern the urogenital microflora in women focus on the vaginal flora and there are only a few reports that regard to the vulva microflora\textsuperscript{13-15}. However, the composition is relatively similar at both sites, except for a higher density of coagulase negative staphylococci in the vulva and \textit{Escherichia coli} in the perineum\textsuperscript{15}. Investigations providing a limited insight into the complete composition of the vaginal flora took place during
the 1970s and have, in recent years, been superseded by studies using advanced quantitative methodology. Lactobacillus spp. are consistently reported to be the predominant species found in the vagina in the majority of healthy menstruating women. Lactobacilli are non-pathogenic, Gram-positive, rod-shaped, facultative anaerobic bacteria that, as well as colonising the genital tract, are often present in the gut and the oral cavity. The genus is large and comprises more than 100 described species. In recent years, L. iners, L. crispatus, L. gasseri and L. jensenii have been reported to be the most common species found in the vagina of healthy females. Vaginal samples often contain only one or a few species, and lactobacilli can be found in concentrations of up to 10^8 cells per ml vaginal fluid. Besides Lactobacillus spp., the bacterial flora in the genital tract is characterised by a mix of Gram-positive cocci and Gram-negative rods (Table I).

In addition to bacteria, up to 20% are colonised with yeast and various colonisation rates have been reported for Mycoplasma spp. and Ureaplasma spp. Microbes found in the vagina are generally believed to ascend from the rectum to the genital tract which is seen as a natural colonisation process. In the vagina, the bacterial flora is regulated by the normal flora through so-called “bacterial interference”. This term describes the situation when the normal flora outcompetes exogenous microorganisms. Bacterial interference is achieved through several contributing mechanisms, for example, the production of antimicrobial substances, competition for the limited amount of nutrition in the ecological niche, pre-emptive attachment to tissue sites and more rapid rate of growth than exogenous microorganisms (reviewed by Larsen & Monif, 2001). Despite evidence that the Lactobacillus flora plays an important protective role against pathogens within the genital ecological niche, healthy women who are not colonised with lactobacilli are not uncommon. This is, however, not considered abnormal or as a sign of disease. Moreover,

### Table I. Prevalence of aerobic and facultative anaerobic species in the vagina of asymptomatic women 19-24 days from onset of menses (published by Eschenbach et al. 23)

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Prevalence in vaginal flora, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactobacillus spp.</td>
<td>88</td>
</tr>
<tr>
<td>Group B Streptococcus</td>
<td>18</td>
</tr>
<tr>
<td>Esherichia coli</td>
<td>6</td>
</tr>
<tr>
<td>Other Gram-negative rods</td>
<td>2</td>
</tr>
<tr>
<td>Gardnerella vaginalis</td>
<td>18</td>
</tr>
<tr>
<td>Ureaplasma urealyticum</td>
<td>38</td>
</tr>
<tr>
<td>Mycoplasma hominis</td>
<td>6</td>
</tr>
<tr>
<td>Candida albicans</td>
<td>12</td>
</tr>
<tr>
<td>Prevotella spp.</td>
<td>28</td>
</tr>
<tr>
<td>Anaerobic Gram-positive cocci</td>
<td>42</td>
</tr>
<tr>
<td>Black anaerobic Gram-negative rods</td>
<td>12</td>
</tr>
<tr>
<td>Bacteroides fragilis group</td>
<td>10</td>
</tr>
</tbody>
</table>


the normal flora changes during different phases of life and during the menstrual cycle. A difference can be seen in the flora as well as the vaginal pH in premenarcheal, premenopausal, postmenopausal and pregnant women. For example, the vaginal *Lactobacillus* flora is foremost prominent in women of reproductive age and the vaginal pH is often low. However, in fertile women, the number of protective *Lactobacillus* are reduced during menses, possibly as a result of a changed physiological environment. Furthermore, the changes in the microbiological flora have been associated with behavioural variables, for example, the number of sex partners and vaginal douching.

### 1.1.3 Defence mechanisms

In addition to the influence of the genital normal flora, several systems are believed to collaborate in order to resist the overgrowth of potential pathogenic exogenous microbes, but the combined system of interaction is still unsolved. The vaginal wall is lined by a stratified squamous epithelium, which is kept moist by vaginal fluid. The fluid contains secretions from cervical and vestibular glands and a plasma transudate secreted through the vaginal wall. In addition to components secreted by the normal flora, the vaginal fluid has a selective antimicrobial activity towards potential pathogenic microbes. Organic acids, peroxides and polypeptides are major elements found in significant concentrations that are high enough to be microbicide. Furthermore, the acidic milieu (pH <4.5) contributes to the continuance of a stable and specific flora. Despite the fact that certain microbes grow at a broad range of pH levels, certain enzymatic systems may be corrupted when they grow in unfavourable milieus, such as low pH. The low pH is maintained by a continuous metabolism of glycogen by *Lactobacillus* species and/or vaginal epithelial cells (VECs) and lactic acid is the end product. In fact, women with high vaginal lactobacilli counts do have a lower vaginal pH. Furthermore, lactic acid present in the supernatant of cultured lactobacilli has been shown...
to cause growth inhibition of *G. vaginalis*, irrespective of the present pH. The impact of hydrogen peroxide producing *Lactobacillus* spp. in the vagina has also been investigated in several studies. Al-Mushrif et al. showed that in women without a history of BV, 75% percent were colonised with lactobacilli that produced hydrogen peroxide. Only 14% of the women with BV had H$_2$O$_2$-producing lactobacilli. In another study, Valore et al. reported antimicrobial polypeptides in the vaginal fluid among healthy women. Lysozyme and calprotectin were present in concentrations that were previously found to be antimicrobial. In the same study, other polypeptides were also identified but not in significant concentrations. However, a better effect of these polypeptides may be achieved by synergistic actions. For example, Singh et al. identified a significant antimicrobial effect when lactoferrin and secretory leukoprotease inhibitor were combined. Additionally, non-specific antimicrobial peptides released by VECs, called defensins, and an immune triggering heat-shock protein, called hsp70, contribute to a healthy vaginal status. Alongside the protective role of the genital normal flora, the innate and acquired immune systems play a considerable role in the prevention of pathogenic microbes. This protection is not fully understood and is attributed to several means of action. In short, the invading microbe expresses certain pathogen associated molecular patterns (PAMPs). Pathogenic, Gram-positive bacteria present peptidoglycan on their surface and Gram-negative bacteria present lipopolysaccharide. The PAMPs are recognized by different Toll-like receptors on the surface of VECs, several of which have been previously identified. The interaction initiates a sequence of events that finally leads to the release of pro-inflammatory cytokines and the activation of the acquired immune system.

### 1.2 Urogenital infections

#### 1.2.1 Introduction

Infections in the genital tract in women can be divided into two groups; (i) sexually transmitted diseases and (ii) non-sexually transmitted diseases. In this thesis, only the most prevalent non sexually transmitted diseases will be discussed: UTI, BV and VVC. UTI occurs due to the entrance of exogenous bacteria into the urinary tract and BV and VVC arise when the vaginal flora is altered. Despite the diversity in these conditions, the composition and characteristic of the genital *Lactobacillus* flora is believed to play a role in the prevention of these events.
1.2.2 Urinary tract infections

UTI is one of the most common bacterial infections in man. Women are significantly more likely to experience UTI than men. According to the 2001 National Ambulatory Medical Survey, UTI account for an estimated 7.8 million office visits in the United States per annum. The annual costs for office visits, prescriptions and hospitalisation totals approximately 1.6 billion dollars. Based on material from the Swedish National Board of Health and Welfare, the number of prescriptions written out to women totals 350,000 per annum. An estimated 30% of the women will suffer a recurrence within one year after the first UTI.

UTI refers to the presence of pathogenic microbes and clinical symptoms in the urinary tract. The primary source of infection is enteric bacteria, foremost *E. coli*, which occasionally colonises the vaginal introitus. In fact, women with recurrent UTIs run a 4.5 fold increased risk of vaginal colonisation by *E. coli* than women without such a history. Among others, sexual habits and the use of spermicidal preparations have been suggested as risk factors for UTI, all of which are summarised in a review by Franco, 2005. However, women who are colonised with hydrogen peroxide producing lactobacilli have been reported to be less prevalent with *E. coli* and thus less prone to UTI. These results were in agreement with previous in vitro studies made by Klebanoff et al. Postmenopausal women seem to have somewhat different risk factors compared to younger women. Women with diabetes, vaginal symptoms, urge incontinence and poor overall health run a higher risk of developing UTI than women without these conditions.

Colonisation of *E. coli* in the periurethral spot along with the presence of a short urinary urethra support colonisation of *E. coli* in the urinary bladder, and thus also UTI. In order to evade the human defence system, these *E. coli* strains are alleged to produce a shielding acidic polysaccharide capsule. This protects the bacteria from being phagocytosed by polymorphnuclear leucocytes. The recommended treatment of symptomatic UTI is antibiotics. However, in the case of recurrent UTIs, particular strategies have been developed with regard to the pathogenic species or special risk factors. An increased consciousness regarding the raised antibiotic resistance among pathogenic species have led to new applications to strengthen the normal vaginal flora by supplying lactobacilli and to prevent UTIs by drinking cranberry juice or eating cranberry extract.

1.2.3 Bacterial vaginosis

BV is referred to as a disturbed vaginal microflora. A dramatic decrease of vaginal lactobacilli and an elevation of *Gardnerella vaginalis* have been observed in women with BV. Additional microbes that have been thought
to give rise to BV are *Mobiluncus* spp., *Mycoplasma hominis*, *Bacteroides* spp. and *Atopobium vaginae* \(^{10,60,61}\). Two standard methods are used to diagnose BV: Amsel’s criteria and Nugent’s score \(^{62}\). In the former, three out of four criteria must be fulfilled: homogenous discharge, positive whiff-amine test, pH > 4.5, and clue cells found on wet-mount microscopy, whereas the latter is done by microscopic evaluation of a gram stained vaginal smear. The score is based on the presence of lactobacilli, gramnegative rods and Gram-variable rods. The Nugent’s score is the preferred method used for research purposes. Factors such as sexual preferences, smoking, douching, race/ethnicity and lack of vaginal hydrogen peroxide producing lactobacilli have been associated with a higher risk of developing BV \(^{10,63-65}\).

Furthermore, BV has been linked to several adverse health outcomes, which include endometritis, pelvic inflammatory disease, preterm delivery and to potentiate transmission of HIV in heterosexuals \(^{66-69}\). However, the latter outcome is very difficult to control. The prevalence of BV in the United States is 29% and among women with BV, only 15% experience symptoms \(^{65}\).

The recommended treatment regimes are either metronidazole or clindamycin and are administered orally or intravaginally. However, the expected cure rates of, for example, oral metronidazole do not exceed 70% after one months follow-up (reviewed by Larsson & Forsum, 2005 \(^{70}\)). A potential explanation for this failure is the significant increase of *Atopobium vaginae* found in BV \(^{61,71}\).

Studies of alternative treatements of BV, which include the oral or vaginal introduction of *Lactobacillus* or by buffered vaginal gels with lactate have been carried out, however, only a small number were placebo-controlled (reviewed by Larsson & Forsum, 2005 \(^{70}\)). One of the placebo-controlled studies was made by Reid et al. and demonstrated a 37% cure rate by oral ingestion of *L. fermentum* and *L. rhamnosus* \(^{72}\). The subjects had asymptomatic BV and the vaginal flora had been improved after six weeks. The interest in this area is increasing but further clinical studies are needed. Furthermore, research that leads to an increased understanding of the pathogenesis of BV has been initiated in order to develop new models for treatment.

### 1.2.4 Vulvo-vaginal candidiasis

Three out of four reproductive aged women experience at least one VVC infection and up to 8% have recurrent VVC (RVVC), which is defined as four or more episodes every year \(^{73,74}\). Most VVCs are uncomplicated and after self-diagnosis often treated with over-the-counter (OTC) antymycotic drugs. An increased concern has been expressed as these women may have been misdiagnosed, the result of which could be untreated severe vaginal infections \(^{75}\). Furthermore, the abuse of OTC preparations may occur in
non-infectious entities. In complicated cases where the patient visits a clinic, diagnosis is set by the evaluation of anamnesis, signs and symptoms together with wet mount microscopy. However, the tests have poor sensitivity \(^{76}\) and the symptoms have unsatisfactory specificity \(^{77}\). A vaginal culture has been suggested as an additional diagnostic tool in patients with negative microscopy \(^{78}\).

*Candida* is found in a relatively high frequency (~20%) of vaginal exudates from healthy and asymptomatic women of reproductive age. Thus, *Candida* can be a vaginal commensal as well as a pathogen. The suggested predisposing factors for vaginal *Candida* colonisation are diabetes, age and pregnancy (reviewed by Sobel, 2007 \(^{79}\)).

An increased risk for VVC has been identified in women directly after antibiotic use and is indicative of a potential protective role of the normal microbiota, which may have been deteriorated due to the drug treatment \(^{80}\). Furthermore, in women who have been treated for VVC with an antmycotic preparation, 25% tested positive for *Candida* after one month \(^{81}\). The *Candida* strains that were isolated during VVC and one month after were identical in more than 60% of the women \(^{82}\). This correlation confirms that recurrent infections are due to a relapse rather than re-infection. The exact pathogenesis of VVC has still not been clarified and the specific mechanism in which *Candida* induces inflammation is still obscure. The transformation from budding cell to hyphae form has been suggested to be an important virulence factor \(^{83}\). By electron microscopy, hyphae have been shown to invade the vaginal mucosa by penetration of the stratum layers \(^{84}\). In order to avoid immune defence mechanisms and antmycotic drugs, *Candida* may stay within the VEC stroma, form a reservoir and subsequently induce another infection.

![Figure 2. Candida hyphae and chlamydospore formations.](image)

*Figure 2.* Candida hyphae and chlamydospore formations.

*Photo: Anna Andersson*

*Candida albicans* is the major species associated with VVC and is responsible for approximately 80% of the cases, whereas *Candida glabrata* is responsible for about 14% \(^{85, 86}\). This statistic is probably the reason why most research is focused on the control of *C. albicans*. The importance of *Lactobacillus* in the
management of vaginal C. albicans is not fully understood. In vitro experiments have shown a reduced adherence of C. albicans to VECs when they are mixed with Lactobacillus than in mixtures without Lactobacillus. Furthermore, specific Lactobacillus strains produce metabolites that are toxic to C. albicans. However, there is a lack of large clinical studies that investigate the significance of the Lactobacillus flora or possible treatment effects with exogenous Lactobacillus therapy.

1.3 Probiotic Lactobacillus

1.3.1 In general
A general definition of a probiotic microbial isolate was established by the Food and Agriculture Organization of the United Nations and World Health Organization, 2002. According to their guidelines, probiotics are defined as “live microorganisms which when administered in adequate amounts confer a health benefit on the host”. Such a microorganism may originate from different genera, for example, Lactobacillus, Bifidobacterium and Saccharomyces. The concept of using probiotics, and in particular Lactobacillus, for the maintenance of health and the treatment of disease is not novel. However, an increase in responsiveness to self-treatment in general may have led to a renewal of people’s worldwide interest. Research in this field is continuously expanding and is probably due to an increased global awareness of antibiotic resistance development among pathogenic microbes. The field of research covers immunological diseases, cancer, allergies, irritable bowel diseases, diarrhoea and urogenital infections, among others. Urogenital infections include UTI, BV and VVC, but research is also expanding to include the prevention of sexually transmitted diseases. Therefore, the area of interest is large and much is left to do, particularly with respect to the urogenital tract.

During the late 80’s, the first Lactobacillus strain with probiotic potential was described. Since then, Lactobacillus strains which effect UTI pathogens, BV pathogens and C. albicans have been reported using in vitro interference test models. However, most strains still lack results from clinical trials. The effect in vivo has mainly been examined using a few specific strains and the studies have mostly been made on women with BV. One study was comprised of 40 women with a present BV infection, the majority of which had previously been treated for BV with oral antibiotics. Half of the test group were given vaginal capsules which contained the probiotic strains Lactobacillus rhamnosus GR-1 and Lactobacillus reuteri RC-14 for five days, whereas the other half were given metronidazole vaginal gel for five days. A significantly better effect, referred to as a lower Nugent’s score after 30 days, was found in the probiotic group. Authors who review
studies for prevention and treatment of UTI with intravaginal or orally
administered probiotic bacteria often conclude that the clinical studies are
small, uncontrolled and without characterised Lactobacillus strains. So far,
there are no published reports describing the prevention of UTI with
probiotic Lactobacillus in large randomised placebo-controlled studies.
However, an uncontrolled clinical trial showed a 73% decreased UTI rate in
women who received vaginal suppositories containing L. rhamnosus GR-1
and L. fermentum B-54 once a week. The UTI was reduced from 6.0 to 1.6
episodes per year 95. Clinical studies of probiotics that have proven the
effect in women with VVC or RVVC are few and often limited due to small
sample sizes, uncontrolled and use of Lactobacillus in general (e.g. yoghurt)
rather than identified potential probiotic strains (reviewed by Falagas et al.
96). In the summary of review articles that cover the potential role of
probiotics for prevention and treatment of urogenital infections, the
conclusions focus on the importance of using well characterised Lactobacillus
strains that harbour properties that is required by potential probiotics.

1.3.2 Modes of action
In order to find a potential probiotic Lactobacillus strain, a screening of a
great number of Lactobacillus isolates is carried out. Through this procedure,
certain desired properties are evaluated. It is of significance to choose
evaluation variables on the basis of the probiotics intended functional
attributes. The screening is often made through several in vitro methods;
however, there are no true or worldwide preferred approaches to conduct
the screening. Over time, certain general characteristics have been identified
as important factors and are often evaluated through the screening process
97. These include (i) production of antimicrobial substances 98; (ii) adherence
to genital surfaces 99 and (iii) good technical growth and stability through
mechanical strain 92, 100. Each factor is introduced below. In order to
function properly in all women, resistance to specific preparations, such as
spermicidals and antimycotics is necessary. Furthermore, the probiotic strain
must have a strong technical growth capacity in the up-scaled production
system and survive long-term storage in the administration products.

1.3.2.1 Growth inhibitory substances
This group includes organic acids, fatty acids, hydrogen peroxide,
biosurfactants, bacteriocins and bacteriocin-like substances (BLSs) 101.
Antimicrobial acids, H2O2 and BLSs are, through different mechanisms, the
primary metabolites produced by probiotics associated with inhibition and
the control of pathogens. Acids with growth inhibiting effect on different
microbes, and in particular moulds, are produced by specific Lactobacillus
strains and have been identified and characterised previously. For example,
Sjögren et al. showed a growth inhibiting effect on spoilage fungi that was
derived to different 3-hydroxy fatty acids produced by *L. plantarum* MiLAB
14102. Corsetti et al. reported that *L. sanfransisco* CB1 produced several
organic acids, which by a synergistic effect inhibited growth of moulds. Caproic acid was the key compound in the anti-mould mixture103.

Hydrogen peroxide is produced by many *Lactobacillus* strains and in
various amounts. Kaewsrichan et al. showed that a number of cultured
H2O2-producing lactobacilli generate hydrogen peroxide at inhibitory levels
to *G. vaginalis* and *E. coli*.93 The particular inhibition mechanism is divided
into two parts: (i) toxicity to microbes or (ii) as a catalyst in the peroxidase
system38. In the latter, peroxidase in the vaginal fluid uses the H2O2 to
catalyse the oxidisation of halides, which form toxic hyphohalous acid or
halogen.

BLSs are defined as proteinaceous antimicrobial components
synthesised by bacteria, e.g. *Lactobacillus*. In contrast to bacteriocins, BLSs
inhibit the growth of a wide range of microbial species. BLSs from
*Lactobacillus* strains of various origins have been described, all with an
inhibition capacity to different species104, 105. Okkers et al. described a BLS
that was produced by *L. pentosus* TV35b which had been isolated from the
posterior fornix secretions. The peptide was designated pentocin TV35b
and it caused a slight repression in *C. albicans* growth in vitro106.
Furthermore, a few other potential genital probiotic *Lactobacillus* strains have
shown production of such substances in vitro93, 107, but the definite role in
vivo remains to be elucidated.

### 1.3.2.2 Adherence and competition

Adherence to VECs by lactobacilli and the out-competition of vaginal
pathogens on VECs is believed to be of major importance in the control of
pathogenic species in the genital tract. Several reports, all of which include
solely in vitro studies, show the adherence of *Lactobacillus* to VECs93, 108, 109
and some reports identify the blockage of urogenital pathogens to VECs11,
110. Whereas some researchers believe that the competitive exclusion mode
of action is caused by a stronger affinity of *Lactobacillus* than pathogens to
VEC receptors111, others suggest that exclusion occurs due to mechanical
hindrance by *Lactobacillus* fragments110. Some studies include competition in
the form of displacement assays11, 99. In these assays, pathogens are mixed
with VECs for 30 minutes, after which *Lactobacillus* are added to the mix.
The number of adhered pathogens per cell has been shown to decrease after
the addition of *Lactobacillus* to the mix. The decrease is thought to occur
through the displacement of pathogens, because of a higher affinity to VEC
receptors harbour by certain *Lactobacillus* strains. Furthermore, it has been
suggested that small amounts of biosurfactants and an aggregation
promoting factor have a significant impact on the interference of adhesion
of common pathogens and *L. acidophilus*112, 113.
In the characterisation of *L. crispatus* CTV-05, Kwok et al. investigated the adherence to VECs in a large number of women \(^{114}\). Adherence to VECs in women with a history of recurrent UTIs was compared to women without such a history. *L. crispatus* CTV-05 adhered to VECs from both groups. There were no differences in adherence rates to VECs from women at menses or in correlation with oral contraceptive use, spermicidal exposure or sexual activity. However, the effect *in vivo* has not yet been thoroughly evaluated. Little is known about the molecular mechanism of adherence but different components, including glycoproteins, carbohydrates and lipoteichoic acids, have been considered to be more or less involved in the action \(^{11,110,115}\).

Additionally, there is constant competition for the limited amount of nutrients in this ecological niche. To sum up, competition for binding sites and nutrients is important in the regulation of pathogenic species in the female genital tract.

1.3.2.3 Growth, stability and resistance to antimicrobial agents and spermicidals

Good technical growth and stability are two of the most important properties that a probiotic strain must comprehend. Sufficient growth capacity in small production systems does not always correspond to the result in up-scaled production systems. Despite the enhanced results achieved in high technological fermentors, a good inherent growth capability of the strain is preferred. Each probiotic *Lactobacillus* strain is selected and aimed at different target areas. As a consequence, strains are administered by different means. Viability of the strain in the specific product is crucial and should therefore be well investigated prior to its

**Figure 3.** Adherence of *C. albicans* to the surface of a VEC.
introduction in products. Assay for stability control is selected with respect to the specific product. However, the long-term storage of products is often carried out in a similar manner. The product is stored at different temperatures and the number of viable bacteria in the product is registered by cultivation on specific agar plates. Results from stability tests are seldom reported in papers. An independent study of commercially available probiotic products showed that only five of ten products were labelled correctly concerning the number of probiotic bacteria. As described by the definition of a probiotic, the probiotic effect is dependent on the number of bacteria in the product, therefore a lack of viable probiotic bacteria will result in a reduced probiotic effect.

In order to select a probiotic strain for use in women with VVC, the strain is examined regarding its resistance to antimycotics. Generally, women with VVC often begin treatment with OTC antimycotics. The effect of probiotics (without a known resistance to antimycotics) taken with the intention of preventing recurrence may thus fail. In women with recurrent UTI and BV, probiotics resistant to commonly used antibiotics for the treatment of each infection may be preferred. However, the transfer of antibiotic resistance genes should be considered before any clinical use of the specific strain takes place.

Spermicidal agents are used as contraceptives in some countries. Resistance to the active substance should thus be investigated in order to enable the use of the probiotic among as many women as possible.

1.3.2.4 Other mechanisms
At present, there is a great deal of interest in investigations regarding cytokine induction by probiotic bacteria and commensal microbiota in general. The major part of the effort is put into the microflora of the intestinal tract. Several studies have identified regulating properties among probiotic Lactobacillus strains in ex vivo and in vitro models where lactobacilli are co-cultured with immune cells. For example, the internationally known L. casei shirata was reported to induce the production of IL-12 and TNF-α in murine primary splenocytes. However, at present there are no published reports of immune induction by urogenital probiotics.

1.3.3 Safety aspects
Lactobacilli are primarily considered as “good” commensal bacteria which participate in the defence against pathogenic bacteria in the genital tract and the gut. Infections caused by Lactobacillus are rare and infections correlated to the intake of probiotics have only occurred in a few cases concerning L. rhamnosus GG (reviewed by Reid & Hammond, 2005). However, products containing Lactobacillus probiotics are considered
harmless in relation to the estimated 20 billion doses sold worldwide per annum. Additionally, no reports of infections caused by genital probiotics have been described. Due to an increased concern regarding conjugative transfer of antibiotic resistance genes, the benefits must be weighed against possible harm. Large clinical trials that position efficacy in relation to harm, are therefore needed.

1.3.4 Commercially available urogenital probiotics

Presently, there are few commercially available probiotic products for the urogenital tract on the Swedish market. One type of product consists of a tampon impregnated with lactobacilli and another type of product consists of vaginal suppositories which contain lactobacilli. However, there are no reports on the characterisation of the \textit{Lactobacillus} strains that are included in these products. Possibly, the scarcity of products is a consequence of the insecurity of governmental authorities regarding the classification of such products. Probiotic products for the urogenital tract can also be found on the international market. In accordance with the products on the Swedish market, few strains have been thoroughly characterised. One exception is the product Fem-Dophilus™, which contain vaginal capsules with \textit{Lactobacillus} GR-1 and \textit{Lactobacillus} RC-14. These strains have been selected with respect to their properties as potential probiotics for the urogenital tract.

Research within the field of urogenital probiotics is expanding and it will probably not be long before the market offers products with different probiotic components for various genital conditions.
Objective
The objective of this thesis is to clarify the ecological role of lactobacilli in the female genital tract and to focus on selected \textit{Lactobacillus} strains with probiotic qualities. The project incorporated \textit{in vitro} studies and \textit{in vitro} method development in order to select and characterise potential probiotic \textit{Lactobacillus} strains. A placebo-controlled clinical study and a number of pilot studies were carried out during the project.

2.0 Subjects and methods

2.1 Microbiology of the urogenital tract [paper I]

\textit{Aim}
To compare the prevalence and quantity between lactobacilli and other microbes and vaginal pH in the urogenital tract of healthy women.

2.1.1 Subjects
The normal vaginal \textit{Lactobacillus} flora and its relation to other microbes and vaginal pH were examined in 191 women. Healthy Swedish volunteers with a lowest age of 18 and with regular menstruation were eligible. The study was carried out as a randomised, double blind and placebo-controlled study. The women who attended the clinic and who were willing to participate were physically examined. Any subjects who had vulvo-vaginal disease were excluded. Further exclusion criteria are stated below.

Exclusion criteria: (i) clinical symptoms of vulvo-vaginal disease at physical examination, (ii) planned pregnancy, (iii) at least four episodes of candida infection the previous year, (iv) at least two episodes of urinary tract infection the previous year.

2.1.2 Study plan
Each woman wore panty liners 24 hours a day during four consecutive menstrual cycles followed by one menstrual cycle without panty liners. Each woman made six visits to the clinic, including enrolment. Half of the study population wore vapour-permeable panty liners and the rest wore the same type of panty liners impregnated with lactobacilli. A statistical analysis of the samples collected at enrolment was made on the samples from both groups. The analysis of the samples collected throughout the study was carried out separately on women in the placebo group and the LB931 group.
2.1.3 Sampling
Two microbiological samples were collected at each visit: (i) vagina and (ii) labial fold. The vaginal sample was taken by rotating a wetted sterile cotton pin three cm into the vagina. A cardboard template was used for the sampling of the labial fold in order to ensure that a comparable area of sampling was used on all women. A wetted cotton pin was gently rubbed five times back and forth between the arms of the template. The sample was mixed on a Vortex shaker and cultured on five different agar plates: Rogosa agar, Rogosa agar with 128 µg ml\(^{-1}\) Vancomycin, blood agar, McConkey agar and Sabouraud dextrose agar. The bacteria and yeast quantity was recorded in CFU ml\(^{-1}\).

Vaginal pH was determined at each visit by gently pressing the top of the indicator paper (Merck, KgaA, Darmstadt, Germany) against the mucosa, three cm into the vagina. The colour response was read according to the colour scheme on the strips box.

2.2 Selection and characterisation of a potential probiotic *Lactobacillus* strain [paper II]

**Aim**
To select, identify and characterise a potential probiotic *Lactobacillus* strain of genital origin to be used in clinical trials, particularly in women with recurrent urinary tract infections and bacterial vaginosis.

**2.2.1 Procedure**
The screening of *Lactobacillus* strains in order to find a potential probiotic strain was performed in three steps as illustrated in Figure 4. The strains were isolated from the vaginal swabs of healthy volunteers from the northern part of Sweden. A total of 511 *Lactobacillus* isolates were initially screened with respect to their survival through freezing at -80°C followed by thawing at 22°C. Strains that survived the procedure without a reduced growth capacity on Rogosa agar plates were grown in MRS broth. Any isolates that were proved unable to reach a concentration of 10^8 bacteria per ml were excluded and the rest were evaluated further with regards to their ability to inhibit the growth of potential urogenital pathogens; designated Interference Test Strains (ITS). The agar overlay method was previously described by Tano et al.,\(^{126}\) and is thoroughly described in paper II under the subheading “Agar overlay interference test”. A strong inhibition (more than 80% of the ITS) was required in order to fit into the final phase of the screening. Finally, the production of hydrogen peroxide was registered and used as a final exclusion method in the screening. H\(_2\)O\(_2\) was detected on
specific MRS agar plates containing tetramethylbenzidine and horseradish peroxidase. The parameters registered were i) time till colour change response and ii) strength of colour. The most efficient producer of H₂O₂ was selected for further characterization.

2.2.2 Characterization of the selected isolate
Initially, the strain was typed using a biochemical method followed by a genetic identification by BCCM/LMG (Gent, Belgium) using 1D-protein gel electrophoresis (SDS-PAGE). The identification was followed by methods to characterise the selected strain by its metabolism, stability and function in order to evaluate its potential for use as a urogenital probiotic strain. A thorough description of each method is found in paper II.

Figure 4. Screening procedure. Five hundred and eleven Lactobacillus isolates were examined for growth and survival through freeze-thawing. Twenty six of these strains were examined with regards to the interference of urogenital pathogens on solid agar. Finally, eleven strains were evaluated for production of hydrogen peroxide.
2.3 Identification of a *Lactobacillus* strain with antifungal properties [paper III]

**Aim**
To select, identify and characterise a potential probiotic *Lactobacillus* strain with strong fungistatic effect on *C. albicans* and *C. glabrata*; significant pathogens in women with vulvo-vaginal candidiasis.

2.3.1 Procedure
*Lactobacillus* strains were isolated from the forehead, throat and teeth of healthy adult volunteers. Another source of strains was faecal samples from newborn infants obtained three to five days after birth. Figure 5 illustrates the screening procedure as a flowchart. Initially, 126 *Lactobacillus* strains were screened for their ability to inhibit growth of a *Candida albicans* strain. The inhibition test was carried out on solid agar by visual evaluation of the growth inhibition zones achieved by each *Lactobacillus* strain. All the strains were matched with the effect of the vaginally isolated *Lactobacillus plantarum*, LB931, and those strains with equal and/or greater inhibition than that strain were selected for the second screening. The chosen *Lactobacillus* strains were grown in MRS broth and the growth inhibiting effect on *C. albicans* and *Candida glabrata* was examined. All *Candida* strains were vaginal isolates.

![Figure 5. Screening procedure.](image)

One hundred and twenty-six *Lactobacillus* strains were screened for their ability to inhibit the growth of *C. albicans* on solid agar. Eighteen strains with visual growth inhibition on *C. albicans* were further evaluated in screening II. Growth inhibition of *C. albicans* and *C. glabrata* was measured in cell-free filtrate from the *Lactobacillus* strains.
2.3.2 Characterisation of the selected isolate
The isolate was initially typed by biochemical methods and by genetic identification using partial sequence analysis of 16S rRNA. Further characterisation involved survival through freeze-drying, survival in the vagina, impact of pH, susceptibility to anti-fungal drugs and finally the effect on vaginal lactic acid bacteria isolates.

2.4 Transfer of *L. plantarum* LB931 from panty liners to the vagina and labial fold [paper I]

*Aim*
To evaluate the possibility to administer a potential probiotic to the genital surface with panty liners impregnated with a L. plantobacillus strain.

2.4.1 Study plan and sampling
The study plan and sampling procedure is previously described under 2.1.2. The panty liners were impregnated with *L. plantarum* LB931 (selection of LB931 is previously described under 2.2) and the detection of LB931 was made at visit 2 and visit 5 only.

2.4.2 Identification of LB931
The identification and quantification of *L. plantarum* LB931 was made in three steps (Figure 6) that were carried out in the following order: i) visual identification, ii) biochemical identification and iii) genetic identification.
Figure 6. The colonies on the Rogosa-Vancomycin agar plates that were morphologically similar to *L. plantarum* LB931 were counted. Five randomly chosen colonies that were similar to LB931 were then isolated and stored in a freezer for further identification. One of the five colonies was directly typed based on its biochemical fermentation pattern. The biochemical fermentation profile of the isolate was registered using the API 50 CHL typing kit and the species belonging was determined using the API LAB software. Isolates typed as *L. plantarum* were further identified with pulsed field gel electrophoresis. The method is thoroughly described in paper I. The PFGE profile from each isolate was matched against the profile of LB931 and isolates that were identical to LB931 were referred to as LB931.
2.5 *Lactobacillus fermentum* Ess-1 administered to women with VVC [paper IV]

**Aim**
To evaluate the intervention of *Lactobacillus fermentum* Ess-1 in women with an acute vulvo-vaginal candidiasis, with regard to the incidence of vulvo-vaginal candidiasis episodes and symptoms, in relation to the previous history. Additionally, information regarding factors that are important for the design of future clinical effect studies in women with VVC was collected.

2.5.1 Subjects, study plan and sampling
The study was designed as a case study and involved six women of reproductive age and with a history of symptomatic VVC episodes the previous twelve months. The women were recruited at Sanden’s Health Care Centre in Boden, Sweden. One oral capsule containing fluconazole 150mg was prescribed at enrolment and all the women started the *Lactobacillus* intervention directly. One *Lactobacillus* capsule a day was ingested and vaginal capsules were inserted in special “routines” (defined as one capsule per day for three consecutive days), at four or five occasions during the study. Detailed information regarding the study plan and capsule administration can be found in Figure 7. A microbiological sample from the front side of the vaginal wall was collected at each visit. Symptoms and signs were recorded at all visits.
Visit 1 occurred at different times during the menstrual cycle for each patient. Visit 2 was planned somewhere between days 16-20 in the menstrual cycle (study period 2, SP2), calculated from the first day of bleeding. Visit 3 was planned for days 16-20 of SP3. Visit 4 was planned for days 16-20 of SP4. Oral capsules: One capsule was ingested once a day, starting at enrolment and was carried out until visit 3.

Vaginal capsules: The short routines of vaginal capsule usage are marked *. At each mark, a vaginal capsule was inserted before bed time, once a day for three consecutive days. During SP 1, the first routine was started the same day as visit 1. If the inclusion visit occurred between days 1-13 of the menstrual period (calculated from the first day of bleeding) another routine of capsules (marked *) was carried out on day 16-18. If the inclusion visit occurred later than day 13, only the first routine of vaginal capsules were carried out during SP1; the one starting at the day of visit 1. Between visit 3 and visit 4 no capsules were used (follow-up period).
3.0 Results and discussion

In order to form a better understanding of the different species, the general microbial flora in healthy women will be initially discussed. Focus will be aimed towards the relationship of the studied species and vaginal pH. Furthermore, the following sections will deal with the process of identifying probiotic candidates and evaluating their particular qualities. Finally, the results from the clinical studies will be discussed.

3.1 Microbial ecology of the genital tract

3.1.1 The vaginal pH in healthy women
Generally, the vaginal pH is a rough index of the status of the vagina and the health of the vaginal flora. The upper limit for a healthy vaginal pH has been set to 4.5 as, according to Amsel’s criteria, a higher vaginal pH than 4.5 is a sign of BV. However, a number of healthy individuals do have an elevated vaginal pH. The median vaginal pH for the women in our study was 4.7, and the range was from 4.0 to 6.3. Therefore, the median vaginal pH does not reflect the common opinion of a healthy vaginal pH. According to these results, more than 50% of the study population had an elevated vaginal pH. The indicator paper method has its limitations as the pH value is visually monitored through the comparison of the test strip to a standard colour scale with fixed values (i.e. 4.0, 4.4, 4.7, 5.0 ...). However, the indicator paper method was chosen because it is an easy and rapid method and it has been compared to measurement by vaginal electrodes with no significant difference in the result.

3.1.2 Relationships between vaginal pH and genital microbes
In our study, women with high lactobacilli numbers correlated to a lower vaginal pH. The median pH in women with more than 6.0 log_{10} CFU ml^{-1} was 4.4 (n=84), whereas, in women with less than 2.0 log_{10} CFU ml^{-1} lactobacilli, the median pH was 5.0 (n=44). The question is if the lactobacilli significantly lower the vaginal pH or if the high number of lactobacilli, which grows favourably in acidic milieus, is due to low vaginal pH? In vitro experiments by Boskey et al. showed that the level of acids produced by lactobacilli is comparable to the one seen in vivo in the vagina. However, this study does not rule out the potential role of acid production by the VECs. Whether or not this question can be answered by the results of this study, the influence of acid production and secretion by VECs is believed to play an important role.
3.1.3 Relationships of genital microbes in healthy women

With regard to any possible relationships between the counts of other microbes and lactobacilli, no significant correlation was found in either the vaginal samples or the labial samples. However, the number of lactobacilli in the vagina was positively correlated to the number of lactobacilli in the labial samples, although the number of lactobacilli in the labial samples was significantly lower. Therefore, in a balanced genital microflora, lactobacilli defence reaches from the vulva through the vagina to protect the host from the overgrowth of potential pathogenic microbes. 70% of the women were persistent carriers of vaginal \textit{Lactobacillus} spp. whereas 7% lacked lactobacilli throughout the study. In actual fact, there were no similar studies to compare these results with, as there were none found. However, it is possible that the women that lacked lactobacilli were colonised with lactobacilli that could not be detected on Rogosa agar. \textit{L. iners} has recently been described as one of the most common \textit{Lactobacillus} species in the vagina. This species cannot be cultured on common \textit{Lactobacillus} agar but it can be cultured on blood agar. Furthermore, at enrolment, 24% of the study population were not colonised with lactobacilli in the vagina, whereas in some specific samples we found up to 9.8 log_{10} CFU ml^{-1}. This variance is hard to interpret as there is a strong global belief that lactobacilli, as part of the commensal flora, strongly contribute to the healthy maintenance of the genital flora. However, co-variates such as hormone levels and sexual habits were not recorded. Decreased oestradiol levels have previously shown to reduce the vaginal flora and a greater number of sex partners have shown to be correlated to an unstable vaginal flora. In addition, a limited identification method may impact on this matter. An extended questionnaire and a genetic identification technique that could identify strict anaerobic lactobacilli would be preferred in future studies. When the women were stratified by vaginal lactobacilli counts, correlations in the prevalence of different species were found. The group of women with high numbers of vaginal lactobacilli (\geq 7 log_{10} CFU ml^{-1}) was less prevalent with Group B streptococci (GBS) than women with low lactobacilli counts (<3 log_{10} CFU ml^{-1}) (Mann-Whitney, p=0.036). Few studies have examined the relationship between vaginal lactobacilli counts and GBS prevalence. However, in a study by Kubota \textit{et al.} \textit{Lactobacillus} were reduced in pregnant women with a GBS-positive vaginal flora, which is a result that is in accordance with our study. Our study also showed that four out of five women were at any time prevalent with GBS and 39% were persistent carriers. Therefore, in conformity with other studies, vaginal colonisation of GBS can be chronic as well as transient. GBS transferred to the infant at delivery is the leading cause of morbidity and mortality in newborns. Therefore, a boosted vaginal lactobacilli flora could reduce the risk of infant infection. Furthermore, 24% of the women were prevalent with yeast at enrolment.
and 18% of the women were persistently colonised. The prevalence of vaginal yeast was significantly higher in women with average numbers of vaginal lactobacilli ($3 - 6.99 \log_{10} \text{CFU ml}^{-1}$) than in women with lower or higher numbers ($p=0.002$ and $p=0.007$). To our knowledge, there are no previously published reports to compare these results with. In cases with *Candida* overgrowth, the cause is possibly due to other factors that influence the genital health, the genital microflora and health in general, for example, the use of antibiotics rather than the vaginal lactobacilli density.

### 3.2 In search of new probiotic strains

#### 3.2.1 Screening of *Lactobacillus* isolates

In this study, two screenings were made in order to find *Lactobacillus* strains that had the properties that are needed to be a urogenital probiotic. However, different properties were sought after among the strains which influenced the choice of screening procedure. The methods that were used were developed from previously published methods. However, a number of the methods were used for the first time and, consequently; there are no previously published reports to compare the results with. In paper III, the work was solely focused on the inhibition of *Candida*. In paper II, a three-step screening procedure was performed in order to identify a strain with stability through mechanical stress (freezing-thawing), a good growth capability, an ability to produce metabolites with a growth inhibition capacity against common potential urogenital pathogens and a strong capability to produce hydrogen peroxide. The vast majority of the isolates (95%) were eliminated from the screening due to bad survival rates through mechanical stress and by poor technical growth ability. Less than half of the remaining isolates (n=11) produced high amounts of inhibitory substances. Of the eleven remaining isolates, the one with the best hydrogen peroxide production was selected. The strain was typed as a *L. plantarum* by the API 50 CH kit. The strain was designated LB931. Actually, the selection of screening parameters has not yet been globally standardised, therefore, different parameters have been used in the past in order to identify strains which have been classified as genital probiotics at a later date. The process, however, most often includes the investigation of metabolites that inhibit growth of the target microbe. The extent of species of which growth inhibition was achieved by LB931 was thoroughly studied using the agar overlay method. The semi-quantitative agar overlay method was chosen because of its advantages: over 20 test strains can be tested on one single plate. The method has been successfully used in the screening of inhibitory substances that were produced by alpha-haemolytic streptococci. Clinical isolates were used as interference test strains, however, *G. vaginalis* was not
included in the test panel as this species is difficult to culture. LB931 generates and releases metabolites that diffuse through the agar and impede the growth of potential uropathogenic bacteria; this happens without any surface contact between the ITS and the *Lactobacillus* itself. The growth was completely hampered in the vast majority of the test strains.

Furthermore, a liquid assay of LB931 filtrate was performed which added quantitative data and a time axis to the inhibition results. Similar results in the liquid assay could eliminate the possibility that components in the agar, by synergistic means, improved the inhibitory effect of the free substances produced by the *L. acidobios* strains. A bactericidal effect was found on all bacteria test strains (*GBS, Klebsiella* spp., *E. coli, Enterococcus* spp. and *S. aureus*) after twelve hours of incubation. The particular mode of action has not yet been established. It is generally believed that these actions are constituted by one particular substance, several metabolites or a complex of metabolites that are reciprocally dependent on one another. The fact that the mode of inhibition comprises Gram-negative bacteria as well as Gram-positive bacteria species indicates the action of a BLS. One future goal is to identify the active substance(s) by fractioning the filtrate using chromatography and to perform inhibition bio assays on the fractions. This method has previously been used successfully in the identification of a BLS produced by a *Lactobacillus* strain and a lactic acid bacteria-produced acid with inhibitory effects on spoilage fungi 106, 138.

There are a few reports that describe *Lactobacillus* strains that generate metabolites with fungistatic effects 93, 139. However, none have shown a growth inhibiting effect against *C. glabrata*, the second most common *Candida* sp. that causes VVC. For that reason, the screening in paper III was based on the inhibition of both *C. albicans* and *C. glabrata*. Furthermore, we chose *Lactobacillus* strains isolated from other spots than the genital tract. This choice may very well be questioned by reviewers who claim, for obvious reasons that a probiotic strain has to originate from the spot from where it is applied. From a microbiological point of view one cannot argue with that statement. However, with previous results and reports in mind, there is little that indicates that *Lactobacillus* strains isolated in the genital tract possess the right properties in respect to the production of fungistatic metabolites. Accordingly, in order to find a good probiotic candidate strain that can be used in women to prevent VVC, the production of inhibitory substances against *Candida* spp. should be prioritised. As a result of our screening, the strain typed and designated *L. fermentum* Ess-1 was found to be the only one with strong inhibitory activity against both *C. albicans* and *C. glabrata*. This is the first reported *Lactobacillus* strain with activity on the two most common *Candida* species that cause VVC. Furthermore, Ess-1 was compared to reference strains with previously recognised production of
antifungal substances. Clearly, our strain showed a stronger effect than all the other strains that is was compared to.

3.2.2 Administration of genital probiotics

The possibility of supplying LB931 to the urogenital tract by using panty liners impregnated with LB931 was investigated in a large placebo-controlled study (paper I). LB931 was found in a high proportion of the women at both of the sample areas: the labial fold (86%) and in the vagina (54%). One of the most important features of a probiotic strain is its capacity to survive on the skin, epithelium or in the vaginal mucosa. Exogenous probiotic bacteria that are introduced to the genital surface are not expected to colonise in such way that only one dose would be required to restore the genital flora for a long time. It has previously been reported that certain Lactobacillus strains can adhere to the surface of VECs in vitro. However, the constant flow of vaginal fluid in women (1-4 ml per day), which also contain free superficial vaginal epithelial cells, may have a negative impact on the colonisation rate of the supplied probiotic bacteria. As a solution, the continuous use of liners with LB931 is suggested to ensure viable probiotic bacteria in the genital tract for a long period: see the pilot study included in paper II. In the latter study, 37 of 40 samples from the perineum and 28 of 40 samples from the urethra contained more than 10^2 LB931 per sample.

Other suggested means for administration are by vaginal capsules and others of which are limited; e.g. impregnated tampons that are restricted to use only during menstruation. With respect to the oral ingestion of urogenital probiotics, a number of factors will probably have an impact on the results, for instance, survival in the gut. As oral administration of probiotics is believed to make it to the vulva by the anus and the perineum, the style of underwear, hygiene and sexual habits, among other things, might have an impact on the number of supplied bacteria to the vulva. A promising study has been carried out by Reid et al. In this study L. rhamnosus GR-1 and L. fermentum RC-14 were ingested by ten women. The bacteria were prepared in a three ml skim-milk solution (>10^9) and the women swallowed two doses per day. After one week, at least one of the probiotic strains was detected in all the women using ribotyping. Interestingly, but as yet unexplored ways to administer probiotics are by sanitary napkins and natural oils. However, administration through the usage of vapour-permeable panty liners is a simple and safe way to supply probiotic bacteria to the vulva and the vagina which does not cause discomfort during continuous use. In the case of Ess-1, the main target is the vagina where Candida invade the mucosa. The results from the case study in women with VVC showed a loss of the supplied Ess-1 after 7-10 days. Ess-1 was only detected on three of 24 visits. In accordance with that
result, the supply/continued existence study showed that a high number of Ess-1 could only be achieved through the continuous supply of Ess-1. With these results in mind, a continuous instillation of vaginal capsules or vagitories is suggested in the administration of these probiotic bacteria. Additionally, a high dose of bacteria (more than $10^9$ probiotic lactobacilli per capsule) is believed to be necessary in order to effectively inhibit the Candida growth by the substance produced by the bacteria. However, such bacteria levels are possibly hard to accomplish, with respect to the technical limits in the production system and the survival of bacteria in the product through long-term storage.

**3.2.3 Other qualities of the probiotic bacteria**

Within the sphere of research groups that are in search of new probiotic bacteria to be used in the genital tract, there are differences in the studies selection process concerning the qualities of the strains. There are no clear guidelines to follow, which has led to a multitude of methods that show the potential of different bacteria strains. As there is a commercial interest in probiotic strains, one might meet with resistance in sharing strains for comparative studies. Furthermore, there is no standard negative control strain used in most studies. Therefore, it is difficult to compare probiotic strains against each other and to evaluate one’s own. Despite this, certain properties should be examined and evaluated before classifying a strain as a probiotic. The selection of qualities that are to be studied should be made with respect to which pathogen(s) the probiotic strain is supposed to act against and how the probiotic strain will be administered.

Several physiological characteristics were studied in LB931 and Ess-1, for example, the susceptibility to antibiotics and antimycotic preparations. Probiotics aimed for a population that most likely uses antimycotic preparations each time a yeast infection occurs should not be susceptible to the active substance in these preparations. Ess-1 was not affected by any of the antimycotic substances tested. Furthermore, LB931 was tested for stability on panty liners and the production of hydrogen peroxide and Ess-1 was examined regarding survival in the vagina over menstruation. All studies showed positive results.

**3.3 Clinical studies**

There are few clinical studies that have been properly designed to evaluate the effectiveness of probiotic *Lactobacillus* in the prevention of urogenital infections in women. In one study, vaginal suppositories that contained *L. acidophilus* failed to cure women with BV [144]. However, this *Lactobacillus* strain had not been selected for its qualities as a probiotic. In another study
made by Hilton et al., 28 women with recurrent VVC infections were given vaginal suppositories impregnated with *Lactobacillus GG*. During the first week, all the women experienced an improvement in symptoms. However, microbiological samples showed that only five of the women were significantly colonised with *Candida* prior to the treatment. The diagnostic tools in our case study also gave various answers. Six women were diagnosed with VVC but only two women had detectable levels of yeast in the microbiological cultures at enrolment. Therefore, future clinical studies need thorough criteria in the diagnosis of genital infections, in order to get a significant result of the effect of the probiotic bacteria in women with a particular symptomatic condition. Furthermore, the choice of bacteria is crucial if a potential effect should be achieved.

One worrying factor is the high occurrence of self-diagnosis and self-medication. Women with an acute VVC and a history of VVC the previous twelve months were recruited in our study. All together, the women had experienced 44 VVC episodes previous year, but only seven episodes resulted in an office visit. As four of the six women in our study were not colonised with yeast (according to the microbiological culture) they may have been self-treated with an antifungal OTC preparation prior to the enrolment or they may have been suffering from another, and therefore unmedicated, condition.

### 3.4 Future aspects

There is no doubt that probiotics, and *Lactobacillus* strains in particular, is a target for a growing global interest among the general public as well as researchers. There are, however, areas within the field that are well explored and others that have hardly been touched. In order to fully understand the possibilities of probiotic bacteria in the female urogenital tract, an understanding of the mechanisms through which the probiotic strains affect the growth of bacteria and yeast is crucial. This matter should also be studied *in vivo* in order to conclude that the production of the inhibitory substances is active. More clinical studies are needed in order to evaluate the prophylactic significance of probiotic bacteria in women and to study the adherence and interaction with the mucosa and VECs. The advantages of using several probiotic strains in a probiotic cocktail in order to accomplish a good result in a larger number of individuals should be examined. Finally, there is a need for further investigations into the communication between the bacteria flora and the defence system regarding the influence of probiotic bacteria.
4.0 Concluding remarks

Throughout the study, several interesting facts were found and will be investigated further in the future. The main concluding remarks from the thesis are stated below:

(i) **L. plantarum** LB931 was identified as a promising urogenital probiotic for use in clinical trials in women with UTI or BV.

(ii) **L. fermentum** Ess-1 is the first potential probiotic strain that secretes metabolites with strong fungistatic effects towards both *C. albicans* and *C. glabrata*.

(iii) The use of panty liners impregnated with **L. plantarum** LB931 is a feasible way to administer probiotic bacteria to the genital surface.

(iv) Vaginal pH was significantly higher in healthy women with very low numbers of vaginal lactobacilli than in women with high numbers.

(v) The prevalence of GBS in the vagina of healthy women was lower in women with high numbers of lactobacilli ($\geq 7 \text{ log}_{10} \text{ CFU per sample}$) than in those with average or low numbers ($< 7 \text{ log}_{10} \text{ CFU per sample}$).

(vi) The prevalence of yeast in the vagina of healthy women was higher in women with an average colonisation of lactobacilli (3-6.99 $\text{ log}_{10}$ CFU per sample) than in women with no, low or very high numbers of lactobacilli ($< 3$ or $\geq 7 \text{ log}_{10}$ CFU per sample).

(vii) Future effect studies with probiotic bacteria in women with vulvo-vaginal candidiasis must include improved VVC specific diagnostic criteria and the probiotic doses should contain approximately $10^9$ bacteria and be taken continuously.
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