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Abstract

Lactobacillus is the predominant species of the vaginal microbiota in women of childbearing age. Lactobacilli are thought to contribute to the local immune defence by producing a variety of antimicrobial substances and, thereby, attenuate growth of other opportunistic bacteria. A disturbed vaginal microbiota, with loss of lactobacilli and an overgrowth of other anaerobic bacteria such as occurs in cases of bacterial vaginosis (BV), increases susceptibility to sexually transmitted infections, risk of ascending infections to the upper genital tract and postoperative infections, which can pose a threat to pregnancy and cause preterm birth (PTB).

Antimicrobial polypeptides (AMPs) are important molecules of the mucosal innate immune defence. Secretory leukocyte protease inhibitor (SLPI) is a multifunctional AMP that is present at high concentrations in the healthy female genital tract. Thus, the presence of a healthy microbiota, such as lactobacilli, and the surveillance by the mucosal innate immune system are suggested to be important factors for the homeostasis of the lower genital tract.

Our aim was to analyse the occurence of virulence features in the opportunistic Gram-negative bacterium *Prevotella bivia*, commonly found in BV, associated with upper genital tract infection and PTB, and to investigate whether vaginal lactobacilli interact with the host innate immune defence, by affecting the regulation of SLPI and pro-inflammatory cytokine responses in host cells.

Studies of the anaerobic bacterium *P.bivia* showed that one out of five strains tested had a high capacity to invade HeLa cells. The lack of adhesion structures on *P.bivia*, as well as a similar capacity of different strains to adhere to HeLa cells, suggested that *P.bivia* are endowed with some other factor important for the intracellular invasion. Only the most invasive strain of *P.bivia* gave rise to a weak IL-6 and IL-8 response. Thus, a high invasion capacity together with a low pro-inflammatory cytokine response in certain strains of *P.bivia* are suggested to be virulence factors in establishing a subclinical upper genital tract infection.

Analysis of isolates of the four most frequent vaginal Lactobacillus spp. (*L.crispatus*, *L.iners*, *L.gasseri* and *L.jensenii*), regarding the capacity to influence the secretion and expression of SLPI in monocytic- (THP-1) and cervix epithelial- (HeLa) cells, showed that *L.iners* could up-regulate the constitutive secretion of SLPI. However, high concentrations of *L.iners* down-regulated the SLPI secretion in both cell types. The largest difference between the four lactobacilli species in their regulation of SLPI was obtained between *L.iners* and *L.crispatus*. At the concentrations tested, *L.crispatus* gave rise to a strong reduction of SLPI in both cell types. A negative correlation was found between SLPI protein and mRNA expression levels in HeLa cells, but not in THP-1 cells. In both cell types, synergy effects in the pro-inflammatory cytokine response were obtained by co-incubation of lactobacilli with *E.coli*. Positive synergy effects were obtained for the IL-8 and TNF-α production in THP-1 cells and for IL-6 and IL-8 in HeLa cells. Negative synergy effects were obtained for IL-6 and IL-18 in THP-1 cells. Moreover, negative correlations were obtained between the cytokines and SLPI levels. Analysing the effects of the addition of recombinant SLPI to HeLa cells prior to the exposure to *E.coli* showed a significant reduction of the IL-6 and IL-8 responses in the cells.

The results indicated that vaginal lactobacilli can contribute to the regulation of SLPI and pro-inflammatory cytokine responses in host cells. However, our data also suggested that a dominating *Lactobacillus* spp., such as *L.iners* or *L.crispatus*, may influence the mucosal innate immune defense in different ways. Moreover, the regulatory effect on the SLPI secretion was inversely associated with the capacity of the bacteria to evoke pro-inflammatory cytokines in the host cells.

Key words: *Prevotella bivia,* bacterial vaginosis (BV), invasion, *Lactobacillus*, secretory leukocyte protease inhibitor (SLPI), cytokines,THP-1 cells, HeLa cells.

LIST OF PUBLICATIONS

The thesis is based on the following studies, which can be referred to in the text by their roman numerals

I. Louise Strömbeck, Jens Sandros, Elisabeth Holst, Phoebus Madianos, Ulf Nannmark, Panos Papapanou and Inger Mattsby-Baltzer
***Prevotella bivia* can invade human cervix epithelial cells, HeLa**
APMIS 115: 241–251, 2007

II. Louise Strömbeck, and Inger Mattsby-Baltzer
Commensal vaginal lactobacilli and their regulatory effects on secretory leukocyte protease inhibitor (SLPI) and cytokine secretion in human monocytic cells
Submitted, 2008

III. Louise Strömbeck and Inger Mattsby-Baltzer
Effects of commensal vaginal lactobacilli on the regulation of secretory leukocyte protease inhibitor (SLPI) and cytokine secretion in human cervix epithelial cells (HeLa)
Submitted, 2008

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ABBREVATIONS

AMPs	antimicrobial peptides/proteins
BV	bacterial vaginosis
FGT	female genital tract
IL	interleukin
LPS	lipopolysaccharide
LTA	lipoteichoic acid
MAMP	microbe-associated molecular patterns
NFκB	nuclear factor kappa B
PRRs	pattern recognition receptors
PTB	preterm birth
SLPI	secretory leukocyte protease inhibitor
STDs	sexually transmitted diseases
TLRs	toll like receptors
TNF-α	tumor necrosis factor – α

1. INTRODUCTION

1.1 The female genital tract

The female genital tract (FGT) (fig.1) is composed of a sequence of cavities, the vagina, endocervix, uterus and Fallopian tubes, which allow for passage in both directions. The upper genital tract comprising the endocervix, uterus and Fallopian tubes, is well protected from vaginal microorganisms unless the cervix becomes infected. The cervical opening is normally filled with a mucus plug that is effective in preventing the entry of microorganisms into the uterus. However, this plug is not always impermeable. In the middle of the menstrual cycle, the cervical mucus changes in viscosity to allow sperm to enter the uterus. Despite the apparent vulnerability of the female upper genital tract, infections of this area do not occur frequently.

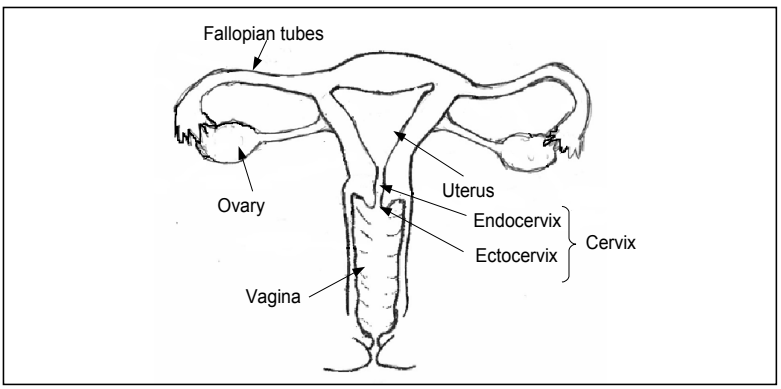

Fig 1. Female genital tract (FGT). Infections in FGT may target the cervix and ascend to the uterus and Fallopian tubes. The most common infection of the vagina is bacterial vaginosis (BV) and yeast infections.

1.2 Infections of the FGT

1.2.1. Sexually transmitted diseases, STDs

Sexually transmitted diseases (STDs) are caused by pathogens that are commonly transmitted through sexual contact. They make up a major portion of infectious diseases and are a significant public-health and financial burden on society worldwide. Examples of STDs are human papilloma-virus (HPV) and *Chlamydia trachomatis,* which are extremely prevalent STDs, while human immunodeficiency virus (HIV) is associated with a high rate of mortality. The major complication associated with HPV (type 16 and 18) infection is cervical cancer [1], and with *C.trachomatis* there is an increased risk of pelvic inflammatory disease (PID), ectopic pregnancy, preterm birth (PTB), and infertility.

Despite a high prevalence of STDs, vaccines against only two viral STDs are available, HPV (cancer and wart-inducing HPV types) and Hepatitis B virus.

C. trachomatis and *N. gonorrhoeae* can overcome the immune defence in the cervix. Once such bacteria have infected the cervix, the chance of an ascending infection increases. Both pathogens are able to grow and multiply in the cervix, uterus, and fallopian tubes.
Even though symptoms of chlamydia or gonorrhea are usually mild or absent, serious complications such as infertility can occur silently before it is recognized as a problem. Both *C. trachomatis* and *N. gonorrhoeae* are intracellular pathogens. This intracellular invasion capacity is probably a prerequisite in order to escape the hostile luminal milieu of the vagina and cervix.
A consequence of gonorrhea and chlamydial disease or the viral disease herpes is the increased risks for acquisition of HIV [2]. It is assumed that the local inflammatory responses in the cervix induced by the primary STD lead to more dense populations of immune cells, which are targets of the HIV viruses.

1.2.2 Bacterial vaginosis, BV

Bacterial vaginosis (BV) is the most common cause of vaginal infection in women of childbearing age. BV is characterised by an imbalance of the vaginal microbiota with a marked reduction of lactobacilli species, an overgrowth of a mix of mainly endogenous obligately anaerobic bacterial spp., and elevated pH in the vagina [3-5]. Women with BV may have an abnormal white or grey thin vaginal discharge with an unpleasant odor. BV may be accompanied by pain, itching, or burning [6], although the occurrence of BV with no signs or symptoms is estimated to account for half of all cases [7]. The total number of bacteria associated with BV is increased 100-1000 fold compared to normal levels. Thus, there is both a qualitative and quantitative change of the microbiota with BV. The presence of anaerobic bacteria gives rise to amines and an elevated pH, which further promotes the growth of anaerobic bacteria [8].
Among the bacterial spp. commonly found in BV are *Gardenerella vaginalis, Atopobium vaginae, Prevotella spp*., *Mobiluncus spp., Mycoplasma hominis and Ureaplasma spp.* [9-11]. However, the list of BV-associated bacteria is growing, since new species are being revealed, due to the use of cultivation independent methods of detection.
A virulence property of *Prevotella* sp. and some other species is the secretion of hydrolytic enzymes. It has been suggested that the capacity to degrade mucins by sialidases would facilitate the adhesion of bacteria to vaginal host cells and thereby colonization of the epithelium [12, 13]. Bacterial hydrolytic enzymes may also affect other secreted host factors such as antibodies and antimicrobial polypeptides/ proteins (AMPs) [14, 15].
In most cases, BV causes no complications, although it does present some serious risks health risks. Several studies have shown an association between BV and an increased susceptibility to STDs such as HIV-1, *Herpes simplex* virus, HPV, *N. gonorrhoeae,* and *C. trachomatis* [16-21].
BV has also been associated with an increased risk of endometritis, PID, and postoperative infections (hysterectomy, legal abortion [22-26]. The cure rate of BV by antibiotic treatment is between 60-70 % [27].

1.2.3 Ascending infections and pregnancy complications

PTB is the major cause of neonatal mortality and long term morbidity [28]. PTB, defined as birth before 37 weeks of gestation, occurs in circa 5-10% of all deliveries in developed countries. [29-33]. Approximately 50% of all premature births are idiopathic (termed “spontaneous PTB”). A growing body of evidence suggests genital infection and/or

inflammation as major causes of spontaneous PTB. Infections from organisms such as *N. gonorrhoeae, C. trachomatis, Trichomonas vaginalis,* PID and BV have been shown to significantly increase the risk of PTB [17, 29, 34, 35]. A strong association has also been found between BV and late spontaneous abortion (gestational week 16-24) [36, 37]. Some of these infections often occur without clinical symptoms. Thus, clinically silent upper genital infections and inflammation are strongly associated with an increased risk of spontaneous PTB [38-40]. Although BV is a marker for increased risk of PTB, it may not be the actual cause. A possible pathogenic mechanism has been suggested to be an ascending subclinical infection, which can lead to a microbial invasion of fetal membranes, bacterial invasion of the amniotic cavity and eventually to a fetal infection [38, 41, 42]. Several bacterial species isolated from the amniotic cavity of patients with spontaneous preterm labour and intact fetal membranes have been shown to be similar to those that are commonly found in BV. Among these bacterial spp. are *Gardnerella vaginalis, Prevotella* sp. *Mycoplasma hominis, Ureaplasma* sp., and *Mobiluncus* sp. [9-11, 43]. *Prevotella* spp., such as *P. bivia*, which is associated with preterm birth and is one of the most frequent species isolated from the amniotic fluid of patients with intra-amniotic infection, have been shown to increase the rate of PTB twofold in women with preterm labor [44-46].

1.3 Innate immune defence of the FGT

The innate immune system of the female reproductive tract is an important factor in the prevention of ascending genital infections that can threaten pregnancy and fetal development. The mucosa of the lower genital tract has to selectively support a habitat for resident commensal microbes and, at the same time, inhibit the growth of potential pathogens, whereas the upper genital tract must remain aseptic.

The components that constitute the innate immune defense of the FGT can be divided into; 1) morphological and physical defence features, 2) commensal bacteria colonizing the vagina, and 3) membrane-associated- and soluble factors [47, 48].

The innate immune system of the FGT is partly under hormonal control. The thickness of the endometrium (the lining of the uterus) (fig. 1), and its immune system change with fluctuating estrogen and progesterone levels during the menstrual cycle and pregnancy [49-51].

1.3.1 Morphological and physical defence features

The FGT can be divided into the lower genital tract (vagina and ectocervix) lined by multilayered nonkeratinized stratified squamous epithelium (fig. 2A), and the upper genital tract (endocervix, uterus, and fallopian tubes) lined by simple columnar epithelium (fig.2B). The lower and upper genital tract is connected via the cervix, which is composed of three different anatomical regions; the ectocervix (the outer part facing the vaginal lumen), a transformation zone (TZ), and the endocervix (facing the lumen of the cervical canal)(fig 1). The TZ constitutes an abrupt junction between the stratified squamous epithelium of the ectocervix and the columnar epithelium of the endocervix. The cervix forms a narrow canal and the cervical mucus is highly microbiocidal due to its content of a variety of AMPs that are active against a broad spectrum of microbes [52, 53].

Epithelial cells and the mucus layer function as a first physical barrier against potential pathogens [54]. The mucus physically protects the mucosa by hindering bacterial attachment and penetration.

Immune cells. Immune cells such as macrophages, dendritic cells (DCs), Langer-

hans cells, NK-cells, B cells and T cells are present throughout the genital tract mucosa with the highest concentrations located in the cervix (fig.2). Although no distinctive lymphoid structures are present in the genital tract, lymphoid aggregations containing B cells, CD8+/CD4- T cells, and macrophages have been reported to form in the transformation zone of the cervix. The highest number of macrophages and T cells are found in the TZ whereas macrophages, DCs, and T cells seem to be relatively sparse in the ectocervix and vagina. Additionally, numerous neutrophils also reside in the TZ. Macrophages, NK cells, and T cells are located in the lamina propria and as intraepithelial cells in the lumenal and glandular epithelium of the endocervical mucosa (fig 2B) [48, 55, 56].

Epithelial cells. The epithelial cell layer in the vagina and ectocervix is continuously sloughed off and is thereby also reducing the amount of attaching microbes (fig.2A) [57]. However, besides being a physical barrier for micro-organisms, mucosal epithelial cells may actively participate in the mucosal immune defence by secreting cytokines in response to pathogens [58] The cytokine response of the mucosal epithelium depends partly on the type of epithelial cells and pathogens involved. [59, 60]. Both invasive and non-invasive strains of *N.gonorrhoeae* induce the pro-inflammatory cytokines interleukin-6 (IL-6) and IL-8 in immortalized epithelial cells of the human ectocervix, endocervix, and vagina [61]. *C. trachomatis* invasion of a cervical cell line (HeLa) has been shown to induce the secretion of the pro-inflammatory cytokines IL-1α, IL-6, IL-8, and IL-18 [62] [63].

1.3.2 Membrane associated factors

A characteristic feature of innate immunity is the ability to recognise structurally conserved molecules derived from microbes and microbe-associated molecular patterns (MAMP) via pattern recognition receptors (PRRs) on the host cells. PRRs can be in either a soluble, membrane-bound or cytosolic form and function either at an extracellular or intracellular level. Examples of PRRs are: CD14 a molecule which can be either soluble or bound to external membrane [64, 65], the membrane bound mannose receptor (MR), dendritic cell-associated C-type lectin-1 (dectin-1)[66], Toll like receptors (TLRs), the intracellular NOD-like receptors (NLRs), and retinoic-acid-inducible gene (RIG)-like helicases (RLHs) [67-70].

TLRs. The TLRs, which are transmembrane proteins localized either at the cell surface or within phagosomes/endosomes, are one of the most extensively investtigated PRRs. Ten different human TLRs have been identified, each with a distinct MAMP specificity. TLRs are evolutionnarily conserved membrane-bound PRRs that recognize a broad spectrum of MAMPs including carbohydrates, lipids, proteins, and nucleic acids [70]. Examples of TLRs and their specific MAMPs ligands are displayed in Table 2. Expressions of TLRs have been found in various tissues and on a variety of cell types such as macrophages, DCs, neutrophils, fibroblasts, and epithelial cells [68, 71]. The distribution of some TLRs in the FGT are summarised in Table 3. Notable, TLR 4, which binds lipopolysaccharide (LPS) does not seem to be expressed in epithelial cells of the vagina, ectocervix, or endocervix regions of the FGT that are constantly exposed to endogenous and exogenous microbes. TLR2, which is another important receptor for bacteria, is expressed in minor amounts in the same epithelial cells. In contrast, the endometrium expresses both TLR4 and TLR2. The co-receptor molecules MD2 and CD14 are both involved in binding bacterial cell wall components. CD14 enhances the sensitivity towards bacteria and bacterial products [72] [73], while

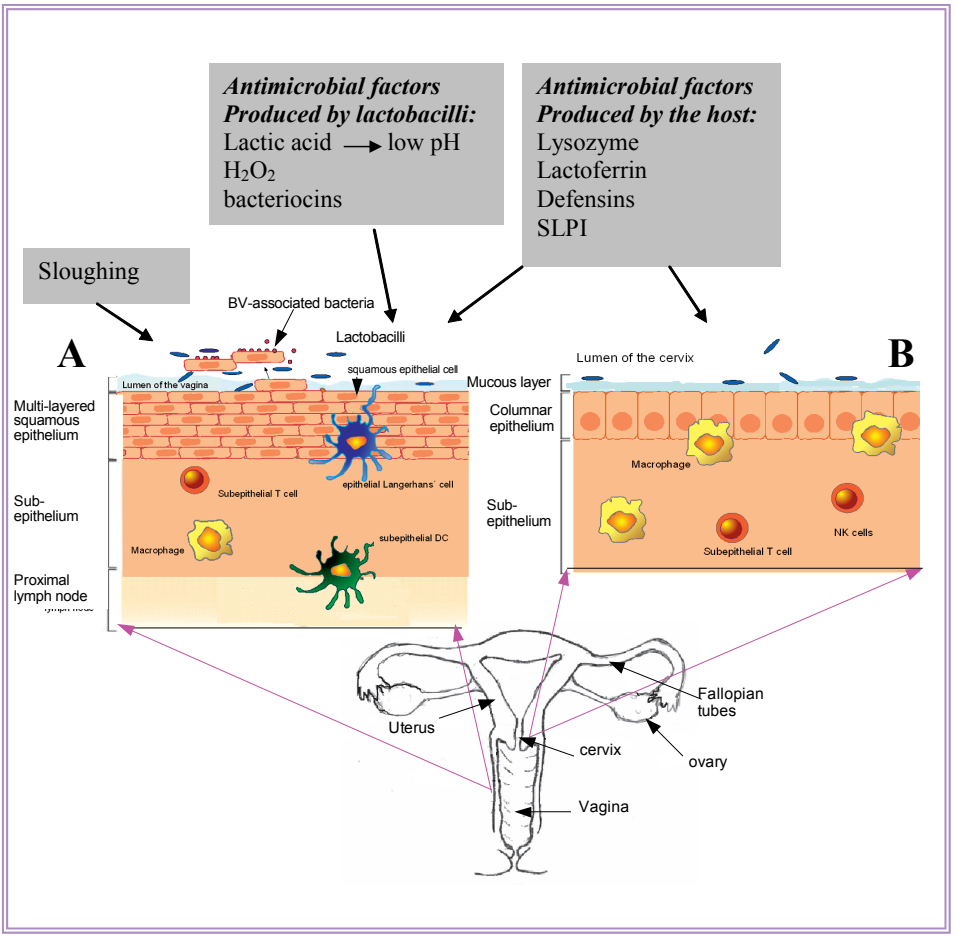

Fig 2. The female genital tract (FGT) with magnified vaginal (A) and cervical (B) epithelium. **A**) a multilayered squamous epithelium is lining the vagina and ectocervix. Macrophages, dendritic cells (DCs) and T cells are mainly present in the sub-mucosal area, although a sparse distribution of these cells also can be found in the epithelium. Epithelial cells are constantly sloughed off from the surface, thereby diminishing the number of bacteria attached to the epithelium. Epithelial cells also contain glycogen, which support the growth of lactobacilli and the production of lactic acid, which lower the pH in the vagina. **B**) the lining of the proximal part of cervix, the endocervix, consists of a single layered columnar epithelium. Macrophages, T cells, and NK cells are present in the epithelium and in the sub-mucosa. However, no dendritic cells appear to be present in the endocervix.

MD2 is required for a LPS-induced signal transduction [74].

1.3.3 Soluble factors

Mucosal secretion of the vaginal and cervical epithelia contain an abundance of soluble factors that participate in a local non-specific innate immune defence. Among the secreted soluble factors are components of the complement system,

cytokines, chemokines, nitric oxide, fibronectin, and AMPs [47, 48, 55, 75].

Antimicrobial factors. AMPs have a non-specific anti-microbial activity to a broad spectrum of bacteria fungi, and viruses. Some AMPs are enzymes (e.g., lysozyme) that disrupt essential microbial structures, others bind essential nutrients denying them to microbes, and some act by disrupting microbial or viral membranes (defensins) [76-78]. Since most AMPs are cationic, they are thought to be attracted to anionic components on the surface lipid membranes of bacteria, viruses, fungi, and protozoa by electrostatic attraction and thereby cause cell wall disrupttion and lysis [47, 48, 79].

The mucus of the FGT contains a variety of AMPs such as defensins, cathelicidins, lactoferrin, lysozyme, calprotectin, elafin, and secretory leukocyte protease inhibitor (SLPI) [80-83].

In women with BV, the levels of AMPs and antibacterial activity have been reported to be decreased, compared to levels in healthy women [84].

Table 2. Microbial ligands to human TLRs

Microbial products (MAMPS):		TLR
• Atypical LPS	*(gramneg)*	TLR2
• Peptidoglycan (PG)	*(grampos and gramneg)*	
• Lipoteichoic acid (LTA)	*(grampos)*	
• Lipoarabinomannan (LAM)	*(Mycobacteria)*	
• Porins	*(Neisseria)*	
• Lipoprotein/lipopeptides	*(a variety of pathogens)*	
• Diacyl lipopeptides	*(mycoplasma)*	TLR6/TLR2
• Triacyl- lipopeptides	*(bacteria and mycobacteria)*	TLR1/TLR2
• LPS	*(gramneg)*	TLR4/MD2[#]
• LTA, chlamydial LPS, HSP60, viral protein	*(gramneg, viruses)*	TLR4
• Flagellin	*(bacteria)*	TLR5
• CpG DNA	*(bacteria, C.albicans)*	TLR9*
• Glucans (Zymosan)	*(fungi)*	Dectin-1/ TLR2
• ssRNA (viral)	(*antiviral compounds)*	TLR7*, TLR8*
• dsRNA (viral)	*(viruses)*	TLR3*

* intracellular

[#] MD2 is a cofactor for LPS induced signal transduction.

Table 3. PRR expression in epithelial cells isolated from different locations of the FGT*.

PRRs	Vagina	Ectocervix	Endocervix	Endometrium
TLR 2	+/-#	+/-	+/-	+/+++
TLR 3	+++	+++	+++	+++
TLR 4	-	-	-	++
CD14	+	ND	+/-	+

* Author's unpublished results
\+ expression of mRNA
\- no mRNA expression
\# individual variation
ND not done

1.3.4 The vaginal microbiota

One of the most important defence mechanisms against infections in the FGT is the composition of the microbiota that colonizes the vagina. Lactobacilli are the predominant species of the vaginal microbiota in healthy women of childbearing age [47, 85, 86]. Glycogen, which is released from vaginal epithelial cells, supports the growth of lactobacilli. Since the production of glycogen is controlled by estrogen a dominance of lactobacilli is found in fertile women [87].

Other bacteria such as *Streptococcus* spp., *Staphylococcus* spp., *Gardnerella vaginalis*, and *Enterococcus faecalis* are examples of bacterial species that occur in low concentrations in the FGT microbiota of healthy women [3, 88]. The presence of *Lactobacillus* spp. in the vaginal microbiota is associated with a reduced risk of BV, ascending genital tract infections, and sexually transmitted diseases (STDs) [89-92].

In 1892 the German obstetrician, Albert Döderlein (1860 – 1941), was the first to describe a rod shaped Gram-positive bacterium in the vagina that, subsequently, was named "Döderlein's bacillus". Lactobacilli which are found primarily in the mucus of the vagina are facultative anaerobes. These belong to the group of lactic acid bacteria, due to their ability to produce lactic acid [93].

L.iners, *L.crispatus*, *L.jensenii* and *L. gasseri* have been reported to be the most predominant *Lactobacillus* species in the vaginal microbiota. Additionally, in the majority of women with a *Lactobacillus* dominated microbiota, only one species appears to dominate [47, 85, 86, 94-97].

Lactobacilli are thought to prevent the growth of non-residential bacteria by several mechanisms. The glycogen released from vaginal epithelial cells is metabolised by lactobacilli into lactic acid, which lower the pH of the vaginal fluid to pH 3.5 – 5 (fig 1A). A low pH is permissive for lactobacilli and other residential commensal bacteria that can grow in an acid environment, but is antimicrobial against non-resident species [53, 57, 80]. Additionally, the production of antimicrobial substances such as hydrogen peroxide (H_2O_2), bacteriocins, and other organic acids by the lactobacilli, together with the competition for adhesion sites and nutrients are all factors that contribute in preventing the growth of other resident and non-resident bacterial species (fig 1A) [89, 98-102].

2.SPECIFIC BACKGROUND

2.1 Intracellular invasion

Adhesion to and invasion of mucosal epithelial cells is a strategy for the establishment of infection used by several bacterial pathogens such as *C. trachomatis* and *N. gonorrhoeae* [103] [104]. Invasion of human oral epithelial cells by anaerobes such as *Prevotella intermedia*, *Porphyromonas gingivalis*, and *Actinobacillus actinomyc-temcomitans* has been suggested as a pathogenic mechanism for periodontal disease, a chronic polymicrobial infection with a slowly progressing inflammation and destruction of tissue [105-108].
Micro-organisms present in the lumen of the cervix are surrounded by high concentrations of AMPs. To survive in such a hostile milieu micro-organisms need to be covered by a protective surface, degrade AMPs by the secretion of proteases, or being able to invade epithelial cells [109, 110]. For example, sialidases produced by *Prevotella* spp. such as *P.bivia* and some other micro-organisms present in the microbiota of patients with BV, are likely to represent virulence factors that act by destroying mucins and proteins, and thereby enhancing the adherence of bacteria to epithelial cells. Adhesion of a pathogenic micro-organism to its host is the first stage in any infectious disease.

2.2 Cytokines and chemokines

Pro-inflammatory cytokines are small proteins that function primarily in the induction of inflammation. The concept is based on the genes coding for the synthesis of small mediator molecules that are up-regulated during inflammation. Examples of pro-inflammatory genes are type II phospholipase A_2, cyclooxygenase-2 and inducible NO synthase. These genes codes for enzymes that increases the synthesis of platelet-activating factor, leukotrienes, prostanoids, and nitric oxide (NO) [111]. Another feature is enhancement of the innate immune responses [112]. NFkB is an important transcription factor in non-lymphoid cells for all the cytokines described below.
IL-1 and TNF-α. Interleukine -1 (IL-1) and tumor necrosis factor – α (TNF-α), proteins with molecular masses of 15- to 18-kDa are particularly effective in inducing the expression of pro-inflammatory genes and may act in synergy in this process. These cytokines initiate a cascade of inflammatory mediators by inducing endothelial adhesion molecules in endothelial cells. This event is essential for the adhesion of leukocytes to the endothelial surface, which is a first step in the migration into the tissue.
IL-6. IL-6 is synthesized as a precursor protein. Monocytes express at least five different molecular forms of IL-6 with molecular masses of 19- to 26 kDa. The promoter gene for IL-6 contains many different regulatory elements allowing the induction of gene expression by many stimuli. IL-6 is a multifunctional cytokine that regulates acute phase response and inflammation, immune response and hematopoesis [113].
IL-8. IL-8 is member of the CXC chemokine family, which primarily mediate the activation and migration of neutrophils into tissue from peripheral blood by acting as chemo-attractants [114, 115]. IL-8 can also activate neutrophils to degranulate and cause tissue damage. IL-8 is produced via processing of a precursor protein. In its processed form IL-8 has a molcular weight of only 8-to 11 kDa. It is produced by various cells including monocytes, macrophages, fibroblasts, and epithelial cells. The synthesis of IL-8 is strongly enhanced by IL-1 and TNF-α [116].
IL-18. IL-18 belongs to the IL-1 family and is synthesiezed as a precursor, requiring caspase-1 for cleavage into the

active form. The active IL-18 molecule has a molecular weight of 18 kDa [117]. IL-18 is a pro-inflammatory and pro-apoptotic cytokine produced by macrophages, monocytes, keratinocytes and epithelial cells. It stimulates the production of IL-1β, TNF-α and IFN-γ [62, 118, 119].

There is a constitutive secretion of IL-1α, IL-1β, IL-6 and IL-8 in cervico-vaginal fluids of women in childbearing age with a *Lactobacillus*-dominated microbiota [120-122]. A local increase of an array of proinflammatory cytokines and chemokines has been reported in BV, chorio-amnionitis [123] [124-126], in spontaneous preterm labour and PTB [29], and in infection with *Neisseria gonorrhoeae* [122].

Despite the lack of clinical inflammation in BV, the levels of IL-1 and IL-8, but not IL-6, are increased in vaginal and cervical secretions [124, 127, 128] [120, 121, 125].

In preterm labor, however, significantly elevated cervical IL-6 and IL-8 levels are associated with microbial invasion of the amniotic cavity or chorioamniotic membranes, and with histological chorioamnionitis [129-131].

2.3 Secretory leukoprotease inhibitor, SLPI

SLPI was originally isolated from the secretions of patients with chronic obstructive pulmonary disease [132]. SLPI is a cationic acid stable serine protease inhibitor of the whey acidic protein-like family with a strong affinity for chymase, chymotrypsin, elastase, proteinase 3, cathepsin G and tryptase (fig. 3) [132-134]. Additionally, it has also been shown to exert antimicrobial, antiviral and antifungal activities [135-137]. SLPI also appears to be an important regulator of the inflammatory response by suppressing NFkB activation with resultant inhibition of peptide mediators, cytokines and chemokines [138-140]. It is a 11.7 kDa protein, consisting of 107 amino acids organized in two homologous, whey acidic protein four-disulfide core (WFDC) domains, of 53 and 54 amino acids, respectively (fig 2) [132, 141].

The gene for SLPI is located on chromosome 20q12–13.1 in humans. The antiprotease active site of SLPI is located on a loop (residues 67–74) of the carboxyl-terminal domain (fig 2) [142, 143] whereas the antimicrobial activity of SLPI has been found to be located in the amino-terminal domain [144, 145] [135]. SLPI is produced by epithelial cells, macrophages, and neutrophils, and is found in large quantities in bronchial-, cervical-, and nasal mucosa, saliva, and seminal fluids [48] [52, 139, 142, 143, 146].

2.3.1 SLPI in diseases

In women suffering from STDs or BV, dramatically reduced SLPI levels have been reported in vaginal fluid [91, 147].

SLPI have been shown to prevent HIV transmission in macrophages by the binding to annexin II, a cellular cofactor supporting macrophage HIV-1 infection [148]. In addition, low levels of vaginal SLPI have been associated with a high transmission rate of HIV to the infant at delivery in pregnant women with a HIV-1 infection [147].

In patients with inflammation in the lungs due to the hereditary disease cystic fibrosis, which affect exocrine glands of the lungs, liver, pancreas and intestines, the inhalation of aerosolized SLPI was shown to reduce IL-8 and neutrophil elastase in the bronchoalveolar lavage fluid [149]. Furthermore, an inverse correlation of the concentration of SLPI and neutrophil elastase in lung secretions has been reported in patients with chronic obstructive pulmonary disease [150, 151].

In the gut, an impaired induction of SLPI as well as other AMPs has been reported in Crohn's disease [152, 153].

Moreover, people with a *Helicobacter pylori* infection exhibited a strong decline

in antral SLPI levels compared to *H. pylori*-negative subjects and subjects from whom *H. pylori* had been eradicated [154].

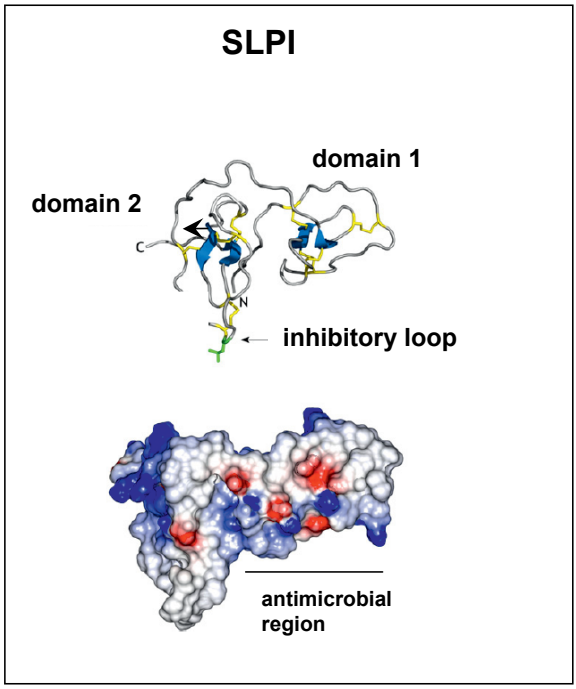

Fig. 3. Upper part: ribbon diagram of the 3D structures of SLPI. The side-chain Leu72 of SLPI is shown in green. The disulfide bonds are colored yellow. Below: molecular surfaces of SLPI in the same orientation as in the figure above colored according to the electrostatic surface potential (blue, positive regions; red, negative regions) (modified from T Moreau et al 2008).

AIMS OF THE STUDY

The major aim of the thesis was to explore the interactions between microorganisms and host cells with regard to vaginal opportunistic bacteria but also with regard to lactobacilli and their influence on local immune defence factors. The specific aims of the studies were:

- To investigate virulence features of *Prevotella bivia,* a gram negative rod commonly found in BV,

- To analyse some of the most pre-dominant vaginal *Lactobacillus* spp in their ability to regulate innate immune factors such as SLPI and cytokines in cervical epithelial and monocytic cells,

- To analyse the effect of SLPI on the pro-inflammatory cytokine response induced in cervical epithelial cells.

4. MATERIALS AND METHODS

Cell lines and cell cultivation conditions (I, II, III)

The cervix epithelial cell line (HeLa) (I, III) and the human monocyte cell line (THP-1) (II) were obtained from the American Type Culture Collection (ATCC). HeLa cells were grown in culture flasks at 37°C in 5 % CO_2 in Dulbeccos MEM (DMEM) (I) (Biochrome, Berlin, Germany) or in Eagle's MEM (III) (PAA Laboratories, AU) both supplemented with 10 % fetal bovine serum, 100 mM sodium pyruvate, and 200 mM L-glutamine (all from Sigma-Aldrich, St.Louis, Missouri, USA).

THP-1 cells were grown in culture flasks at 37°C in 5 % CO_2 in RPMI 1640 medium (Invitrogen, Paisley, UK) supplemented with 10 % fetal bovine serum, 100 mM sodium pyruvate, and 200 mM HEPES buffer (all from Sigma-Aldrich St.Louis, Missouri, USA).

Bacterial strains, culture conditions and concentration determination (I, II, III)

The bacterial strains and culture conditions used in the studies are listed in Table I.

The bacteria were harvested for cell stimulation assays, transferred into 10 ml phosphate buffered saline (PBS) and washed once by centrifugation (2000 x g for 10 min.). Determinations of bacterial concentrations were done spectrophotometrically (WPA CO75 Colorimeter, Cambridge, UK), at 590 nm, using standard-curves for each bacterial species, wherein the OD correlated to the number of bacteria per ml.

In some experiments, lactobacilli were killed by ultra violet (UV) light, in which the bacterial suspension was transferred to a 6-well plate and placed on a shaker in UV light for 18 minutes. A verification of UV inactivation of the bacteria was performed by culturing.

Bacterial adhesion assay (I)

The bacterial adhesion analysis was performed according to Adlerberth et. al.[155] with some modifications. In short, HeLa cells were cultured to a confluent cell layer in DMEM medium supplemented with 10 % Cosmic calf™ serum (HyClone laboratories inc., Utah, USA), 200 mM L-glutamine and 100 mM sodium-pyruvate, in 25 ml bottles. The cells were detached by incubation with 54mM EDTA solution for 10 minutes at room temperature and washed twice in DMEM. Finally the cell concentration was adjusted to 5 x 10^6 cells /ml. *P. bivia* strains were cultured on Brucella agar (BBL Microbiology system, Cockeysville, MD, USA), anaerobically, at 37°C, in 95 % N_2 and 5 % of CO_2, for two days. The bacteria were harvested and washed once in Hanks balanced salt solution (HBSS). The bacterial concentration was adjusted to 5 x 10^9 bacteria per ml by determination of the concentration by spectrophotometry, where OD 0.2 corresponded to 2 x 10^8 bacterial cells per ml. A volume of 100 µl of each of the cell and bacterial suspensions were mixed together in a tube with 300 µl of HBSS and incubated, with rotation, for 30 minutes, at 4°C or 37°C. The cells were subsequently washed twice by centrifugation, at 1000 rpm. The cells were subsequently washed twice by centrifugation at 1000 rpm for 10 minutes and re-suspended in HBSS. The remaining cell pellet was mixed with one drop of Histofix (Histolab AB, Sweden). Bacterial adhesion to the cells was evaluated by interference microscopy (Nikon Optiphot, Nikon PlanApo 500 x magnification), using the calculation of the mean number of adhering bacteria to forty cells.

Table I

Bacterial strains	Source	CCUG* number (name in papers)	Culture medium**
Prevotella bivia	FGT ◊	34045 (P45)	Brucella agar
- ” -	- ” -	34046 (P46)	- “ -
- ” -	- ” -	34047 (P47)	- “ -
- ” -	- ” -	33961 (P61)	- “ -
- ” -	- ” -	9557♣ (P57)	- “ -
Lactobacillus iners	- ” -	28746♣	Chocolate agar
- ” -	- ” -	44030 (003)	- “ -
- ” -	- ” -	44065	- “ -
- ” -	- ” -	44119 (119)	- “ -
Lactobacillus jensenii	- ” -	35572♣	- “ -
- ” -	- ” -	44003 (003)	- “ -
- ” -	- ” -	44054	- “ -
- ” -	- ” -	44149	- “ -
- ” -	- ” -	44151 (151)	- “ -
Lactobacillus gasseri	not known	31451♣	- “ -
- ” -	FGT	44072	- “ -
- ” -	- ” -	44076	- “ -
- ” -	- ” -	44081	- “ -
	- ” -	44082	- “ -
Lactobacillus crispatus	- ” -	44016	MRS agar
- ” -	- ” -	44073	- “ -
- ” -	- ” -	44117 (117)	- “ -
- ” -	- ” -	44118	- “ -
Escherichia coli O6:K13	Örskov. Su 4344	11308	Horse blood agar
Escherichia coli HB101	Dr N. Strömberg (University of Umeå).	-	- “ -

◊ Female genital tract

* Culture Collection, University of Gothenburg

** All bacteria except *E.coli* were grown under anaerobic conditions in 95 % N_2 and 5 % CO_2, at 37°C. *E.coli* was cultured under aerobic conditions, at 37°C.

♣ Type species strains

A modification of the adhesion assay was performed in order to simulate the invasion assay. In the modified assay, the HeLa cells were incubated on a slide chamber (Lab-Tek, Chamber Slide System, US) and the cells remained adherent during the incubation with the bacteria.

Invasion assay (I)

The assay is based on the varying bactericidal effect of antibiotics on extra- and intracellular located bacteria [156]. All experiments with different *P.bivia* strains were performed under anaerobic conditions while control experiments with the non-adhesive *E.coli* HB101 were accomplished in normal atmosphere. Bacteria were scraped off agar plates, washed once by centrifugation in phosphate buffered saline, pH 7.2 (PBS), at 3000 x g, and suspended in DMEM without serum and penicillin-streptomycin (PEST) at a concentration of $1x10^7$ bacteria/ml. HeLa cells were washed in PBS, infected with 500 µl bacterial suspension per microtitre well of either *P. bivia* or *E. coli* and incubated, at 37°C, for 2 h. The epithelial cells were subsequently washed three times with PBS and incubated for 3 h with DMEM containing metronidazole (0.1 mg/ml) and gentamicin (0.5 mg/ml), in order to prevent extracellular bacteria from further multiplication. HeLa cells were thereafter washed three times in PBS and incubated with 1 ml sterile distilled water, for 10 min, to lyse the membranes of the epithelial cells. Released intracellular *P. bivia* was plated on Brucella agar and *E. coli* on blood agar. Cultures on Brucella agar plates were incubated for 7 days and those on blood agar plates were incubated for 24 h. The invasion capacity was quantified by counting colonies and expressed as colony forming units (CFU) per well.

In order to estimate the effects of antibiotics and medium on bacterial survival, the same number of bacteria of each strain was incubated without epithelial cells, in DMEM supplemented with metronidazole and gentamicin for 3 h. All the experiments were performed in triplicates with, at least, three independent runs per strain.

Cell stimulation assays (I, II, III)

HeLa cells (I, III). HeLa cells were grown in supplemented medium and transferred to 24-well plates, at a density of 10^5 cells per well the day before cell experiment. Prior to cell-stimulation by bacteria, the cells were washed once in fresh homologous medium. Thereafter, 450 µl of homologous medium, supplemented as above except for the addition of 5 % heat-inactivated FCS instead of 10% normal FCS, and 10 µg/ml of metronidazole (I) or 200 U/ml of penicillin-streptomycin (PEST) (I, III), to prevent bacteria from further multiplication, were added to each well. Bacterial suspensions, 50 µl of various concentrations, were then added to each well and the cells were subsequently incubated at 37° C, in 5 % CO_2, for different time periods. In one experiment, the stimulation of the cells with bacteria was performed in culture flasks (250 ml), instead of a 24-well plate, in order to obtain a higher number of cells.

THP-1 cells (II).THP-1 cells were pre-stimulated for 16 hours with 100 U/ml of interferon gamma (IFN-γ) (Roche Diagnostic, Mannheim, Germany) in RPMI medium supplemented with 10 % fetal bovine serum, 100 mM sodium pyruvate and 200 mM HEPES buffer (Sigma-Aldrich St.Louis, Missouri, US). The medium was removed by centrifugation at 160 x g, and the cell pellet was re-suspended in assay medium (RPMI 1640 medium supplemented with 5 % heat-inactivated fetal bovine serum. The cell concentration was adjusted to $1x10^6$ cells/ml and 450 µl of the cell suspension were transferred to 24-well plates prior to stimulation with bacteria.

Cell supernatants and cells were subsequently collected for further analysis by ELISA or PCR. The supernatants were

centrifuged, at 10'000 rpm, and kept frozen at -70°C until analyses were performed. For PCR, HeLa cells (III) were detached, by adding 0.1 ml of 0,54 mM EDTA solution per well, for 10 minutes, at room temperature. Collected non-adherent THP-1 cells (II) and detached HeLa cells were washed once in 1 ml PBS, centrifuged at 160 x g, and the pellet was re-suspended in RNAlater (Ambion, Cambridgeshire,UK) and stored at -20°C until cDNA preparation for PCR was performed.

In some experiments (III), recombinant human SLPI (rh-SLPI) (R&DSystems, Abingdon, UK) was added to the HeLa cells prior to the addition of bacteria. Cells and supernatants were then collected and treated as described above.

Electron microscopy (I)

Transmission electron microscopy (TEM) was performed in order to confirm the intracellular location of internalized bacteria. HeLa cells cultured in 35 mm petri-dishes were infected with *P.bivia* P47 and treated as described for the invasion assay. After a total of 5 h incubation, the cultures were washed four times in PBS. The HeLa cells were detached from the plastic surface with trypsin/EDTA (0.05% /0.5 mM) solution. Fixation was performed by addition to the cell suspension, 2.5% glutaraldehyde in 0.05 M Na cacodylate, for 45 min. Cells were thereafter pelleted and fixed, with 1% OsO_4 in 0.1 M Na cacodylate, for 30 min. Ethanol dehydration and propylene oxide treatment were subsequently performed by successive gentle re-suspensions and centrifugations. The cells were finally infiltrated with Agar 100 resin in BEEM capsules. The ultrathin sections were contrasted with lead citrate and uranyl acetate before examination, using a Zeiss CEM 902 electron microscope.

Scanning electron microscopy (SEM) and TEM were used for detection of adhesion structures on *P. bivia* P47. Bacteria were grown anaerobically, for 3 days, at 37°C, on trypticase soy agar plates supplemented with hemin and menadione. Bacteria were then gently scraped off the plates and suspended in reduced transport fluid, according to the methods of Leyng et. al. and Salam et. al. [157, 158]. Prior to TEM, bacteria were fixed in a suspension containing an aldehyde mixture (2% paraformaldehyde + 2.5% glutaraldehyde in 0.05 M Na cacodylate buffer, pH 7.2) and diluted 1:1 with phosphate buffered saline (PBS, pH 7.4). The suspension was then washed and kept in PBS. Five µl droplets of the suspension were applied on Formvar coated copper grids for 1 min. After a brief rinse the adhering bacteria were negatively stained, with 0.5% uranyl acetate in water, for 10-30 sec, after which the staining solution was removed with a filter paper. The stained specimens were examined, using a Zeiss 902 electron microscope. Prior to SEM, 250 µl drops of bacteria, fixed and washed as above, were transferred to poly-l-lysine treated gold coated Thermanox cover-slips, for 30 min, to allow the bacteria to adhere to the surface. After rinsing with PBS, the cells were prepared for SEM with the OTOTO method, according to Friedman et. al. [159], comprising repeated treatments with osmium tetroxide and thiocarbhydrazide. The cover-slips were dehydrated in ethanol and infiltrated with hexamethyldisilazane, which was allowed to evaporate in a fume hood. The dried specimens were mounted on aluminum SEM stubs and examined, without thin film metal coating, using a Zeiss 982 Gemini SEM.

Cytokine and SLPI detection by Enzyme-Linked ImmunoSorbent Assay (ELISA) (I, II, III)

Enzyme-Linked Immuno Sorbent Assay (ELISA) was used for quantification of cytokines and SLPI in the cell supernatants. Primary mouse monoclonal anti-human and secondary polyclonal biotinylated goat anti-human antibodies against IL-1α, IL-6, IL-8, IL-18 and TNF-α and their respective recombinant

standards were obtained from R&D Systems (Abingdon, UK) for cytokine quantification. The sandwich ELISA assay was done according to the manufacturer's description except that alkaline phosphatase conjugated streptavidin (Extravidin, Sigma-Aldrich, St.Louis, Missouri, USA) diluted 1:1000 was used instead of streptavidin Horse-Redish-Peroxidase (HRP), and as substrate solution, para-nitrophenyl phosphate (pNPP, 1 mg/ml, Sigma-Aldrich, St.Louis, Missouri, USA) in diethanolamin buffer (pH 9,8) was used. Colour development was analysed spectrophotometrically at 405 nm. An ELISA assay for SLPI quantification was set up with the following antibodies from R&D Systems; a primary monoclonal mouse anti-human SLPI (MAB1274) at a concentration of 4 µg/ml; a secondary biotinylated goat anti-human SLPI (BAF1274), at 20 ng/ml; and recombinant human SLPI (1274-PI), as a standard. The detection limit was 78 pg/ml. The assay procedure was performed as described for the cytokines.

RNA extraction and cDNA synthesis (II, III)

Total cellular RNA was prepared by using the RNeasy Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer's protocol. Samples were lysed and homogenized in the presence of a guanidine isothiocyanate buffer. To remove contaminating DNA, eluted total RNA was treated with RNase free DNase I, according to the manufacturer's description (DNA-free Kit, Ambion, Cambridgeshire,UK). Total RNA was subsequently quantified by measuring the ratios of optical densities at 260 nm and 280 nm. The RNA integrity for each sample was visualised by electrophoresis on a 3 % agarose gel containing 0.02 % SybrGreen.

cDNA was prepared from 1 µg of total RNA, using a mixture of 100 pmol of random hexamer, pd$(N)_6$ (GE Healthcare, UK), first strand reaction buffer (Invitrogen AB, Sweden), 0.5 mM of dNTP mix (GE Healthcare, UK), 1 U/µl of ribonuclease inhibitor (RNasin, Promega, US) and 13.3 U/µl of reverse transcriptase (SuperScript, Invitrogen, Paisley, UK), followed by incubation at 42°C, for 1 hr, and termination of reaction by incubation at 70°C for 10 min.

Polymerase chain reaction (PCR) analysis (II, III)

Relative quantification of SLPI, IL-8, MD-2, CD-14, TLR-2, -3,-4, and -5 mRNA expression was analysed by real time PCR (LightCycler, Roche). The primers for the PCRs are listed in Table II. Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was used as a house-keeping gene (referred to as reference). The primers for IL-8 were obtained from Clontech Laboratories (Paolo Alto, CA, US) and all the other primers were obtained from TibMol (Berlin, Germany). A LightCycler FastStart DNA Master SYBER Green 1 Kit (Roche Diagnostics, Mannheim, Germany) was used for the PCR, according to the manufacturer's protocol. The PCRs were carried out in a total volume of 20 µl, containing 2 µl cDNA, 3.5 mM $MgCl_2$ and 0.5 µM of each primer.

Relative quantification calculation of the samples was performed by using the Relative Quantification LightCycler software (Roche), by which the results were normalized to a calibrator and expressed as the sample/reference ratio of each sample normalized by the sample/reference ratio of a calibrator. A macrophage cell line (MonoMac) was used as a positive control in analyses of the MD-2, CD-14, and TLR-2,-4, -5 mRNA expression.

Table II. Primer pairs used in the PCR

Product	Forward primer	Reverse primer	Product size (bp)
TLR 2	GCCAAAGTCTTGATTGATTGG	TTGAAGTTCTCCAGCTCCTG	347
TLR 4	AAGCCGAAAGGTGATTGTTGT	ATTGCATCCTGTACCCACTGTT	310
TLR 3	AAATTGGGCAAGAACTCACAGG	GTGTTTCCAGAGCCGTGCTAA	320
TLR 5	CCATCCTCACAGTCACAAAGTT	TCTAAGGAAGTGTCTGCTCACAA	326
MD-2	CTCAGAAGCAGTATTGGTC	GTTGTATTCACAGTCTCTCCCT	295
CD-14	GGTGCCGCTGTGTAGGAAAGA	GGTCCTCGAGCGTCAGTTCCT	454
SLPI	GCTGTGGAAGGCTCTGGAAA	TGCCCATGCAACACTTCAAG	298
IL-8	ATGACTTCCAAGCTGGCCGTGGCT	TTCTCAGCCCTCTTCAAAAACTTCTC	289
GAPDH	GGCTGCTTTTAACTCTGG	GGAGGGATCTCGCTCC	190

Absorption Assay (III)

L. iners 119 and *E. coli* were used at a concentration of 10^9 bacteria per ml, in combination with different doses of recombinant human (rh) SLPI (R&D Systems, Abingdon, UK), to assess bacterial absorption of SLPI. The bacteria were grown and treated as described for the cell assays. The combination of bacteria and SLPI was performed in cell assay medium (Eagle's MEM complemented as for the cell assay) and incubated at 37°C, for 2 h, and then at 4°C, for 16 h. The samples were then centrifuged at 10'000 rpm and the supernatants were collected for analysis of SLPI by ELISA.

Western Blot (III)

HeLa cell- supernatants from cells unstimulated or stimulated with *L.iners* 119, at concentrations of 10^7 or 10^9 bacteria per ml, for 20 h, were analysed for SLPI by Western Blotting. Electrophoresis of samples was performed, using precast NuPage Novex 10 % Bis-Tris mini gel (Invitrogen, Carlsbad, CA,USA) in 1 X SDS NuPage MES Running buffer (Invitrogen), according to the manufacturer's description. Recombinant human SLPI (R & D Systems, Abingdon, UK) was used as a positive control, at a concentration of 0.5 µg/ml. A pre-stained standard, SeeBlue Plus2 (Invitrogen) was used, as a molecular weight marker. Protein transfers to nitrocellulose membranes were conducted, using the iBlot Dry Blotting System (Invitrogen), according to the manufacturer's description. The nitrocellulose membrane was blocked, using Blocking Buffer (Sigma, St. Louis, MO, USA), for 60 min, and was thereafter and in sequential steps washed three times, for 5 min each, in Tris-buffered saline containing 0.1 % Tween 20 (TTBS). The membranes were incubated for 120 min, with an affinity purified goat anti human SLPI antibody (R & D Systems, Abingdon, UK), at a concentration of 0.1 µg/ml, followed by incubation for 45 min, with biotinylated rabbit anti goat immunoglobulins (Southern Biotechnology Associates, Ink. Birmingham, AL, USA), diluted 1:1000 in TTBS. The membranes were then incubated for 45 min with Extravidin Alkaline Phosphatase (Sigma), diluted 1:5000 in TTBS. Band visualization was performed, using Western Breeze Chemiluminescent Substrate (Invitrogen) containing 5 % Chemiluminescent Substrate Enhancer (Invitrogen) with a sub-

sequent imaging of membranes in Gene Gnome Bio Imaging (Syngene) for 20 min.

5. RESULTS

Adhesion, invasion and intracellular location of *Prevotella bivia* in HeLa cells (I)

Five strains of the opportunistic pathogen *P.bivia*, all isolates from women with BV, were investigated for their capacity to adhere to and invade cervix epithelial cells (HeLa).

Invasion

The number of intracellular bacteria isolated from infected HeLa cells varied between the five *P.bivia* strains (fig. 1). The *P. bivia* strain 46 (P46) exhibited the lowest number of intracellular bacteria per well corresponding to 60 bacteria per $1x10^5$ HeLa cells. The strains P61 and P57 showed a 3-10 fold higher invasion capacity, corresponding to approximately $2\text{-}6x10^2$ bacteria per $1x10^5$ cells, while the number of intracellular P45 ($3x10^3$ bacteria per $1x10^5$ cells) was 40 times higher than that of P46. Strain P47 gave rise to the highest number of bacteria per well (approximately $7x10^3$ bacteria per $1x10^5$ cells), being 120-fold higher than that of P46. The invasion efficiency ranged from 0.002% (P46) to 0.2% (P47) (the ratio of the number of intracellular bacteria to the total inoculum) (fig. 2).

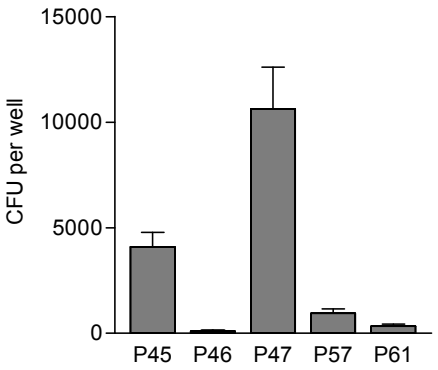

Fig.1. Recovery of intracellular P.bivia in HeLa cells. The mean number of CFU in HeLa cells infected by *P.bivia* per well. The bars represent the standard error of the mean (SEM).

Adhesion

The five isolated strains of *P.biva* were investigated for their capacity to adhere to HeLa cells by interference microscopy. The adhesion capacity ranged between 14-22 bacteria/cell (fig 3). There was no significant difference in the adhesion capacity between the most invasive (P47) and the least invasive strain (P46) when the bacteria were added in different concentrations to the HeLa cells. The numbers of adhering bacteria per cell increased in a dose-dependant manner for both strains and reached a plateau at a ratio of 100:1 bacteria per cell (fig. 4).

No correlation was established between intracellular survival and adhesion for the five strains. Thus, the strain giving rise to the highest number of intracellular bacteria, P47, showed no increased adhesion capacity in comparison with the other strains (fig. 1 and 3).

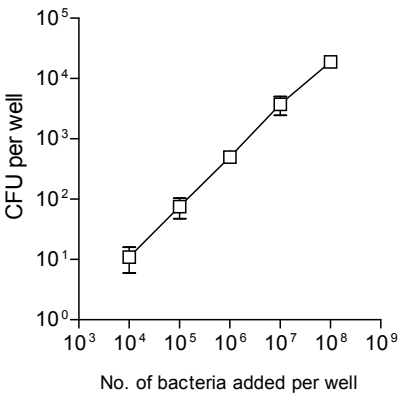

Fig.2 Invasion of HeLa cells by *P.bivia* (P47). The mean number of cells per well was 4,5 x 10^5. The mean number of CFU recovered from HeLa cells infected by P47 is indicated. The bars represent the standard error of the mean (SEM).

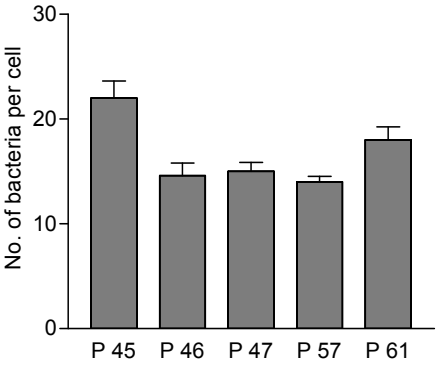

Fig.3. Adhesion of *P.bivia* to HeLa cells, ratio 1000:1 bact./cell. The number of bacteria adhering to HeLa cells was analysed by interference microscopy. The bars represent SEM.

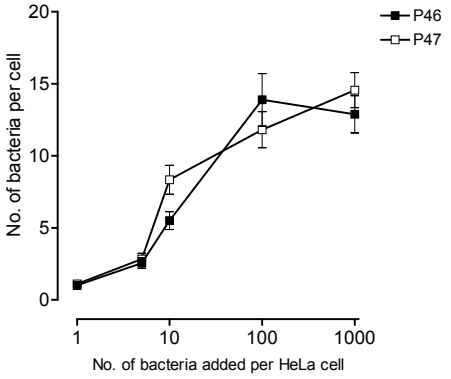

Fig.4 Adhesion of P.biva (P46 and P47) to HeLa cells.The mean nuber of adhering bacteria per cell is indicated.The bars respresent the standars error of the mean (SEM).

Intracellular location of P.biva

The intracellular location of the most invasive strain P47 was investigated by electron microscopy. Incubation of HeLa cells with P47 was performed as described for the invasion assay. An intracellular location of P47 was seen in phagosome-like vesicles, as analyzed by transmission electron microscopy (TEM) (fig. 5).

Adhesion features of P.bivia

The P47 strain was further investigated for morphological adhesion features on the cell surface by TEM. No visible adhesion structure like fimbriae was visible on the surface of P47 (fig.6).

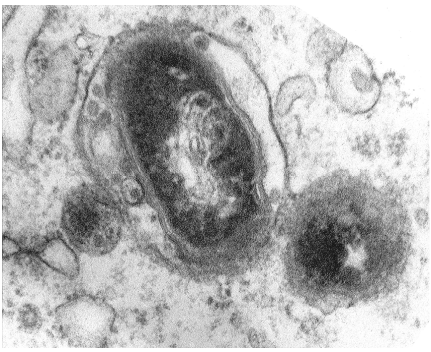

Fig.5. TEM micrograph of intracellular localization of *P.bivia* (P47) in HeLa cells.

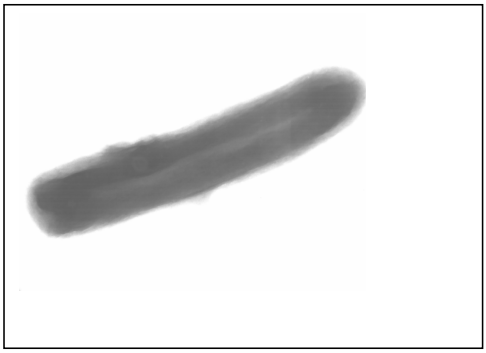

Fig. 6 TEM analysis of adhesion structures on *P.bivia* (P47).

Cytokine inducing capacity of *P.bivia* (I)
Since *P.bivia* is associated with upper genital tract infection and BV, which often is a subclinical syndrome, the ability of this Gram-negative anaerob to induce a pro-inflammatory cytokine response in HeLa cells was investigated by ELISA. P46, P47, P57, and P61 were analysed for their cytokine inducing capacity in HeLa cells. Of the four tested *P.bivia* strains only P47 was able to stimulate, to a low but significant IL-6 and IL-8 response in HeLa cells, at a concentration of 100 bacteria per cell. This cytokine-inducing capacity of P47 was, however, approximately 2 % of that induced by *E. coli*. No detectable cytokine levels in the cell supernatant were obtained by any of the other *P. bivia* strains tested (Table1).

Table 1. The levels of IL-6 and IL-8 in supernatants from HeLa cells stimulated with *P. bivia* or *E. coli*.

Strain		IL-6 (pg/ml)		IL-8 (pg/ml)	
	bacteria per cell	6 h	20 h	6h	20h
P46	10	<	<*	<	<*
	100	<	<	<	<
P47	10	<	<	<	<
	100	<	17 ± 5	<	57 ± 17
P57	10	ND	ND	ND	ND
	100	<	<	ND	ND
P61	10	ND	ND	ND	ND
	100	<	<	ND	ND
E.coli	10	75 ±14	430 ±26	343 ±91	470 ± 45
	100	ND	730 ±110	252 ±115	2380 ± 120

ND= not determined
* Background levels of IL-6 or IL-8

SLPI regulation in THP-1 cells (II) and HeLa cells (III) stimulated with vaginal lactobacilli
In order to investigate the effects of lactobacilli on the regulation of SLPI secretion we incubated THP-1- (II) or HeLa cells (III) together with the four most predominant *Lactobacillus* species (*L,crispatus, L.jensenii, L.gasseri,* and *L.iners)* of the female genital tract of healthy women.

THP-1 cells (II)
THP-1 cells were incubated with isolates of *L.iners, L, crispatus, L. jensenii* and *L. gasseri* at different doses for 20 h and the SLPI levels were subsequently measured in cell supernatants.
A significant up-regulation of the mean SLPI secretion, compared to constitutive levels, was observed when stimulating the cells with *L. iners* at 10^6 – 10^8 bacteria per ml (fig.7 and 8). In contrast, a down-regulation of the SLPI levels was seen in

cells incubated with *L.crispatus* at 10^8 and 10^9 bacteria per ml or with *L. gasseri* at 10^9 bacteria per ml (fig.7).

L.jensenii did not alter the constitutive secretion of SLPI in the cells. The most pronounced differences in the induction of SLPI secretion were seen between *L. iners* and *L. crispatus* at concentrations of 10^8 and 10^9 bacteria per ml ($p = 0.0002$ and $p = 0.0001$ respectively).

The capacity of one *L. iners* strain to induce a SLPI response in THP-1 cells was also compared to that of Gram-negative bacteria, such as the uro-pathogen, *E. coli*. THP-1 cells were incubated with *L. iners* or *E. coli* at different doses of bacteria per ml for 20 hours (fig 8). An up-regulation of SLPI, over constitutive levels, was obtained by *L. iners* at 10^6 to 10^8 bacteria per ml. At 10^9 bacteria per ml the SLPI levels decreased below constitutive levels when stimulated with either *L. iners* 119 or *E. coli,* although the decrease was not significant for *E. coli*. A significant difference between *L. iners* 119 and *E. coli* was obtained in their capacities to regulate the SLPI secretion in the cells, at 10^8 bacteria per ml.

The kinetics of the SLPI secretion in THP-1 cells stimulated by *L. iners*, at a concentration of 10^7 bacteria per ml, showed a significant increase of SLPI secretion over constitutive at 6 and 20 h of incubation (fig.9 a). Analysing the kinetics and dose response of the SLPI mRNA expression in cells stimulated with *L. iners* revealed an initial increase over constitutive level already at 1 h of incubation for all tested concentrations of bacteria. However, at 2 h and 20 h of incubation the mRNA expression returned to the same level or below that of the constitutive expression for all the doses tested (fig.9 b). This was in contrast to the up-regulation of SLPI protein levels at these time points by *L. iners* at 10^7 bacteria per ml (fig.9 a).

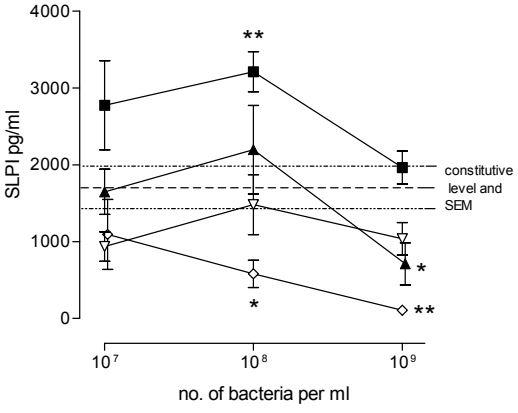

Fig.7. SLPI levels analysed by ELISA in supernatants from THP-1 cells stimulated for 20 h with isolates of *L.iners* (■, four isolates), *L.gasseri* (▲, five isolates), *L.jensenii* (∇, five isolates), and *L.crispatus* (◊, four isolates) The broken line shows the constitutive secretion level in the cells at 20 h. The bars in each point represent SEM.

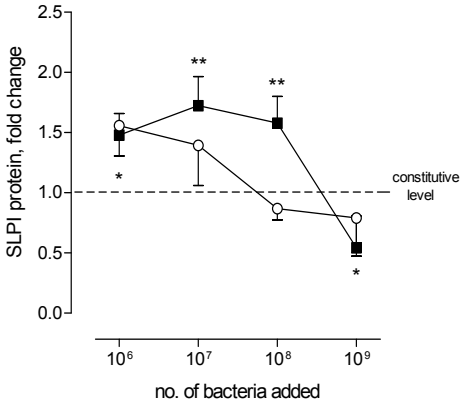

Fig.8. SLPI secretion in THP-1 cells stimulated for 20 h with *E.coli* (○) or *L. iners* 119 (■) at different doses. The SLPI secretion is presented as fold change over constitutive levels. The bars in each point represent SEM.

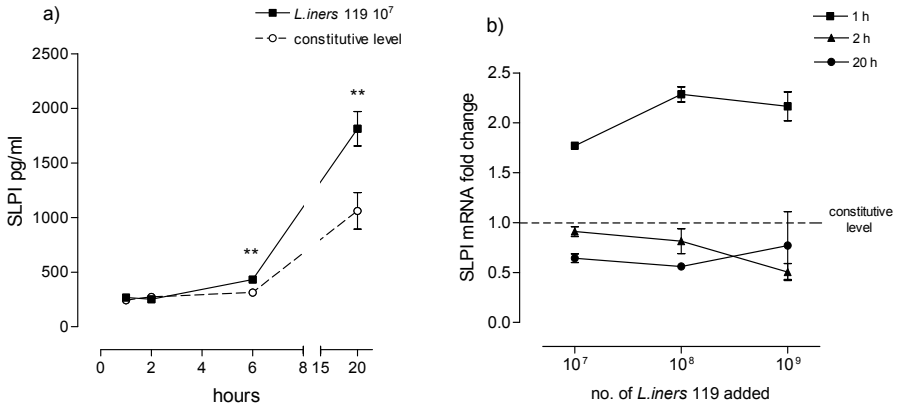

Fig. 9. SLPI protein secretion and mRNA expression in THP-1 cells stimulated with *L.iners* 119. a) Kinetics of SLPI protein secretion in cell supernatants un-stimulated (○) or stimulated (■) with *L.iners* 119 at 10^7 bacteria per ml for 1, 2, 6 and 20 h analysed by ELISA. b) Relative SLPI mRNA expression calculated as fold change over the constitutive expression level in THP-1 cells stimulated for 1, 2 and 20 h with different doses of *L.iners* 119. The bars in each point represent SEM.

Table 2. SLPI and cytokine levels analysed by ELISA in supernatants from HeLa cells stimulated with each *Lactobacillus* strain alone or in combination with *E.coli* at a total concentration of 10^8 bacteria per ml. The control is supernatant from un-stimulated cells. The standard error of mean (SEM) of triplicates are indicated.

Bacterial strain		SLPI ng/ml		IL-6 ng/ml		IL-8 ng/ml	
		Single strain	Combined with *E.coli*	Single strain	Combined with *E.coli*	Single strain	Combined with *E.coli*
L.jensenii	003	48,8 ± 5,8	39,8 ± 2,1	0,17 ± 0,03	5,05 ± 0,36	0,20 ± 0,01	5,16 ± 0,21
	151	57,8 ± 0,9	39,6 ± 0,4	0,07 ± 0,01	6,38 ± 0,34	0,10 ± 0,01	4,33 ± 0,36
L.crispatus	117	30,8 ± 0,5	22,0 ± 0,3	0,46 ± 0,05	4,69 ± 0,10	0,66 ± 0,03	6,87 ± 0,42
L.iners	030	52,6 ± 1,7	40,8 ± 1,8	0,25 ± 0,01	6,68 ± 0,06	0,18 ± 0,01	9,66 ± 0,25
	119	59,9 ± 2,4	40,7 ± 1,0	0,09 ± 0,01	6,79 ± 0,39	0, 07 ± 0,01	8,39 ± 0,13
E.coli		42,1 ± 1,2	NA*	4,98 ± 0,9	NA	3,13 ± 0,19	NA
Control		66.8 ± 4,1		0		0	

*NA = not applicable

HeLa cells (III)

Since *L.iners* up-regulated and *L. crispatus* down-regulated the secretion of SLPI in THP-1 cells at a concentration of 10^8 bacteria per ml we investigated whether SLPI would be regulated in the same way in HeLa cells.

HeLa cells were incubated with isolates of *L. iners, L. crispatus or L. jensenii* at 10^8 bacteria for 20 h (Table 2). None of the isolates of *L. iners* (119 and 030) or *L.jensenii* (151) altered the constitutive secretion levels of SLPI in the cells. In contrast, *L. crispatus* (117) and *L. jensenii* (003) significantly reduced the SLPI levels. *L. crispatus* (117) reduced the SLPI secretion as much as 54 %, compared to the constitutive level, and by 49 %, compared to the levels obtained by *L.iners* (119) (p=0.0005 and p=0.003, respectively). *L.jensenii* (003) also significantly reduced the SLPI secretion, compared to the constitutive level (p = 0,0143).

L. iners was also compared with *E .coli* in their effects on SLPI secretion in HeLa cells (fig.10). There were no differences observed in the SLPI levels in supernatants from cells stimulated with either *L.iners* or *E.coli* at 10^6 and 10^7 bacteria per ml compared to the constitutive levels in the cells (fig.10 a). A significant decrease in SLPI levels was, however, observed upon incubation with 10^8 and 10^9 *E. coli* per ml, and with 10^9 *L. iners* per ml (fig.1a). Thus, a tenfold higher concentration of *L. iners* than *E. coli* was required to inhibit the secretion of SLPI to the same extent.

The kinetics of SLPI secretion in HeLa cells incubated for different time periods with *L. iners* (10^7 or 10^9 bacteria per ml) or with *E.coli* (10^9 bacteria per ml) revealed a significant decrease in SLPI levels with 10^9 bacteria per ml at 6 h (*L. iners*, p= 0.0007; *E. coli*, p=0.0056) and at 20 h (*L. iners* p > 0.0001; *E. coli* p > 0.0001), compared to constitutive levels (fig. 10 b).

The expression of SLPI mRNA in HeLa cells stimulated with *L. iners* or *E. coli* did not differ, compared to constitutive SLPI mRNA expression at 1 or 2 h of incubation (fig.11). However, at 20 h, the mRNA expression was significantly higher than the constitutive levels in cells incubated with *L. iners* or *E. coli* at 10^9 bacteria per ml (p= 0.0191 and p=0.0133, respectively). Additionally, there was a significant difference in the mRNA expression of cells incubated with 10^7 or 10^9 bacteria per ml at this time point. A negative correlation (r = - 0.9805, p = 0.0195) was established at 20 h between SLPI mRNA and secreted SLPI protein levels.

Cytokine-induction in THP-1- and HeLa cells stimulated with *L.iners, L.crispatus, L.jensenii* and *L.gasseri* (I, II)

As cytokines are a part of the local immune defence at mucosal surfaces we also investigated the ability of the chosen vaginal lactobacilli to evoke a cytokine response in THP-1 (II) and HeLa cells (III).

THP-1 cells (II)

THP-1 cells were incubated with isolates of *L.iners, L.crispatus, L.jensenii* and *L.gasseri*, at different doses, for 20 h.

All isolates of the four *Lactobacillus* species were capable to induce a cytokine response in THP-1 cells, although the levels varied among the species. At concentrations of 10^7 and 10^8 bacteria per ml the isolates of *L.crispatus* induced the highest secretion of TNF-α and IL-8, whereas isolates of *L. iners* induced the highest levels of TNF-α and IL-6 at 10^9 bacteria per ml (fig. 12 a, b and c).

Significant differences between *L. crispatus* and *L. iners, L. gasseri* or *L. jensenii* were obtained for the induction of TNF-α and IL-8, at 10^8 bacteria per ml, and between *L. gasseri* and *L. crispatus*, for IL-6, at the same bacterial concentration. At 10^9 bacteria per ml, significant differences were observed between *L.iners* and *L.crispatus* for TNF-α and between *L.iners* and

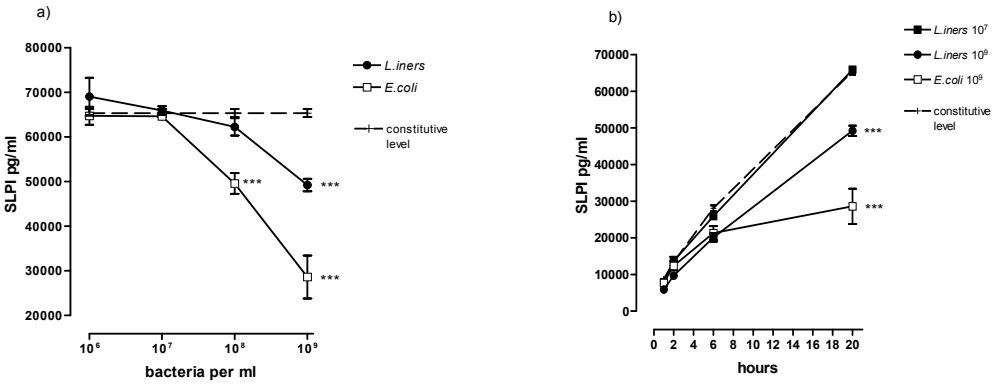

Fig.10. SLPI secretion in HeLa cell supernatants stimulated with *L.iners* or *E.coli*. a) SLPI levels in cell supernatants analysed by ELISA after 20 h of incubation with different doses of *L.iners* or *E.coli.* b) Kinetics of SLPI levels in cell supernatants analysed by ELISA. The cells were incubated with *L.iners* at 10^7 or 10^9 bacteria per ml or *E.coli* at 10^9 bacteria per ml for different time points. The bars in each point represent SEM of triplicates of three independent experiments. ***$P < 0.0001$

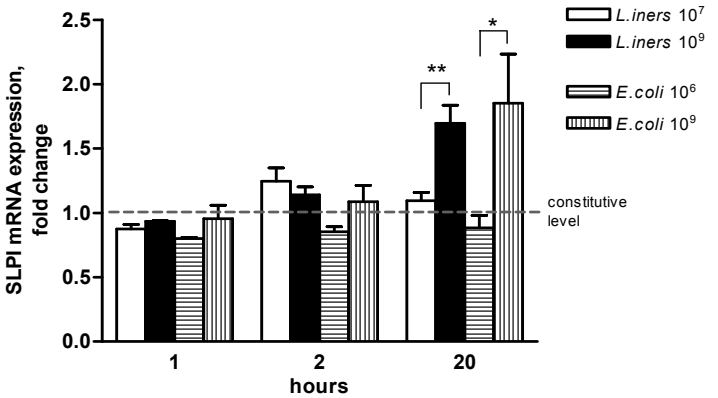

Fig.11. SLPI mRNA expression in HeLa cells incubated with different doses of *L.iners* or *E.coli* for 1, 2 or 20 h. The mRNA levels were analysed by PCR and expressed as fold change over constitutive level. The bars in each point represent SEM of triplicates of two independent experiments.
** $P = 0{,}0043$, * $P = 0{,}0288$

L. crispatus or *L. gasseri* for IL-6. Significant differences were also obtained between *L. jensenii* and *L. gasseri* or *L. crispatus* for the IL-18 at 10^9 bacteria per ml (fig. 12 a-d).

HeLa cells (III)

HeLa cells were incubated with isolates of *L. iners, L. crispatus,* and *L. jensenii* at 10^8 bacteria per ml, for 20 h. All the different lactobacilli strains induced in general very weak IL-6 and IL-8 responses (0.07 – 0.7 ng per ml) in the HeLa cells stimulated with 10^8 bacteria per ml (Table 2).

Since HeLa cells are known to secrete no or very low levels of cytokines in response to LPS we compared the cytokine response induced by *L.iners* to that of *E.coli*, which has previously been shown to evoke a cytokine response in HeLa cells (fig. 13)

In comparison with *E. coli,* all the tested concentrations of *L. iners* induced very low levels of IL-6 and IL-8 in HeLa cells (fig. 13 a). There was approximately a 10 – fold difference between *E. coli* and *L. iners* in the induction of IL-6 in cells stimulated with 10^7 to 10^9 bacteria per ml, and *E. coli* also induced a very strong IL-8 response at 10^8 to 10^9 bacteria per ml. The differences between the two bacterial species were however less in the induction of IL-6 than it was for IL-8 (fig, 13 b).

SLPI and cytokine responses in THP-1 cells (II) or HeLa cells (III) co-stimulated with *Lactobacillus* species and *E. coli*

Since LTA from lactobacilli of gut origin previously has been shown to down-regulate a TNF-α response induced by LPS in THP-1 cells, we wanted to investigate whether the presence of lactobacilli could influence SLPI and cytokine responses induced by *E. coli* in our cells. Lactobacilli were added prior to *E. coli* to THP-1 cells or HeLa cells. The cells were subsequently incubated with a total concentration of 10^8 bacteria per ml for 20 hours.

THP-1 cells (II)

SLPI secretion and mRNA expression

The SLPI levels decreased significantly for all combinations of lactobacilli and *E. coli* compared to the levels induced by the lactobacilli alone except for *E. coli* in combination with *L. crispatus,* wherein the SLPI levels were unaffected (fig.14a). The combination of *L. jensenii* 003 or *L. iners* 119 with *E. coli* also gave rise to SLPI levels below that induced by *E. coli* alone. While *L. iners* induced an up-regulation of the SLPI mRNA expression above the constitutive level at 1 h of incubation (fig.14 b), no such up-regulation was seen for the combination of *L .iners* 119 and *E. coli* at the same time point. At 2 h the SLPI mRNA expression in the cells did not differ from the level induced by the stimulation of *L. iners* alone or in combination with *E. coli*. At 20 h of incubation however, the mRNA expression was reduced compared to the constitutive expression for both *L. iners* 119 alone and in combination with *E. coli*.

Cytokines

In general, the co-stimulation of lactobacilli with *E.coli* induced stronger TNF-α, IL-8, IL-6 IL-18, and IL-1α (not shown) responses in THP-1 cells than those induced by each bacteria alone (fig.15). Synergy effects, i.e., significantly higher levels than the additive levels derived from each of the bacterial strains, were obtained for TNF- α by the combination of *E.coli* with either *L. jensenii* 151 ($p=0.0011$), *L. iners* 030 ($p=0.0004$) or *L. iners* 119 ($p<0.0001$) (fig.15 a). Positive synergy effects were also obtained for IL-8 responses by *E. coli* in combination with either *L. jensenii* 003 ($p=0.0157$), *L. jensenii* 151 ($p=0.0046$) or *L. iners* 119 ($p=0.0358$) (fig.15 b). The induction of IL-1α showed a similar pattern as for TNF-α and IL-8, with positive synergy effects by the

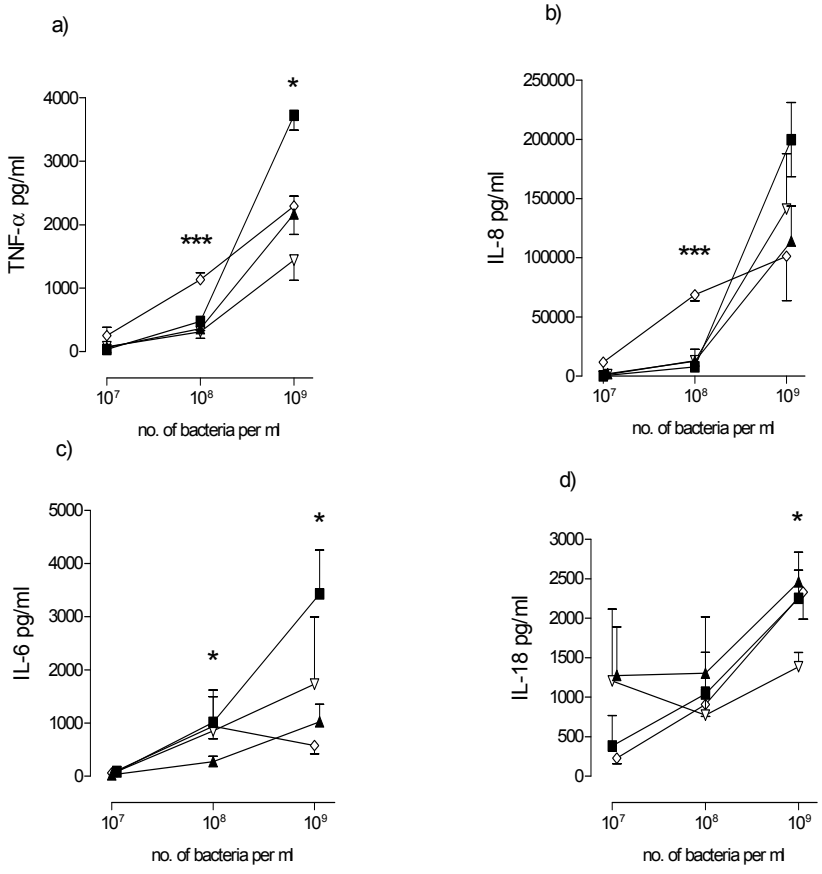

Fig. 12. Cytokine responses in THP-1 cells stimulated with isolates of *L.iners* (■, n=4), *L.gasseri* (▲, n=5), *L.jensenii* (∇, n=5), *L.crispatus* (◊, n=4) at bacterial concentrations of 10^7, 10^8 and 10^9 per ml after 20 hours of incubation. The a) TNF-α, b) IL-8, c) IL-6 and d) IL-18 levels in THP-1 cell supernatants were measured by ELISA. The bars in each point represent SEM.

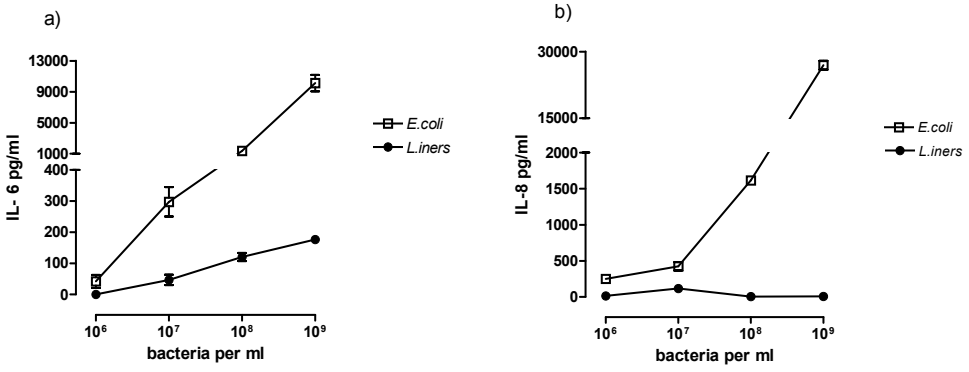

Fig.13. Cytokine secretion in HeLa cells stimulated with *L.iners* or *E.coli* at different concentrations for 20 h. a) IL-6 - and b) IL-8 levels in supernatants analysed by ELISA. The bars in each point represent SEM of triplicates of one representative experiment.

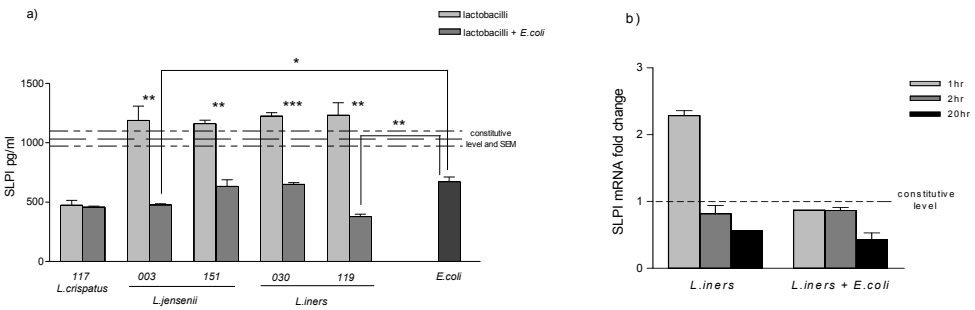

Fig. 14. SLPI secretion and mRNA expression in THP-1 cells. a) The SLPI secretion in cell supernatants was analysed by ELISA at 20 h of incubation with isolates of *L.crispatus, L.jensenii,* or *L.iners* alone or in combination with *E.coli* at a total bacterial concentration of 10^8 per ml. b) SLPI mRNA expression in THP-1 cells stimulated with *L.iners* 119 or *E.coli* alone or *L.iners* 119 and *E.coli* in combination for 1, 2 and 20 h at a total bacterial concentration of 10^8 per ml. Results are presented as fold change over constitutive expression. The bars represent SEM.

obtained by the combination of *E.coli* with *L. crispatus* 117 in the IL-8 response and for *E. coli* together with all lactobacilli except *L. jensenii* 151 in the IL-6 response with either *L. jensenii* 003 (p=0.0182), *L. iners* 030 (p=0.0499) or *L. iners* 119 (p=0.0301) (fig.15 d).

Correlations
A negative correlation was obtained between SLPI and the TNF-α, IL-1α, IL-6, IL-8, or IL-18 levels (Table 3).

HeLa cells (III)
SLPI secretion
The presence of lactobacilli in *E. coli* stimulated cells did not alter the reduction in SLPI secretion compared to that induced by *E. coli* alone, except for *L. crispatus* (Table 2). *L. crispatus* gave rise to lower levels of SLPI than that induced by *E. coli* alone ($p = 0.0007$), and the SLPI secretion was further reduced, compared to constitutive levels by the combination *L. crispatus* and *E. coli* ($p < 0.0001$).

Cytokines
Although the IL-6 and IL-8 responses induced by the different *Lactobacillus* strains were weak in comparison to that induced by *E. coli* (Table 2), the presence of either *L. iners* 030, *L. iners* 119 or *L .jensenii* 151, in combination with *E. coli*, gave rise to significant synergy effects in the induction of IL-6, with an increase of 28 – 36 % ($p < 0.0001$, $p = 0.0116$ and $p = 0.0165$ respectively). The combination of either of the *L. iners* strains with *E. coli* induced approximately three – fold higher levels of IL-8 ($p < 0.0001$) than *E. coli* did alone. The combinations of *L. crispatus*, *L. jensenii* 003 or *L. jensenii* 151 with *E. coli* roughly induced IL-8 1.5–2 times more than that induced by *E. coli* alone ($p = 0.0023$, $p = 0.001$ and $p = 0.0388$ respectively).

Correlations
Negative correlations were obtained between the levels of SLPI and IL-6 ($r = -0,9472$ and $p = 0,0145$) or IL-8 ($r = 0,9862$ and $p = 0.0019$) in HeLa cells incubated with the various lactobacilli.

Effects of exogenous administrated SLPI on the cytokine secretion in HeLa cells (III)
Since we had found a negative correlation between SLPI and the IL-6 or IL-8 levels induced by the *Lactobacillus* species, we proceeded to investigate if added recombinant SLPI (rhSLPI) could alter the secretion of these cytokines in HeLa cells.
rhSLPI was added to HeLa cells prior to the incubation with *E. coli.* The IL-6 and IL-8 protein secretion was subsequently measured in cell supernatants by ELISA and IL-8 mRNA expression by RT-PCR (fig 16).
Both the IL-6 and IL-8 responses induced by *E.coli* were significantly down-regulated at 20 h in cells pre-treated with rhSLPI (fig.16 a). Further analysis of the IL-8 mRNA expression showed that the reduction in the cytokine protein levels was reflected in the corresponding mRNA expression (fig.16 b).

Expression of Toll-like receptors (TLRs), MD-2 and CD14 on HeLa cells (III).
The mRNA expression of TLR-2, -4, -5, MD2 and CD14 in un-stimulated HeLa cells was analysed by PCR, in order to evaluate the capacity of HeLa cells to respond to the MAMPs of lactobacilli and *E. coli*. There was no detection of TLR-2 or MD-2 expression in the cells, and a low expression of TLR-4, TLR-5 and CD-14 (11 %, 19% and 2 % respectively of positive control).

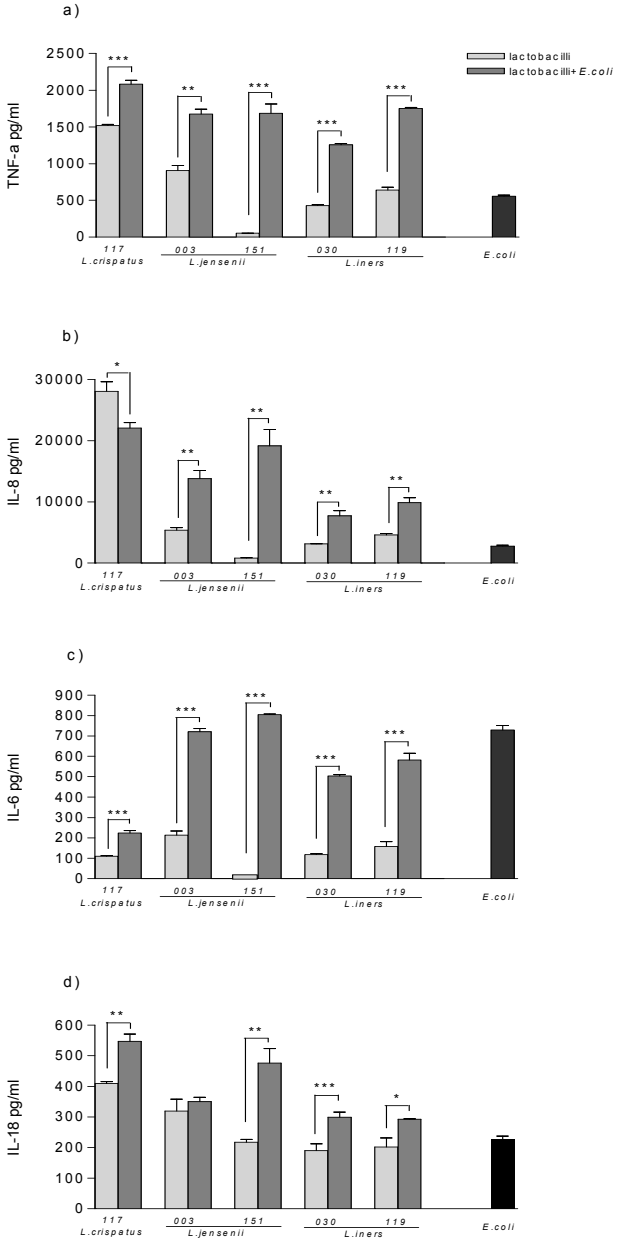

Fig. 15. The cytokine levels of a) TNF-α, b) IL-8, c) IL-6 and d) IL-18 in THP-1 cells stimulated for 20 h with isolates of *L.crispatus*, *L.jensenii* or *L.iners* alone or in combination with *E.coli* at a total bacterial concentration of 10^8 per ml. The cytokine levels in THP-1 cell supernatants were analysed by ELISA. The bars represent SEM.

Table 3

Correlation between SLPI and cytokine levels in THP-1 cells supernatants stimulated with *L.iners, L.jensenii. L.gasseri, L.crispatus* or *E.coli* alone or each of the *Lactobacillus* isolates in combination with *E.coli*.

	Cytokine				
	TNF-α	IL-8	IL-1α	IL-6	IL-18
Corr.coeff.*	r = -0,8456	r = -0,7163	r = -0,6079	r = -0,5777	r = -0,5944
P-value	p = 0,0005***	p = 0,0088**	p = 0,036*	p = 0,0492*	p = 0,0415*

* Spearman Rank Correlation.

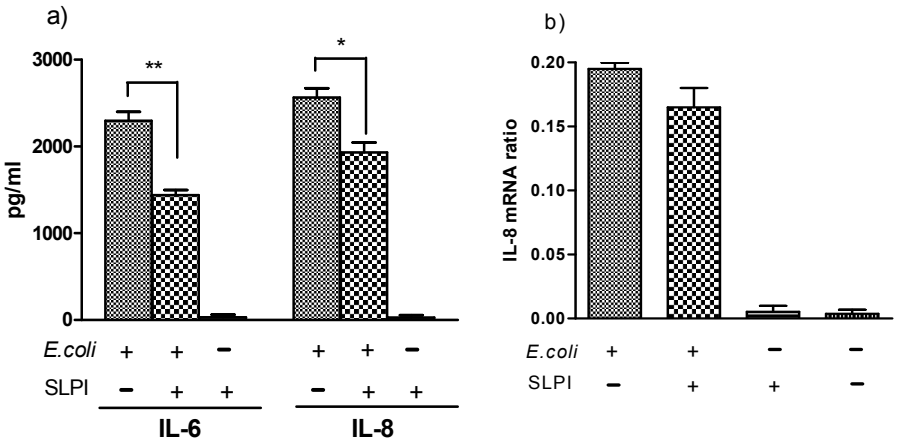

Fig. 16. Effects of added rhSLPI on IL-6 and IL-8 responses in HeLa cells stimulated with *E.coli.* a) IL-6 and IL-8 secretion in HeLa cells incubated for 20 h with *E.coli* at 10^8 bacteria per ml with or without rhSLPI 10 µg per ml and analysed by ELISA. b) IL-8 mRNA expression in HeLa cells incubated for 20 h with *E.coli* at 10^8 bacteria per ml with or without rhSLPI added at a concentration of 10 µg per ml. The IL-8 mRNA expression level in HeLa cells are compared to control. The bars in each point represent SEM of triplicates

Absorbtion of SLPI

To exclude the possibility that SLPI was absorbed to bacterial surfaces or degraded by bacterial proteases and, thereby, reducing the SLPI available for measurement by ELISA, *L.iners* 119 or *E.coli* was incubated with rhSLPI. No significant differences of the SLPI levels, as compared to the control, were obtained when *L. iners* 119 or *E. coli* at 10^9 bacteria per ml, were incubated with rhSLPI at concentrations of 20 or 4 ng per ml for 20 hours (fig. 17).

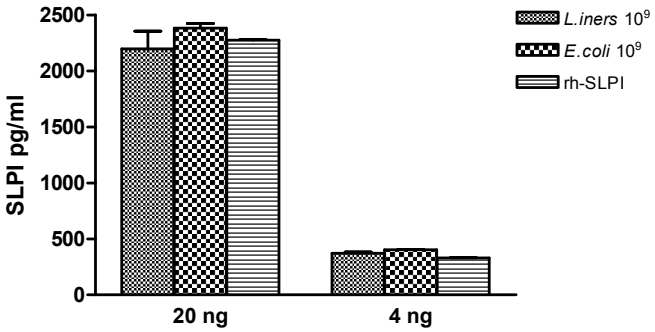

Fig. 17. Absorption experiment, simulating the cell assay procedure. *L.iners* or *E.coli* at 10^9 bacteria per ml was incubated with rhSLPI at 4 or 20 ng per ml in the absence of HeLa cells and incubated over night. The supernatants were analysed by ELISA. The bars in each point represent SEM of triplicates.

6. DISCUSSION

In these studies we have explored some commensal bacteria isolated from the lower FGT for their interaction with host cells regarding their regulation and induction of innate immune factors and an opportunistic species (*P. bivia*) for virulence properties.

In the first paper (I) we investigated possible pathogenic characteristics of *P.bivia,* a gram negative obligate anaerobic rod, frequently isolated in and associated with BV, PID, intra-amniotic infection and PTB [44, 45].

BV is a disturbance in the normal vaginal microbiota defined by a loss of lactobacilli and overgrowth of anaerobic bacteria, increasing the risk for uterine infections [30, 160, 161]. However, the mechanisms by which opportunistic bacteria such as *P.bivia* may ascend to the upper genital tract and cause infection are less well studied. Epithelial cell invasion is a strategy for the establishment of infection used by several bacterial pathogens such as *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, and enteropathogenic species of *Salmonella, Shigella, Campylobacter, Yersinia* and *E.coli* [59, 103, 104, 162-165]. Also, anaerobic Gram negative bacteria such as *Prevotella intermedia*, *Porphyromonas gingivalis*, and *Actino-bacillus actinomycetemcomitans* have been shown to invade human epithelial cells, and it has been suggested as a pathogenic

mechanism for a chronic infection with a slowly progressing inflammation and destruction of tissue [105-108].

In paper (I), five strains of *P.bivia* were tested for adherence, invasion and cytokine stimulation in human cervix epithelial cells (HeLa). The results demonstrated that *P.bivia* is capable to adhere to and invade HeLa cells *in vitro*. However, the invasion capacity varied strongly among the five isolates of *P.bivia*, where the number of intracellular bacteria differed more than 100-fold (fig.1). This variation appeared not to be due to the adherence of bacteria to the epithelial cells, since all strains adhered to approximately the same extent. Rather, it indicated that some strains may be endowed with factors that promote internalization. To our knowledge, nothing is known about the expression of fimbriae or any other outer membrane factors of *P.bivia* that could mediate an attachment to the host cell surface and induce a subsequent internalization. Different types of fimbriae have previously been identified on the periodontal pathogen *Prevotella intermedia* as examined by electron microscopy (EM) [157]. However, in our study, no visible evidence of fimbriae could be detected on the surface of the most invasive *P.bivia* strain (P47) by EM. Despite that we could not identify any adhesion structures on P47, the number of intracellular P47 per HeLa cell was close to that previously reported for the piliated *N. gonorrhoeae* [61], and the invasion efficiency of P47 (0.2 %) was in the same range of that reported for *P.intermedia*, *N. gonorrhoeae* or group B streptococci (~ 0.2 %- 0.4%) in epithelial cells [61, 105, 166].

Both invasive *C. trachomatis* and *N. gonorrhoeae* have been reported to induce a release of IL-1α, IL-6 and IL-8 in cervical cells, but only a piliated *N. gonorrhoeae* induced IL-1α while neither the presence of pili nor invasiveness of *N. gonorrhoeae* was shown to be essential for the IL-6- or IL-8 responses [61, 63]. In our study, internalization of *P. bivia* but not the adherence capacity appeared to correlate to the secretion of IL-6 and IL-8, since none of the *P. bivia* strains except P47 was able to elicit detectable cytokine levels in the cells. In comparison with *E.coli*, however, the cytokine response to P47 was quite weak.

Increased levels of IL-1 and IL-8, in the lower genital tract have been associated with BV, whereas no significant difference in the IL-6 levels in cervico-vaginal fluids have been observed between healthy women and women with BV [125, 127, 128, 167]. The weak induction of IL-6 induced by *P.bivia* in HeLa cells in our study support the lack of a significant IL-6 response clinically observed in BV [125, 127, 128, 168, 169].

In summary, we demonstrated in this study that the opportunistic bacterium, *P.bivia*, can invade human cervix epithelial cells *in vitro* and that this capacity varies widely between different isolates of *P.bivia*. Furthermore, the results suggest that a strong intracellular invasion capacity could be a virulence factor for an ascending route of infection of the upper FGT by *P.bivia*. An intracellular localization may be a defence mechanism by which *P.bivia* could escape the hostile milieu that the cervical canal represents [170]. Hypothetically, the capacity to invade epithelial cells could also be a mechanism for *P.bivia* to enter into the amniotic cavity of pregnant women, giving rise to a subclinical infection. The very weak pro-inflammatory cytokine response induced by the bacterium in cervix epithelial cells may represent another important property of *P.bivia* that may facilitate its spread and persistence in other areas.

In paper (II) and (III) our aim was to explore the role of vaginal lactobacilli as possible regulators of innate immune factors such as SLPI and cytokines in cervix epithelial cells (III) and in monocytes (II).

Since SLPI is present in high concentrations in cervico-vaginal secretion

in women colonized by commensal lactobacilli but decreased in BV and STDs, together with our findings that *L.iners* correlated with levels of SLPI [91, 171], we reasoned that lactobacilli may influence the regulation of constitutive and inducible innate immune factors such as SLPI and pro-inflammatory cytokines in host cells.

L. iners, L. crispatus, *L. gasseri*, and *L. jensenii,* which have been reported in several studies as the four most pre-dominant vaginal *Lactobacillus* species in the lower genital tract of normal healthy women of fertile age [47, 85, 95] [171] were analysed for their abilities to regulate SLPI and cytokine responses in THP-1(II) and HeLa cells (III).

L.iners was the only one of the four tested lactobacilli spp. that gave rise to an up-regulation of the SLPI secretion in THP-1 cells. This effect was not observed in HeLa cells incubated at the same concentration of *L.iners.* The SLPI secretion in HeLa cells remained at a constitutive level, which was approximately 50 times higher than the constitutive level in THP-1 cells. However, high concentrations of *L.iners* induced a down regulation of the SLPI levels in both cell types.

A large difference was observed between *L.iners* and *L.crispatus* in their induction of SLPI secretion irrespective of the bacterial concentration tested. While *L.iners* did not affect or up-regulate the SLPI secretion, all concentrations of *L.crispatus* gave rise to a considerable down-regulation of SLPI in both cells. Compared with *L.crispatus* and *E.coli*, a tenfold higher concentration of *L.iners* was needed to down-regulate the constitutive SLPI secretion in both HeLa and THP-1 cells.

In the host cells there are both extracellular and intracellular pattern recognition receptors (PRRs) such as Toll like receptors (TLRs) and NOD-like receptors (NLR). PRRs may be expressed differently in HeLa cells and THP-1 cells, which may be one explanation to the differences in SLPI regulation observed in the cells.

In addition, the difference in SLPI secretion in response to different bacterial species, or strains thereof, may reflect differences in the exposure of various conserved microbial associated molecular patterns (MAMPs) such as peptidoglycan, LTA and LPS by the bacteria. Various expression patterns of these molecular structures may lead to differently modulated host responses depending on which receptor or combination of receptors the bacteria bind to [172]. Furthermore, it is likely that more than one type of PRR is activated by the bacteria. This implies that microbes individually may tailor collaboration between different PRRs and hence the signalling response in the host cell [173]. The activation of intracellular signalling pathways by the bacteria may subsequently directly or indirectly affect the regulation of SLPI.

The negative correlation obtained between the levels of cytokines and SLPI in both HeLa and THP-1 cells stimulated with lactobacilli or *E.coli* alone or in combination suggests that cytokines may take part in a regulation of SLPI. The inverse correlation may reflect an "inflammatory state" in the cells, in which SLPI is directly or indirectly down-regulated in response to high levels of microbes and/or by the cytokines which they may induce. This inverse relayion between SLPI and cytokines in our study are in line with the observations *in vivo* where the SLPI levels are reported to be decreased and the cytokine levels elevated in vaginal fluids in women with STDs and/or BV [91, 125, 147].

In addition, exogenous administration of cytokines have been reported to influence the expression of SLPI in murine macrophages where SLPI mRNA was up-regulated by IL-6 and IL-10 and down-regulated by IFN-γ [139, 161]. Since a high concentration of bacteria induced quite strong cytokine responses including IL-6, it could be an explanation for the up-regulated mRNA levels observed in both THP-1 and HeLa cells in our study.

An inverse relationship between SLPI and cytokines has been reported by several studies. Exogenously added rhSLPI was shown to down-regulate the TNF-α secretion in LPS activated human monocytes/ macrophages [174], and reduced an inflammatory reaction in response to the deposition of IgG immune complexes in an acute lung injury model [175]. Based on several studies the evidence indicates that SLPI down regulates the activation of NF-kB [140, 174-178]. In our study of the anti-inflammatory effect of SLPI in HeLa cells, there was a significant down-regulation of both the IL-6 and IL-8 responses by the addition of rhSLPI to the cells prior to the exposure to *E.coli.* Thus, cervix epithelial cells behave similar to innate immune cells.

There was no obvious correlation between the SLPI protein secretion and mRNA expression in THP-1 cells stimulated with *L.iners*. In HeLa cells, however, there was an inverse correlation between SLPI protein and mRNA levels. For example, at 1 h of incubation, all tested concentrations of *L.iners* gave rise to an up-regulation of the mRNA expression above constitutive levels in THP-1 cells followed by a subsequent decrease at 2h and 20 h to a constitutive level or below. The up-regulation of mRNA in THP-1 cells at 1 h may reflect the up-regulation of SLPI protein secretion seen at 20 h when stimulating the cells with *L.iners* at 10^7 or 10^8 bacteria per ml. At the same time points however, there was an inverse correlation between the SLPI mRNA and protein levels by the incubation of *L.iners* at 10^9 per ml. A similar discrepancy between mRNA and SLPI protein levels were obtained in the HeLa cells, where the reduced SLPI secretion observed at 20 h with 10^9 *L.iners* or *E.coli* 10^8 bacteria per ml were not reflected in the mRNA expression (fig 11). Both cell types seemed to have the ability to up-regulate SLPI mRNA in response to *L.iners,* albeit at different time points and at different concentrations of *L.iners*.

The contradicting results of high SLPI mRNA levels but reduced protein levels could not be explained by absorption of secreted SLPI to the bacteria, since incubation by the same concentration of bacteria with rhSLPI did not result in reduced SLPI levels. In this experiment, a degradation of SLPI by a direct role of secreted proteases from the various bacterial species could also be excluded. Furthermore, the binding of SLPI to proteases secreted by the cells could also be ruled out since the analysis of SLPI levels in cell supernatants by our immunoassay gave identical results when compared to results obtained by a SLPI kit tested for cross reactivity and interference with elastase, trypsin or chymotrypsin.

The SLPI secretion may be regulated by an intracellular post-translational degradation mechanism similar to that of *Helicobacter pylori* infected human gastric tumour cell lines in which an inverse relationship between the SLPI protein levels and its corresponding mRNA expression has been shown [179]. In their study the incubation with *H.pylori* resulted in decreased or unchanged SLPI protein levels but with a corresponding up-regulation of the SLPI mRNA levels. However, as the intracellular SLPI levels did correlate with secreted SLPI levels an intracellular post-translational mechanism of the regulation of SLPI secretion was suggested.

All tested combinations of lactobacilli together with *E.coli* gave rise to synergy effects in the induction of cytokines in both THP-1 and HeLa cells. Positive synergy effects were obtained for the IL-8 and TNF-α production in THP-1 cells and for IL-6 and IL-8 in HeLa cells. Negative synergy effects were obtained for IL-6 and IL-18 in THP-1 cells (fig. 15 and table 2). Overall, the vaginal lactobacilli enhanced the IL-6 and IL-8 responses in HeLa cells, and the IL-8 and TNF-α response in the THP-1 cells. A possible explanation to the positive synergy effects in the cytokine

induction may be a simultaneous activation of different PRRs such as TLRs and/or NLRs. In several studies, positive synergy effects in cytokine responses have been demonstrated by simultaneous activation of different TLRs as well as the activation of a combination of TLRs and NLRs by various bacterial cell-wall factors [180-183]. The negative synergy effect seen in the IL-6 and IL-18 responses is not easily explained. There is one report on the down-regulation of the transcription of a number of pro-inflammatory mediators by the intestinal *Lactobacillus*, *L.casei,* in response to *Shigella flexneri* in cultured intestinal epithelial cells [184]. However in this study the expression of all cytokines and chemokines were down-regulated.

In conclusion, our results indicate that vaginal *Lactobacillus* species may contribute to a regulation of the host innate immune defence by the modulation of SLPI and cytokine responses. *L.iners* appeared to be the only species that consistently enhanced or did not change constitutive levels of SLPI whereas the opposite was true for *L.crispatus*.
SLPI was down-regulated in response to the combination of lactobacilli and *E.coli* or by the exposure to high doses of either bacteria alone. However, much higher concentrations of *L.iners* than *E.coli* were required to initiate a decrease of the SLPI secretion in both cell types.
It is possible that the down-regulation of SLPI secretion seen in THP-1 and HeLa cells is a general phenomenon in non-sterile body areas in response to interaction with pathogens or at a threshold concentration of any bacteria,. also commensal lactobacilli. Our results indicate that the triggering factor for the downregulation of SLPI secretion may be associated with the induction of a proinflammatory response.

The presence of high SLPI levels *in vivo* may indicate a genital tract mucosa homeostasis including a balanced colonization of certain commensal bacteria such as *L.iners.*

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