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Therapeutic potential of vaginal lactobacilli for women's health: Interaction with pathogens and epithelium cells.

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ABSTRACT

The vaginal microbiota of healthy reproductive-age women is dominated by *Lactobacillus* spp., which protect against numerous uropathogens. This study aims to identify lactobacilli with antagonist activity toward *Candida*, *Chlamydia*, and HIV that mostly affect women's health.

From vaginas of healthy women, we isolated seventeen *Lactobacillus* strains, highly represented in the vaginal microbiota: *L. crispatus* (BC1-BC8), *L. gasseri* (BC9-BC14), and *L. vaginalis* (BC15-BC17).

The broadest anti-*Candida* activity was observed for *L. crispatus* (BC1, BC4, BC5) and *L. vaginalis* BC15. Most of lactobacilli reduced *Candida* adhesion to HeLa cells by multiple mechanism including exclusion, competition, and displacement. Histone deacetylases inhibition was hypothesized to support the antifungal activity of *Lactobacillus*.

Next, mainly *L. crispatus* strains inhibited *Chlamydia* by secreting metabolites in a concentration/pH dependent mode at short contact times. Lactate production, vaginal acidification, and glucose consumption seemed to be crucial for the anti-*Chlamydia* activity. The metabolic profiles of *Lactobacillus*-conditioned medium (CM) also correlated with the anti-*Chlamydia*/*Candida* activity.

Finally, lactobacilli inhibited HIV-1 replication in human tissues *ex vivo* by multiple mechanisms: Acidification. The pH of *Lactobacillus*-CM was ≤ 4.6. Tissue culture acidification with HCl to this pH abrogated HIV-1 replication. However, *Lactobacillus*-CM, diluted 5-fold (neutral pH), also suppressed HIV-1 infection, as opposed to HCl-treated medium at the same pH, suggesting the existence of other anti-HIV factors. Lactate. Addition of lactate isomers D and L to tissue culture, at the average titers found in all *Lactobacillus*-CM, inhibited HIV-1 replication. Isomer L was produced in higher quantities and was mostly responsible for HIV-1 inhibition. Virucidal effect. Incubation of HIV-1 in *Lactobacillus*-CM suppressed virus infectivity. Lactobacilli cells adsorbed HIV-1, decreasing the number of virions.

This results support role of lactobacilli in protecting the female genital tract from uropathogens, and are prerequisites for the development of new probiotic agents as an effective strategy to enhance vaginal health.

RIASSUNTO

Il microbiota vaginale delle donne sane in età riproduttiva è dominato da specie di *Lactobacillus*, che proteggono contro numerosi patogeni urogenitali. Il presente studio ha come scopo l'identificazione di ceppi di lattobacilli con attività antagonistica verso agenti patogeni che maggiormente incidono sulla salute delle donne, in particolare *Candida*, *Chlamydia* e HIV. Da tamponi vaginali di donne sane abbiamo isolato diciassette ceppi di *Lactobacillus* altamente rappresentate nel microbiota vaginale: *L. crispatus* (BC1-BC8), *L. gasseri* (BC9-BC14) e *L. vaginalis* (BC15-BC17).

Lo spettro anti-*Candida* più ampio è stato osservato per *L. crispatus* (BC1, BC4, BC5) e *L. vaginalis* BC15. La maggior parte dei *Lactobacillus* si sono dimostrati efficaci nell'inibire l'adesione di *C. albicans* sulle cellule epiteliali attraverso meccanismi di esclusione, competizione e spiazzamento del patogeno. L'inibizione dell'enzima istone deacetilasi è stata anche ipotizzata come meccanismo alla base dell'attività antifungina osservata.

Posteriormente, abbiamo osservato che la maggior parte dei ceppi di *L. crispatus,* inibiscono efficacemente anche l'infettività di *C. trachomatis,* principalmente attraverso i metaboliti secreti nei surnatanti dei lattobacilli in maniera concentrazione/pH dipendente e nei brevi periodi di contatto tra lattobacilli e patogeno. La produzione di lattato da parte dei lattobacilli e la conseguente acidificazione dell'ambiente vaginale sembrano essere cruciali per la loro attività anti-*Chlamydia*, insieme al consumo di glucosio. I profili metabolici dei surnatanti dei lattobacilli sono stati correlati con la loro attività anti-*Chlamydia*/*Candida*.

Infine, abbiamo dimostrato che lattobacilli vaginali inibiscono la replicazione di HIV-1 in tessuti umani *ex vivo*: Riduzione del pH. L'acidificazione del terreno di coltura tissutale con HCl a valori di pH pari a quelli misurati nel surnatante delle culture di lattobacilli ($\leq$ 4.6) si è dimostrato sufficiente per abrogare la replicazione di HIV-1. Tuttavia, il surnatante dei lattobacilli, diluito per neutralizzarne il pH, è risultato mantenere l'attività anti-HIV-1, a differenza del terreno di cultura tissutale acidificato con HCl allo stesso valore di pH. Ciò suggerisce l'esistenza di altri fattori responsabili dell'inibizione di HIV-1. Produzione di acido lattico. L'aggiunta degli isomeri dell'acido lattico, D e L, al terreno di coltura tissutale alla concentrazione corrispondente alla quantità rilasciata nei mezzi di coltura dei lattobacilli, è risultato nell'inibizione di HIV-1, in particolare si è sottolineato il ruolo dell'isomero L, che viene prodotto in quantità maggiori rispetto all'isomero D. Effetto virucida. L'incubazione di HIV-1 con il surnatante dei lactobacilli si è dimostrato inibire significativamente l'infettività del virus. In più i lattobacilli stessi sono in grado di assorbire le particelle di HIV-1 sulla loro superficie, diminuendo il numero di virioni liberi.

I nostri risultati supportano l'idea di un ruolo attivo da parte dei lattobacilli nella protezione della mucosa genitale femminile contro patogeni urogenitali, e forniscono un razionale per lo sviluppo di nuovi agenti terapeutici basati sui probiotici come strategia efficace per migliorare la salute vaginale.

INDEX

1. INTRODUCTION 01
1.1 The mucosa of the female reproductive tract as physical barrier against pathogens 01
1.2 Vaginal microbiota 02
1.2.1 Composition of the vaginal microbiota 03
1.2.2 Changes of vaginal microbiota during a women's lifespan 05
1.3 Vaginal *Lactobacillus* spp. 08
1.3.1 *Lactobacillus*-associated protective mechanisms 09
1.3.1.1 Lactic acid 09
1.3.1.1.1 d- l-Lactic acid isomers 10
1.3.1.2 Hydrogen peroxide 11
1.3.1.3 Adhesion to host cells 12
1.3.1.4 Coaggregation 13
1.3.1.5 Bacteriocins 13
1.3.1.6 Biosurfactants 14
1.4 Common gynecological infections 15
1.4.1 Bacterial vaginosis and aerobic vaginitis 15
1.4.2 Vulvovaginal candidiasis (VVC) 16
1.4.2.1 Role of vaginal *Lactobacillus* against VVC 17
1.4.3 *Chlamydia trachomatis* infection 18
1.4.3.1 Lactobacilli and *Chlamydia trachomatis* infection 20
1.4.4 Human immunodeficiency virus infection 21
1.4.4.1 HIV infection in female genital tract 23
1.4.4.2 Role of vaginal *Lactobacillus* against HIV 24
1.4.4.3 Human *ex vivo* tissues to study HIV transmission and pathogenesis 26

2. AIM OF THE RESEARCH 30

3. MATERIALS AND METHODS 32
3.1 Isolation of vaginal lactobacilli and characterization of anti-*Candida* activity 32
3.1.1 Isolation and taxonomic characterization of vaginal lactobacilli 32
3.1.2 Determination of lactobacilli hydrogen peroxide production 33
3.1.3 Preparation of lactobacilli fractions 33
3.1.4 ^{1}H-NMR analysis 33

3.1.5 Evaluation of fungistatic/fungicidal activities of lactobacilli supernatants 34
3.1.6 Adhesion assays 35
3.1.7 Histone acetylation profile analysis 36
3.1.8 Statistical analysis 37
3.2 Antagonistic effect of vaginal lactobacilli toward *Chlamydia trachomatis* infection 37
3.2.1 Preparation of *Chlamydia* elementary bodies (EBs) 37
3.2.2 *C. trachomatis* inhibition test 38
3.2.3 Correlation of metaboloma and anti-*Chlamydia* activity 39
3.2.4, Statistical analysis 39
3.3 Role of vaginal lactobacilli against HIV-1 replication in human tissues *ex vivo* 39
3.3.1 *Lactobacillus* culture conditions 39
3.3.2 *Ex vivo* tissue cultures and HIV-1 infection 40
3.3.3 *Lactobacillus* colonization on tonsillar explants and evaluation of tissue cell depletion 40
3.3.4 HIV-1 infection of human tissues *ex vivo* treated with *Lactobacillus*-CM 41
3.3.5 Virucidal effect 41
3.3.6 Lactic acid quantification, pH measurement of *Lactobacillus*-CM, and evaluation of their effect on HIV-1 replication 42
3.3.7 Evaluation of HIV-1 replication 42
3.3.8 Statistical analysis 42

4. RESULTS 43

4.1 Isolation of vaginal lactobacilli and characterization of anti-*Candida* activity 43
4.1.1 Taxonomy of vaginal lactobacilli and production of antimicrobial compounds 43
4.1.2 Fungistatic and fungicidal activities of vaginal lactobacilli 44
4.1.3 Lactobacilli metabolome correlates with taxonomy and fungistatic/fungicidal activity 47
4.1.4 Vaginal lactobacilli interfere with *C. albicans* adhesion to HeLa cells 49
4.1.5 Lactobacilli effects on *C. albicans* histone acetylation 52
4.2 Antagonistic effect of vaginal lactobacilli toward *C. trachomatis* infection 54
4.2.1 Effects of *Lactobacillus*-CM on *C. trachomatis* infectivity 54
4.2.2 Effects of *Lactobacillus*-CP on *C. trachomatis* infectivity 57
4.2.3 *C. trachomatis* inhibition by lactic acid and hydrochloric acid 59
4.2.4 *Ranking of lactobacilli in relation to anti-Chlamydia activity* 60
4.2.5 Identification of *Lactobacillus* metabolic profiles associated with anti-*Chlamydia* activity 61

4.3 Role of vaginal lactobacilli against HIV-1 replication in human tissues *ex vivo* 65
4.3.1 *Lactobacillus* colonization of tissue explants 65
4.3.2 *Lactobacillus*-CM inhibits HIV-1 replication 67
4.3.3 Effects of lactic acid and pH on HIV-1 replication 69
4.3.4 Virucidal capacity of *Lactobacillus*-CM against HIV-1 72
4.3.5 Virucidal capacity of *Lactobacillus* cells against HIV-1 73

5. DISCUSSION AND CONCLUSIONS 75

6. REFERENCES 85

1. INTRODUCTION

1.1 The mucosa of the female reproductive tract as physical barrier against pathogens

The upper female reproductive tract, which consists of the cervix, uterus, and fallopian tubes, is characterized by the presence of type I mucosa, a simple columnar epithelium formed by a single layer of ciliated columnar cells connected by tight junctions, and is generally considered to be sterile [Heinonen et al., 1985]. The mucosal epithelium in this upper habitat should thus be extremely efficient in recognizing and subsequently responding to microorganisms, and at the same time avoiding chronic inflammation. Introduction of bacteria into these tissues is typically associated with identifiable disease (endometritis or pelvic inflammatory disease). However, the results of several culture-based investigations, documenting recovery of organisms from the endometrium of healthy asymptomatic women, challenge the notion of sterility [Hemsell et al., 1989].

The lower female reproductive tract comprises the vaginal canal and the ectocervix. They are constituted by type II mucosa, characterized by multiple layers of non-keratinized-stratified squamous epithelium [Quayle, 2002]. In contrast to the upper female genital tract, cells of the squamous epithelium are not connected with tight junctions. This permits the transport of small molecules between the cells within the epithelial space, including small viruses and toxic compounds from pathogens [Hickey et al., 2011]. The mucosa of the lower genital tract is a habitat where normally numerous endogenous microorganisms coexist in dynamic equilibrium with the host.

The layer of epithelial cells of the female genital mucosa are not only a physical barrier against pathogens, they are also able to recognize conserved microbe-associated molecular patterns via the expression of pattern recognition receptors, such as toll-like receptors and NOD-like receptors, which mediate the secretion of cytokines, chemokines, and antimicrobial peptides [Schaefer et al., 2004].

The epithelial cells from type I and type II mucosa in the female genital tract are also covered by a layer of mucus, consisting of mucins, which are complex high molecular mass

O-glycoproteins [Andersch-Bjorkman et al., 2007]. The mucus in type I mucosa is produced by specialized goblet cells, similar to the ones in the gastrointestinal tract, while the mucus in type II mucosa is produced by local mucus-secreting epithelial cells [Andersch-Bjorkman et al., 2007]. An important characteristic of the cervico-vaginal mucus is the low pH, between 4 and 5, that protect the host from pathogenic bacteria, yeast, and viruses.

1.2 Vaginal microbiota

The mucosa of lower female reproductive tract is an ecological habitat where several aerobe and anaerobe microorganisms coexist in a dynamic equilibrium; these communities of microorganisms are denominated as "vaginal microbiota". The vaginal microbiota plays an important role in women's health, influencing their development, physiology, immunity, and nutrition. It constitutes the first line of defense for the host by excluding invasive nonindigenous organisms that may cause disease [Chen et al., 2015; Hickey et al., 2012]. The homeostasis of the vaginal communities results from complex interactions and synergies between the host and different microorganisms that colonize the vaginal mucosa (Figure 1) [Larsen and Monif, 2001; Sobel, 1997].

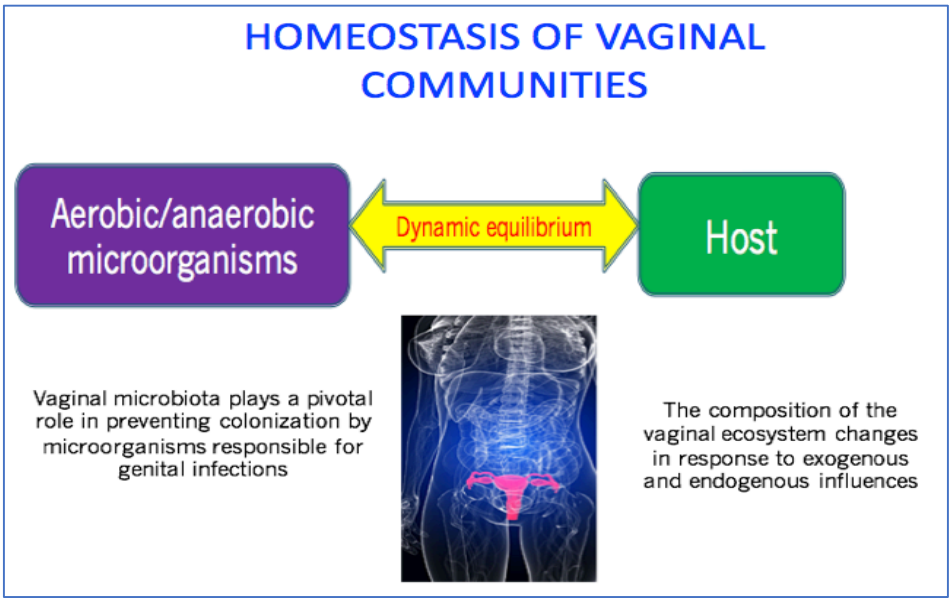

Figure 1. Homeostasis of vaginal communities

1.2.1 Composition of the vaginal microbiota

Numerous studies have been done to characterize the vaginal microbial communities of healthy, asymptomatic, reproductive age women. Although these studies were based on various analytical methodologies sampling different regions of the female genital tract, women from different ethnic groups and geographical area, all consistently demonstrated that the vaginal microbiota composition may vary within and among women. Factors influencing the normal vaginal microbiota may include age, hormone levels, menstrual cycle stage, pregnancy, genetic background, exposure to sexually transmitted agents, immune status, use of antibiotics, sexual intercourse, vaginal lubricants, douching, and possibly diet and nutritional status [Eschenbach et al., 2000; Larsen and Galask, 1982; Smith et al., 1982; Vasquez et al., 2002].

Despite marked differences in the species composition of the vaginal microbiota among women, it appears that all are probably dominated by homofermentative lactic acid bacteria. This suggests that in reproductive age women, despite differences in the vaginal microbiota, the ecological function of various bacterial communities in creating a low pH environment through the production of organic acids is conserved [Ravel et al., 2011; Zhou et al., 2007; Zhou et al., 2010].

The most detailed study published so far used deep sequencing of bacterial 16S ribosomal RNA PCR products to probe the vaginal microbiota in 396 women of childbearing age from different ethnic groups (white, black, hispanic, and asian in North America) [Ravel et al., 2011]. In this study, the microbial communities of reproductive-aged women were clustered into five groups (Figure 2): community groups I, II, III, and V were dominated by *Lactobacillus* species, *L. crispatus*, *L. gasseri*, *L. iners*, and *L. jensenii*, respectively, while community group IV contained a diverse assemblage of facultative and strictly anaerobic bacteria, sometimes associated with vaginal symptoms, including *Gardnerella vaginalis, Atopobium vaginae, Mobiluncus* spp*., Prevotella* spp. and other taxa in the order *Clostridiales;* this latter group was over represented in black and hispanic women [Ravel et al., 2011]. Comparable results were obtained in a study of healthy, reproductive age Caucasian, black, and Japanese women [Zhou et al., 2007; Zhou et al., 2010]. The findings

of these studies indicate there is a limited number of different kinds of vaginal microbial communities in asymptomatic, apparently healthy women. Moreover, from studies of 90 menarcheal adolescent women (13–18 years), it appears that these bacterial communities are established in puberty and may reside in women until menopause [Yamamoto et al., 2009].

An important finding from these studies is that the distribution of bacterial community types varies significantly among women from different ethnic backgrounds.

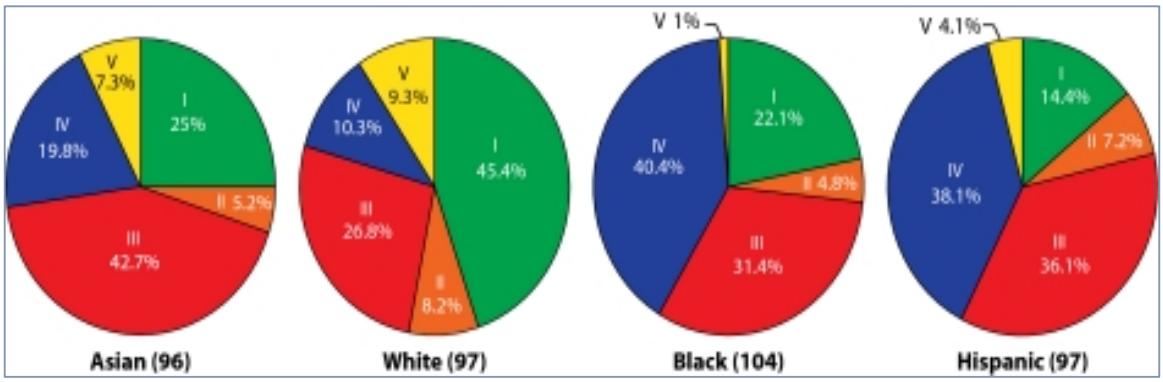

Figure 2. Representation of vaginal bacterial community groups within 4 ethnic groups of women. The number of women from each ethnic group is in parentheses. The roman numerals indicate the 5 common vaginal bacterial community groups [Ravel et al., 2011].

In addition, diversity in the vaginal microbiota in different geographic area was also observed. For example, the vaginal microbiota of Nigerian, Belgian, and Brazilian women appear to be dominated mainly by *L. iners* [Anukam et al., 2006; Martinez et al., 2008; Vitali et al., 2007], whereas in Swedish, German, and Turkish women, *L. crispatus* was the most dominant species [Kilic et al., 2001; Thies et al., 2007; Vasquez et al., 2002]. Furthermore, the vaginal microbiota of Indian and Bulgarian women is dominated by *L. reuteri, L. gasseri,* and *L. fermentum* [Dimitonova et al., 2008; Garg et al., 2009].

Most of the studies to characterize the vaginal microbiota have employed cross sectional designs at a single time point. However, a longitudinal study to characterize daily fluctuations in the composition of the vaginal microbiota has been also reported by Gajer and colleagues [Gajer et al., 2012]; they analyzed the temporal dynamics of the

composition of vaginal bacterial communities in 32 reproductive age women over a 16-week period. The study showed that some bacterial communities change markedly over short time periods transitioning between groups and most frequently to the group IV, whereas others are relatively stable. In some cases, group transitions were triggered by menstruation or sexual behaviors, but in other cases they seem to be driven by uncharacterized factors [Gajer et al., 2012]. Similar results were reported in normal and disturbed vaginal microbiota [Brotman et al., 2008; Brotman et al., 2010; Ravel et al., 2013; Srinivasan et al., 2008]. These longitudinal studies highlight the highly dynamic nature of vaginal microbial communities and emphasize the need to better understand the underlying biological factors modulating fluctuations in composition and functions that affect host physiology.

1.2.2 Changes of vaginal microbiota during a women's lifespan

The vaginal microbial communities experiences significant structural changes at various stages in a women's life and are directly linked to the level of estrogen in the body [Farage and Maibach, 2006]. Major changes in the vaginal physiology and microbiota over a women's lifetime are largely influenced by transitional periods such as puberty, menopause and pregnancy, while daily fluctuations in microbial composition are more likely to be the results of daily life activities and behaviors [Smith and Ravel, 2017].

The initial bacterial colonization occurs at birth, when the newborn is first exposed to her mother's vaginal tract if delivered vaginally, or by the skin bacteria in the case of a caesarian-section delivery [Dominguez-Bello et al., 2010]. Nevertheless, the majority of the vaginal bacteria originate from the gastro intestinal microbiota through a natural ascension independent of hygienic practices or from the surrounding skin epithelium [Dominguez-Bello et al., 2010]. During perinatal development, residual maternal estrogen induces thickening of the vaginal epithelium and the deposition of glycogen in the epithelial cells. Through the exfoliation of epithelial cells, glycogen is released, thereby favoring the colonization of glucose-fermenting microorganisms resulting in a lowering of the vaginal pH. However, this effect is transitory, since the subsequent metabolism of

maternal estrogen is accompanied by thinning of the vaginal mucosa, a reduction of the level of glycogen, and a concomitant increase in vaginal pH [Farage and Maibach, 2006].
In prepuberal girls, the pH of the vagina is nearly neutral, and cultivation-dependent methods have shown vaginal colonization by diverse assemblages of aerobic, strictly anaerobic, and enteric species of bacteria (Figure 3) [Alvarez-Olmos et al., 2004; Hammerschlag et al., 1978].
Between the ages of 8 and 13 years, the production of estrogen increases and consequently the thickness of the vaginal epithelium and the production of glycogen are incremented as well [Farage and Maibach, 2006]. Glycogen is directly or indirectly nutritionally necessary for the maintenance of *Lactobacillus* spp. [Brotman et al., 2010; Galhardo et al., 2006]. These new environmental conditions permit the colonization of microorganisms capable of fermenting glycogen to lactic acid and the concomitant acidification of the vaginal environment that is characteristic of reproductive age women (Figure 3) [Farage and Maibach, 2006; Hammerschlag et al., 1978]. Interestingly, *Lactobacillus* spp. was originally thought to directly ferment glycogen in the vagina. However, recent evidence suggests that human α-amylase catabolizes glycogen into smaller polymers, namely maltose and maltotriose, which can then be used by *Lactobacillus* spp. for metabolism, even in newborns who have residual circulating maternal estrogen [Spear et al., 2014].
During the menopause, estrogen levels again decrease, and this is accompanied by atrophy of the vaginal epithelium and reduced cervico-vaginal secretions [Farage and Maibach, 2006]. In postmenopausal women, the levels of estrogen once again decline, reducing the deposition of glycogen thereby selecting for a high diversity of bacterial species (Figure 3) [Larsen and Galask, 1982].

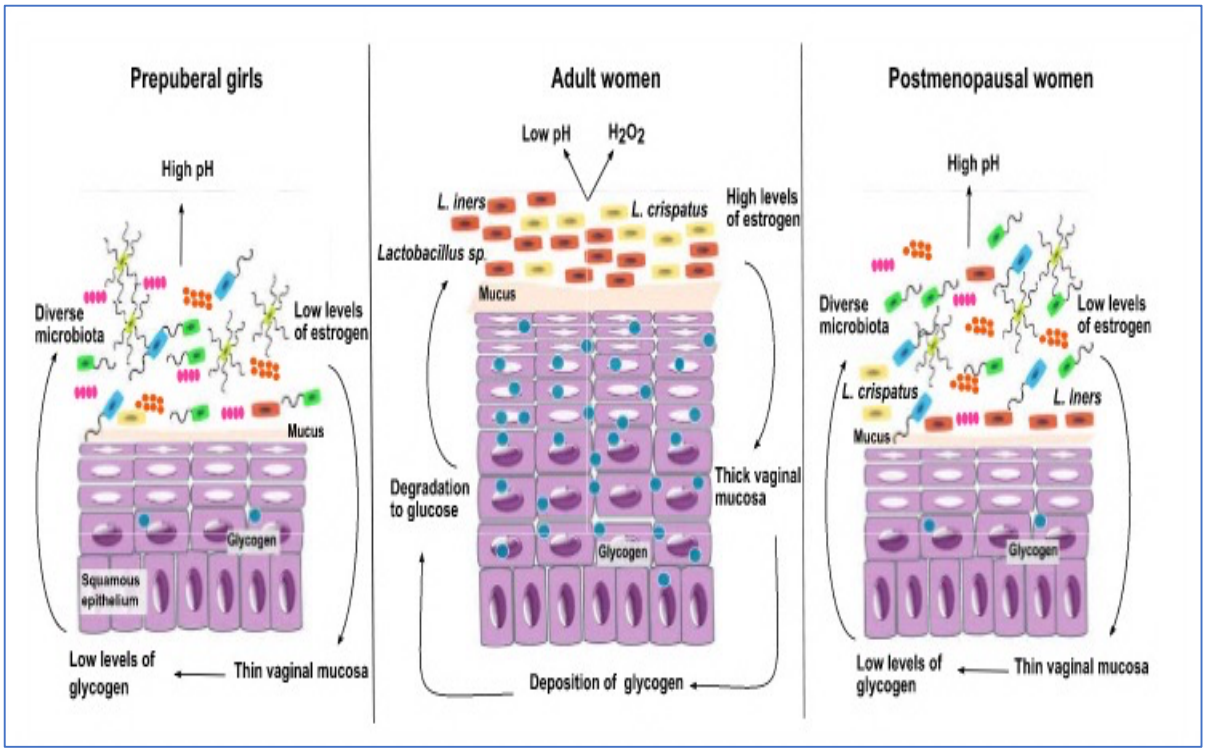

Figure 3. Vaginal microbiota during women's life [Petrova et al., 2013].

1.3 Vaginal *Lactobacillus* spp.

Healthy vaginal microbiota is generally dominated by the Gram-positive bacteria, *Lactobacillus* spp. [Hyman et al., 2005; Ravel et al., 2011]. Lactobacilli form a critical line of defence maintaining the normal vaginal microbiota by preventing overgrowth of pathogenic and opportunistic organisms [Macklaim et al., 2011; O'Hanlon et al., 2013; Ronnqvist et al., 2006].

In general, the presence of high numbers of lactic acid producing bacteria in the vagina is often associated with "healthy" and low numbers or absence as being "abnormal". The members of the genus *Lactobacillus* are considered as a keystone species because of their well-known ability to produce lactic acid through the fermentation of sugars. Albert Döderlein reported for the first time their existence by culturing these bacteria from vaginal secretions. He found that they produced lactic acid, which in turn inhibited growth of pathogens both *in vitro* and *in vivo* [Döderlein, 1892]. "Döderlein's bacillus" was later classified in 1928 as *Lactobacillus acidophilus* [Thomas, 1928]. Subsequent advances in culture techniques and in biochemical characterization of microorganisms have led to important additional insights. In the 1980s, it was determined that *L. acidophilus* was not a single species, but rather a group of closely related, obligatory homofermentative species collectively known as the *L. acidophilus* complex [Lauer et al., 1980].
As a result, the group was divided into DNA-homologous groups, to form many separate species, which are *L. acidophilus*, *L. amylolyticus*, *L. amylovorus*, *L. crispatus*, *L. gallinarium*, *L. gasseri*, *L. iners*, *L. jensenii*, and *L. johnsonii* [Du Plessis and Dicks, 1995]. However, the culture-dependent methods did not permit to identify several *Lactobacillus* species in the vagina. For example, most culture-dependent methods fail to identify *L. iners*, that was only first described in 1999 because it is not able to grow in the media typically used to isolate *Lactobacillus* [Falsen et al., 1999].

Major advances in DNA sequencing technology over the last decade have fundamentally changed the way to assess microbial community structure and composition. For investigations of bacterial diversity, these methods commonly utilize 16S rRNA gene

sequences to compare and classify taxa. This approach investigates DNA sequences extracted directly from samples. Typically, partial 16S rRNA gene sequences are amplified using primers that anneal to highly conserved sequences, and the resulting amplicons are sequenced. Phylogenetic analyses of the sequences allow the classification of phylotypes and determination of the numerically dominant taxa in a community. Other methods that rely on other conserved genes (cpn60, rpoC, uvrB, or recA) have also been developed but are not as widely used [Schellenberg et al., 2009; Van der Lelic et al., 2006].

1.3.1 *Lactobacillus*-associated protective mechanisms

Dominant vaginal *Lactobacillus* species exert important health-promoting effects to maintain the homeostasis of the host and their dominance in the vaginal habitat. This is accomplished by various direct and/or indirect mechanisms such as: the reduction of the vaginal pH by producing organic acids, especially lactic acid; the production of antimicrobial substances (bacteriocins, hydrogen peroxide, etc); the competition with other microorganisms for the nutrients and for adherence to the vaginal epithelium; maintenance of the vaginal epithelium integrity by stimulating mucus secretion and modulating the immune response. These principal mechanisms are described below.

1.3.1.1 Lactic acid

Lactic acid is the principal metabolite produced by vaginal microbiota, thanks to its ability to metabolize glycogen-derived products under anaerobic conditions.

Numerous studies reported that lactic acid is able to maintain healthy host physiological functions since it has been shown to directly inhibit the vaginal colonization of numerous pathogenic microorganisms such as *Chlamydia trachomatis* [Gong et al., 2014; Nardini et al., 2016] and potentially both HSV-2 and HIV *in vitro, ex vivo,* and *in vivo* if there is sufficient lactic acid to acidify the vagina to pH < 4 [Aldunate et al., 2013; Conti et al., 2009; Nahui Palomino et al., 2017]. Lactic acid also inhibits a broad range of bacterial vaginosis-associated microbes at pH < 4.5 [O'Hanlon et al., 2011, 2013]. Other studies showed that lactic acid can stimulate host immune responses, for example by inhibiting the

pro-inflammatory mediators IL-6, IL-8 and IL-1RA; by inducing a Th17 response via IL-23; and by facilitating the release of mediators from vaginal epithelial cells and stimulating antiviral response by the release of antimicrobial effector molecules from epithelial cells [Hearps et al., 2017; Mossop et al., 2011; Witkin et al., 2011].

1.3.1.1.1 d- l-Lactic acid isomers

Lactic acid isomers may also play a role in determining host response and the subsequent host microbiota relationship. Lactic acid exists in the vagina in both "d" and "l" isomers; lactic acid is also produced by vaginal epithelial cells under the control of estrogen only. However, vaginal microbiota, and not host epithelial cells, contributes to the production of most of the lactic acid present in the vagina [Boskey et al., 2001; Boskey et al., 1999]. Lactobacilli can produce both the d- and l-chiral isomers of lactic acid, while humans produce only the l-isomer, except for a small quantity of the d-isomer released via the methylglyoxal pathway [Ewaschuk et al., 2005]. Of importance, among the four most common lactobacilli species in the human vagina *L. iners* does not produce d-lactic acid and is not able to produce the l-lactic acid in abundance, in contrast to *L. crispatus* and *L. gasseri,* while *L. jensenii* produces only d-lactic acid [Witkin et al., 2013], suggesting potential *Lactobacillus* species-specific effects on the host.

Nevertheless, few studies reported on the role of lactic acid isomers. According to the study conducted by Witkin et al., d-lactic acid down-regulated the production of matrix metalloproteinase (MMP)-8. MMP-8 in the vagina alters cervical integrity favoring the entrance of bacteria to the upper genital tract. Therefore, a high d-lactic acid level, present when *L. crispatus, L. jensenii* and/or *L. gasseri* are abundant in the vagina, may lower the bacterial transport to the uterus protecting the woman from upper genital tract infections and infection-related preterm birth [Witkin et al., 2013]. Nunn et al. showed that HIV-1 virions were generally trapped in cervico-vaginal mucus with relatively high concentrations of d-lactic acid from a *L. crispatus*-dominant microbiota. In contrast, HIV-1 virions diffused rapidly through cervico-vaginal mucus with low concentrations of d-lactic acid from *L. iners*-dominant microbiota or significant amounts of *G. vaginalis*, suggesting that the vaginal microbiota, including different species of *Lactobacillus*, can

alter the diffusional barrier properties of cervico-vaginal mucus against sexually transmitted viruses through the production of d-lactic acid [Nunn et al., 2015].
On the other hand, Aldunate and colleagues, observed that l-lactic acid, and not d-lactic acid, at physiological concentrations, exerted potent HIV virucidal activity [Aldunate et al., 2013]. Moreover, by analyzing vaginal fluids from women with vaginal disorders, Beghini et al., observed that women with bacterial vaginosis were found to be deficient in both isomers, while those with vulvovaginal candidiasis had elevated l-lactic acid [Beghini et al., 2015].

1.3.1.2 Hydrogen peroxide

Another property of most vaginal lactobacilli is their ability to release hydrogen peroxide (H_2O_2) in appreciable amounts *in vitro* [Eschenbach et al., 1989]. The H_2O_2 of microbial origin and halide (Cl^-, Br^-, I^-) or pseudohalide (SCN^-) ions is oxidized by peroxidase forming the corresponding hypohalous acid or halogen, which exerts potent toxic properties against bacteria, fungi, viruses or mammalian cells [Klebanoff and Coombs, 1991; Klebanoff et al., 1991].
It has been determined that 96–98% of cultivable vaginal *Lactobacillus* isolates from healthy women are H_2O_2-producers *in vitro* [Eschenbach et al., 1989; Rabe and Hillier, 2003], suggesting that H_2O_2 could be an important bacterial metabolite for protection against pathogenic microorganisms. In fact, Wilks and colleagues reported that the presence of H_2O_2-producing *Lactobacillus* strains such as, *L. jensenii*, *L. crispatus*, and *L. gasseri* is associated with a reduced risk of bacterial vaginosis, preterm birth, and chorioamnionitis [Wilks et al., 2004]. The presence of H_2O_2-producing lactobacilli also correlates with a reduced risk of acquiring sexually transmitted pathogens, such as *Neisseria gonorrhoeae, C. trachomatis,* and HIV [Baeten et al., 2009; Saigh et al., 1978; Wiesenfeld et al., 2003]. Moreover, Fitzsimmons and Berry reported the inhibition of *Candida albicans* by H_2O_2-producing *L. acidophilus* [Fitzsimmons and Berry, 1994], although this result was not confirmed by other studies [Hawes et al., 1996; Sobel and Chaim, 1996].

However, the H_2O_2-production by lactobacilli has not been measured *in vivo*; therefore, the biological relevance of H_2O_2 in the prevention of urogenital diseases remains still unknown. Martin and Suarez work showed that H_2O_2 is only produced by lactobacilli when cultures are agitated, indicating that H_2O_2 is produced only in the presence of oxygen. Furthermore, H_2O_2 was degraded when the bacteria were grown in the presence of iron-containing compounds such as hemin or hemoglobin, and Fe^{3+} ions also induced degradation of H_2O_2 [Martin and Suarez, 2010]. These two findings, in addition to the fact that the vagina is microaerophilic and an iron-rich mucosal site, raises the question of whether lactobacilli can produce enough H_2O_2 amounts to inhibit pathogens *in vivo* [Martin and Suarez, 2010].

1.3.1.3 Adhesion to host cells

Another postulated mechanism, by which lactobacilli would prevent pathogen colonization of vaginal epithelium, is through the adhesion to host cells. Lactobacilli bind to the surface of vaginal epithelial cells and compete for the adhesion sites with other microorganisms to prevent them from attaching and infecting host cells. Several vaginal *Lactobacillus* strains have been shown to block adhesion of pathogens to the vaginal epithelial cells *in vitro*, such as *Escherichia coli*, *G. vaginalis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Trichomonas vaginalis* [Mastromarino et al., 2002; Osset et al., 2001; Phukan et al., 2013; Zarate and Nader-Macias, 2006]. Additionally, it was observed that lactobacilli have a higher affinity for the host cell receptors than pathogens, displacing even already host-adhered pathogens, such as *G. vaginalis, N. gonorrhoeae* and *C. albicans* [Boris et al., 1998; Mastromarino et al., 2002; Parolin et al., 2015; Spurbeck and Arvidson, 2008]. However, little is known about molecular mechanisms underlying host-bacteria physical interactions. Multiple components of the bacterial cell surface are likely to participate in this process, such as glycoproteins, carbohydrates, and lipoteichoic acid, as previously shown for *Lactobacillus* adherence to the vaginal epithelium [Boris et al., 1998; Chan et al., 1985].

1.3.1.4 Coaggregation

Lactobacilli could also protect the vaginal tract from infection by coaggregating with the pathogen, thereby sequestering the pathogen and preventing it from adhering to the host epithelium, thus facilitating its discharge by vaginal fluids and/or killing by antimicrobial metabolites secreted by lactobacilli [Mastromarino et al., 2002]. Coaggregation, as a mechanism of defense was first studied by Reid et al., observing for example that *L. rhamnosus* strongly coaggregated with *E. coli* strains, inhibiting their growth [Reid et al., 1988]. Some vaginal lactobacilli, including *L. gasseri, L. jensenii*, and *L. crispatus*, can coaggregate with *E. coli*, although with different efficiency [Kmet and Lucchini, 1999]. This phenomenon is not restricted to *E. coli*, for instance *L. salivarius*, *L. brevis*, and *L. gasseri* can coaggregate with *G. vaginalis* and *C. albicans* [Mastromarino et al., 2002]. Although coaggregation can be an effective defensive mechanism adopted by some strains, it seems that is not utilized by all vaginal lactobacilli.

1.3.1.5 Bacteriocins

Bacteriocins are defined as small bactericidal proteins with a narrow spectrum of activity, inhibiting strains of the same or closely related species [Cotter et al., 2013]. These proteins appear to be capable to inhibit the colonization of pathogenic and opportunistic microorganisms and perhaps provide an advantage to maintain the wellness of the female genital tract. Strains of the most dominant vaginal *Lactobacillus* species in healthy women (*L. gasseri, L. crispatus*, and *L. jensenii*), as well as the most well characterized vaginal probiotic strain, *L. rhamnosus* GR-1, were found to produce bacteriocin-like compounds, these bacterocin-like compounds have been showed to exert microbicidal activity against *G. vaginalis*, *C. albicans* and *E. coli* [Kaewsrichan et al., 2006; McGroarty and Reid, 1988]. On the other hand, some extensive studies of human vaginal lactobacilli isolates, such as, *L. crispatus*, *L. jensenii*, *L. gasseri*, *L. vaginalis,* and *L. plantarum* showed that none of these strains produced bacteriocins [Martin et al., 2008; Siroli et al., 2017]. Thus, bacteriocins may not be universally produced by vaginal lactobacilli, and their association with protection against pathogens remains to be verified.

1.3.1.6 Biosurfactants

Biosurfactants are amphipathic molecules, which contain both hydrophobic and hydrophilic domains. These compounds, which can be proteins, carbohydrates, or glycoproteins, are employed by microbes for a variety of purposes, such as emulsification of hydrocarbons, quorum-sensing, biofilm regulation, antimicrobial activity, and regulation of adhesion and detachment [Rodrigues et al., 2006].

Reid et al. reported that numerous lactobacilli strains from gastrointestinal and vaginal origin produce biosufactants. Some of these biosurfactants are secreted, whereas others are associated with the surface of the bacterium [Reid et al., 1999]. In another study, using the extract of surface-associated components from *L. jensenii,* it was shown that these bacteria have biosurfactant activity capable to inhibit the adherence of *N. gonorrhoeae* to epithelial cells *in vitro* [Spurbeck and Arvidson, 2010]. Therefore, biosurfactants produced by lactobacilli could play a significant role in reducing microbial infections, and also should be studied further to determine the involvement of these molecules in the inhibition of vaginally acquired pathogen colonization.

1.4 Common gynecological infections

Pathogen invasion of the lower female reproductive tract may result in local infections, as well as widespread to the upper female reproductive tract, potentially leading to fertility and pregnancy disorders. A shift in the composition of the vaginal microbiota, which is dominated by lactobacilli in homeostatic conditions, is associated with several disorders such as bacterial vaginosis, aerobic vaginitis, vulvovaginal candidiasis, urogenital tract infections, or sexually transmitted infections, which can coexist and mutually support each other by fueling local inflammation in the female reproductive tract. *C. albicans*, *C. trachomatis,* and human immunodeficiency virus (HIV) are among the infectious agents that most significantly affect women's health worldwide.

1.4.1 Bacterial vaginosis and aerobic vaginitis

Bacterial vaginosis (BV) is a condition characterized by replacement of the normally protective *Lactobacillus* spp. with a massive overgrowth of anaerobic and facultative organisms, including *G. vaginalis*, *A. vaginae, Bacteroides* spp*., Molibincus* spp*.,* and genital mycoplasmas [Eschenbach et al., 1989; Fredricks and Marrazzo, 2005; Srinivasan and Fredricks, 2008]. BV is the most common vaginal disorder of reproductive age women and is associated with serious adverse sequelae including infertility, endometritis, and pelvic inflammatory disease, spontaneous abortion, as well as an increased risk of acquiring HIV, *N. gonorrhoeae*, *C. trachomatis*, and other sexually transmitted infections [Haggerty et al., 2004; Leitich et al., 2003; Sweet, 1995; Taha et al., 1998; Wiesenfeld et al., 2003].

Aerobic vaginitis (AV) is an alteration in the vaginal microbiota associated with aerobic microorganisms; mainly group B *Streptococcus*, *S. aureus*, *E. coli*, and *Enterococcus* [Donders, 2007; Donders et al., 2002]. AV is differentiated from BV mainly by the presence of inflammatory response associated with aerobe microorganisms [Cauci, 2004]. Interestingly, Donders et al. observed that 20% of women with AV also exhibited an

overgrowth of *G. vaginalis*, indicating that there may be a degree of overlap with BV or that the two entities may coexist [Donders et al., 2002].

1.4.2 Vulvovaginal candidiasis (VVC)

VVC is a common infection compromising the quality of life of many women. *Candida* infection affects 70-75% of women at least once during their lives, whereas 40-50% of them experience at least one recurrence and about 5-8% of these women suffer from recurrent VVC [Peters et al., 2014]. *C. albicans* is the most frequent etiologic agent of VVC [Workowski et al., 2010] causing almost all acute uncomplicated cases and about 70% of complicated cases of recurrent VVC. The remaining 30% are caused by *C. glabrata*, *C. parapsilosis*, *C. kruzei*, or *C. tropicalis* [Nyirjesy et al., 1995].

Candida has an extraordinary dual lifestyle capacity, which permits to adapt to different environmental and host habitats, allowing host-colonization either as a commensal or opportunistic pathogen. This duality is due to their morphological capacity to change from a round ovoid typical yeast cell (Y) to a hyphal mycelial-growing organism (H). This transition is crucial for its pathogenicity. There is sufficient evidence that the Y form is predominantly associated with commensalism, while the H form is associated with pathogenicity. In the Y form, *Candida* can be found in the intestine and vagina of more than 50% of healthy asymptomatic subjects, whereas the H form is invariably found in pathologic specimens obtained from invaded tissues. It remains to be determined whether the presence of commensal *Candida* confers a benefit to the host in terms of balanced microbiota composition and maintenance of local homeostasis. However, when tolerance mechanisms become defective, the Y form changes into the H form and expresses its virulence capacity [Harriott et al., 2010; Wang, 2009].

Factors associated with the transition from asymptomatic colonization to symptomatic infection may be intrinsic to the host, the environment, the host behavior, or related to the organism itself. For instance, diabetes-associated glycosuria and the use of antibiotic and estrogen-based drugs may contribute to *Candida* colonization and infection [Fischer and Bradford, 2011; Nyirjesy et al., 2012; Pirotta and Garland, 2006]. Behavioral factors such as orogenital sex can facilitate *Candida* infections [Geiger and Foxman, 1996]. The use of

hormonal and contraception methods has also been associated with an increase in incidence of VVC [Nyirjesy, 2008].

Symptomatic *C. albicans* infections are commonly treated with azole antifungal drugs. Since azoles are fungistatic for *C. albicans*, cells repetitively exposed to these drugs may become azole resistant [Coste et al., 2007]. Moreover, elevated healthcare costs due to the high incidence of VVC together with the growing problem of antibiotic resistance, urge the development of new effective probiotic agents for the prevention and therapy of this gynaecological infection [Falagas et al., 2006].

1.4.2.1 Role of vaginal *Lactobacillus* against VVC

Although the pathogenesis of VVC remains a controversial issue, it seems that, upon the disruption of the balance existing between host and vaginal microbiota, the overgrowth of *Candida* is facilitated. In some studies, VVC was associated either with a reduced number of lactobacilli or with species of lactobacilli not producing H_2O_2 [Hawes et al., 1996; Hillier et al., 1992; Vitali et al., 2007]. For example, Vitali and colleagues showed that women with VVC hosted reduced numbers of H_2O_2 producer lactobacilli species (*L. acidophilus*, *L. gasseri,* and *L. vaginalis*) and increased numbers of non-H_2O_2 producer bacteria (*L. iners*) [Vitali et al., 2007]. On the contrary, several studies based on molecular methods have shown how vaginal colonization by *Candida* spp. is more common in women with a microbiota dominated by lactobacilli, than the microbial profile associated with bacterial vaginosis [Drell et al., 2013; van de Wijgert et al., 2014; van de Wijgert et al., 2008]. In addition, it was demonstrated that *Candida* infection did not alter the bacterial composition of the microbiota itself, which remained homogeneous and stable over time, and possibly dominated by lactobacilli [Biagi et al., 2009; Zhou et al., 2009].
In vitro studies reported that lactobacilli exert antagonistic effects on *Candida* infection. Lactobacilli can compete with *Candida* for nutrients and/or adherence to host epithelial cells [Osset et al., 2001; Parolin et al., 2015]. *L. acidophilus*, *L.* gasseri, and *L. jensenii*, were shown to coaggregate with *C. albicans* [Boris et al., 1998]. The production of antimicrobial substances produced by specific lactobacilli strains can also prevent VVC,

in particular organic acids, H_2O_2, bacteriocins, and biosurfactants [Okkers et al., 1999; Parolin et al., 2015; Velraeds et al., 1998].

Some clinical studies supported the effectiveness of orally or locally administered probiotic lactobacilli as well. For example, Vujic group treated patients diagnosed with VVC with capsules containing probiotics *L. reuteri* RC-14, *L. rhamnosus* GR-1, and placebo, for 6 weeks. Their result showed that the microbiota was restored by 61.5% in the probiotics group compared to 26.9% of the placebo group [Vujic et al., 2013]. Comparable results were obtained administering vaginal suppositories of *Lactobacillus* GG twice per day for 7 days [Hilton et al., 1995]. Moreover, it was also studied the effect of probiotics associated with azole in treating recurrent VVC. Martinez et al. evaluated one-month therapy with probiotics that were added to a single dose of 150 mg fluconazole. Beneficial effect of lactobacilli was observed compared to the patients that received only fluconazole [Martinez et al., 2009]. Additionally, De Seta et al. observed that local *L. plantarum* administered intravaginally, together with local clotrimazole, offers potential benefits for resolution of vaginal discomfort [De Seta et al., 2014].

1.4.3 *Chlamydia trachomatis* infection

The obligate intracellular Gram-negative bacterium, *C. trachomatis*, is a leading cause of sexually transmitted infections (STIs) with more than 100 million new cases per year according to global estimates [Senior, 2012]. Strains of *C. trachomatis* are divided into three biovars and are further subtyped by serovar. The trachoma biovar (serovars A–C) is the leading cause of non-congenital blindness, the genital tract biovar (serovars D–K) is the most prevalent sexually transmitted bacterium, and the lymphogranuloma venereum biovar (serovars L1–L3) causes invasive urogenital or anorectal infection [Elwell et al., 2016].

In women, 70–80% of genital tract infections with *C. trachomatis* are asymptomatic, but 15–40% ascend to the upper genital tract, which can lead to serious sequelae, including pelvic inflammatory disease, infertility, and ectopic pregnancy [Malhotra et al., 2013]. Moreover, infection with *C. trachomatis* has been shown to facilitate the transmission of HIV and is also associated with the incidence of cervical cancer [Malhotra et al., 2013].

Although chlamydial infection is treatable with antibiotics, no drug is sufficiently cost-effective for the elimination of the bacterium, and an effective vaccine has been elusive so far [Howie et al., 2011].

Chlamydia has a unique cycle of development, alternating between two distinct bacterial forms (Figure 4). The elementary bodies (EBs) are infectious but non-dividing. In contrast, the reticulate bodies (RBs) are non-infectious but replicative [Moulder, 1991]. After attachment and penetration in cells, EBs remain internalized in vacuoles permitting the escape to phago-lysosomal fusion. Within these vacuoles, named inclusion-forming units, EBs differentiate into RBs after several transformations. Unlike EBs, RBs are larger, less compacted, metabolically active, and capable to divide by binary fission. Around 18 h post-chlamydial infection, RBs resulting by binary fission differentiate into EBs, then EBs are expelled from the cell, either by exocytosis or cellular lysis between 48–72 h post-infection [Wyrick, 2000].

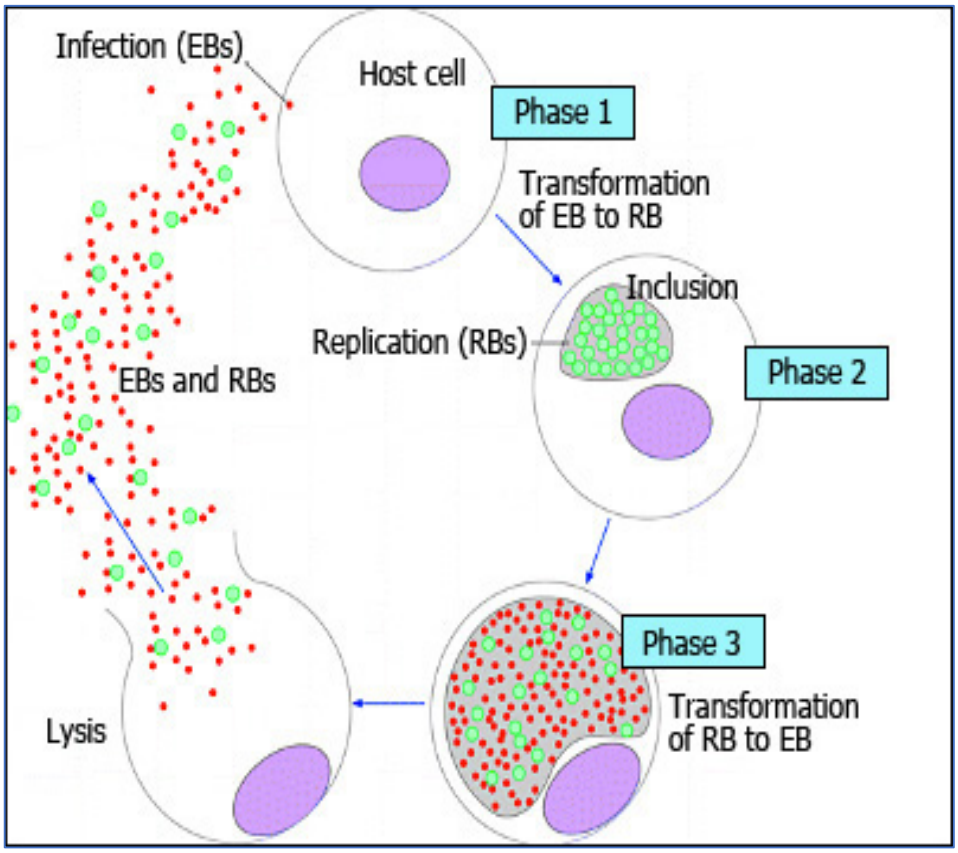

Figure 4: The life cycle of Chlamydia trachomatis (from www.cytologystuff.com).

1.4.3.1 Lactobacilli and *Chlamydia trachomatis* infection

Lactobacilli are the predominant microorganisms of the healthy women's vaginal microbiota and numerous evidences indicate that the presence of a lactobacilli lacking vaginal microbiota facilitates the acquisition of various sexually transmitted diseases, including *C. trachomatis* [Brotman et al., 2010; Hillier et al., 1992; Wiesenfeld et al., 2003].

Despite the importance of a healthy vaginal microbiota in preventing genital infections, only a few studies have been focused on the protective effects of vaginal lactobacilli towards chlamydial infection. Antagonistic effects by lactobacilli on chlamydial EBs, chlamydial absorption to epithelial cells, and intracellular phases of chlamydial replication have been demonstrated. However, the molecular mechanisms underlying the interactions between *Lactobacillus* and *C. trachomatis* in the vaginal environment have not yet been elucidated.

Mastromarino and colleagues investigated the effects of two vaginal strains of *Lactobacillus* (*L. brevis* and *L. salivarius*) against *C. trachomatis* infection on HeLa cells. They observed that both vaginal lactobacilli significantly inhibit the replication of *C. trachomatis,* independently of pH variations, inhibiting their adhesion to host epithelial cells and their intracellular replication as showed by the significant reduction of *Chlamydia* inclusion forming units in HeLa cells compared to the control. A strong inhibition was observed especially when lactobacilli were present during early stages of infection. In addition, the inhibitory effect of lactobacilli against *Chlamydia* infection was dose dependent, suggesting that the concentration of lactobacilli in the vagina contributes to *Chlamydia* inhibition. In the same study, the effects of *L. brevis* and *L. salivarius* were also evaluated against the persistent form of *C. trachomatis* induced by co-infection with HSV-2, observing that *L. brevis* has the ability to inhibit *Chlamydia* even in the persistent form of *Chalmydia* infection [Mastromarino et al., 2014].

Gong et al. investigated the effect of two metabolites produced by lactobacilli (*L. crispatus*, *L. gasseri,* and *L. jensenii*) against *C. trachomatis* infection, lactic acid and H_2O_2. Their results indicate that the anti-*Chlamydia* activity of lactobacilli is primarily due to their ability to acidify the vaginal pH by producing lactic acid. Contrarily, H_2O_2 production

seems to not be relevant for *Chlamydia* inhibition. The authors suggested various mechanisms by which lactic acid may inhibit the development of *Chlamydia*: (i) lactic acid could be involved in the inactivation of *Chlamydia* EBs surface molecules which represent their virulence factors for adhesion and penetration to the host cell; (ii) lactic acid could compromise the integrity of the outer membrane by reducing the number of disulphide bonds between the protein complexes; (iii) and the entry of hydrogen ions into the *Chlamydia* EBs could alter cellular metabolism, essential for early development of *C. trachomatis* [Gong et al., 2014].

Another study investigated the immunomodulatory effect of *L. crispatus* on HeLa and J774 cells subjected to *C. trachomatis* infection by studying the expression of the inflammatory cytokines IL-6, IL-8, TNF-α and IL-10. They observed that, *L. crispatus* specifically enhances the expression of the anti-inflammatory cytokine IL-10 and inhibits the expression of the pro-inflammatory IL-6, IL-8, and TNF-α cytokines in the host cells. These results suggest a potential mechanism by which *L. crispatus* may protect against pathological inflammatory conditions. Additionally, *L. crispatus* inhibited *C. trachomatis* adhesion and infectivity in human epithelial cells and macrophages [Rizzo et al., 2015].

1.4.4 Human immunodeficiency virus infection

The human immunodeficiency virus (HIV) is a retrovirus belonging to the family of *Retroviridae*, genus lentivirus. HIV is the etiologic agent of the acquired immunodeficiency syndrome (AIDS) that results in extremely variable clinical outcomes, such as a severe immunodeficiency accompanied by the establishment of opportunistic infections and tumors, organ decay, and central nervous system degeneration. Two different HIV viruses exist: HIV-1, the pandemic type, and HIV-2, more represented in West Africa [Sharp and Hahn, 2011].

As shown in figure 5, the replication cycle of HIV-1 begins with a high affinity binding between the HIV gp120 and the receptor CD4 expressed on the target cell surface. CD4 is a protein mainly expressed by lymphocytes with ‘helper-inducer’ activity, but it is also present on monocytes, macrophages, dendritic cells, Langerhans cells, and some circulating and bone marrow-resident hematopoietic progenitors. The interaction CD4-

gp120 induces a conformational change in the structure of gp120 that leads to the exposure of the binding site for another receptor, called co-receptor. There are two main co-receptors which HIV gp120 recognizes: CXC-chemokine receptor 4 (CXCR4) or CC-chemokine receptor 5 (CCR5) [Rizzuto et al., 1998]. Upon HIV-1 entry into the target cell, the core dissolves and a reverse-transcription complex assembles in the cytoplasm allowing the reverse transcription of the two identical molecules of ssRNA+ in double stranded DNA and then evolves in the pre-integration complex that crosses the nuclear membrane [Davenport et al., 2002]. Inside the nucleus, the HIV-1 integrase promotes the insertion of the viral DNA in the host cell genome, where it can remain transcriptionally silent for years. The establishment of a pool of long-lived latently infected CD4 T cells, called HIV reservoir, that is not visible to the immune system and continuously revive the infection despite antiretroviral treatment, represent the major obstacle to HIV eradication strategies [Chun and Fauci, 1999]. Antigenic stimulation through activation of the T-cell receptor signaling or stimulation of the host cell by pro-inflammatory cytokines can lead to activation and nuclear translocation of host transcription factors, such as NF-kB, that turn to activate or enhance HIV-1 provirus transcription by the host enzyme RNA polymerase II [Piret et al., 1995]. When a complete transcript is produced, it translocates to the cytoplasm where the synthesis of viral proteins with important regulatory functions takes place. The final step of the viral replication cycle occurs on the plasma membrane of the host cell. Viral components are transported and anchor to the plasma membrane along with the HIV-1 genomic ssRNA+ as the forming virion begins to bud from the host cell. Finally, the structural components of HIV assemble to produce a mature virion [Bukrinskaya, 2004]. These include the molecule $p24_{gag}$ incorporated into the protein core that surrounds the viral RNA, also known as capsid. Immunoassays targeting this protein have been extensively used to measure HIV-1 replication in *in vitro* infection assays as well as to diagnose HIV-1 infection *in vivo.*

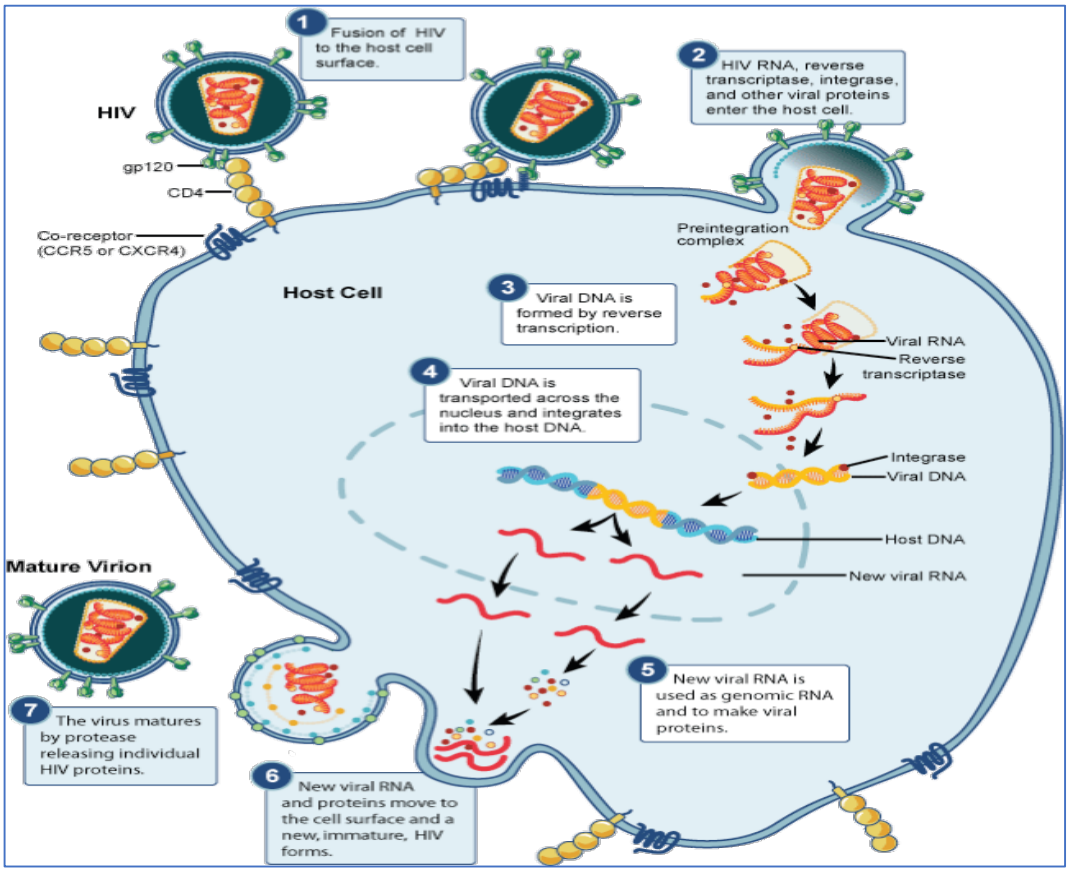

Figure 5. Replication cycle of HIV (from http://www.niaid.nih.gov).

1.4.4.1 HIV infection in female genital tract

The female genital tract mucosa is the portal of entry for several clinically relevant sexually transmitted viruses, including HIV. Women appear to be more easily infected with HIV than men. Differences in social rank, behavior, sex hormone regulation, and especially organization of the mucosal surface appear to be involved [Iwasaki, 2010].
Male to female sexual transmission of HIV is mediated by the exposure of genital mucosa to infectious virions and/or infected lymphocytes and monocytes present in the semen. The ratio of transmissibility of cell-free vs. cell-associated viruses is still uncertain, but it seems that both are sources of infections and should be targeted by intervention strategies.

Under normal circumstances, the incidence of HIV transmission from males to females via vaginal intercourse is very low, within the range of one productive infection for every 200–

2000 exposures [Schellenberg and Plummer, 2012]. This can probably be explained, in part, by the presence of the protective type II mucosa of the female vaginal epithelium cells (see section 1.1). Nevertheless, free HIV virions can infect the female reproductive tract in many different ways: (i) free HIV virions and/or infected cells can penetrate the epithelium through gaps between the epithelial cells or lesion on the vaginal mucosa layer and reach the basal layer where the majority of target cells reside (i.e. CD4 cells); (ii) HIV-infected donor cells trapped in the mucus layer can release new virions; (iii) virions can be captured by Langerhans cells or other antigen presenting cells, located in the epithelium layer and subsequently transferred to target cells in the deeper layers of the mucosa or in lymphoid organs (transinfection); (iv) virions can penetrate the epithelium through epithelial cells (transcytosis) and infect the underlying CD4 T cells or macrophages; (v) virions can also make contact with dendritic cells and subsequently get transported to CD4 T cells [Hladik and McElrath, 2008; Iwasaki, 2010; Lederman et al., 2006]. Regardless of the mechanism of transmission, the establishment of a founder pool of productively infected CD4 T cells in the female reproductive tract precedes for virus dissemination to secondary lymphoid organs, as demonstrated in pathogenic animal models of HIV-1 infection [Haase, 2001].

1.4.4.2 Role of vaginal *Lactobacillus* in protection against HIV

Lactobacilli have been reported to protect against vaginal transmission of HIV [O'Hanlon et al., 2013; Ronnqvist et al., 2006]. Although many hypotheses have been formulated regarding the protective effects of lactobacilli, their exact mechanism of HIV inhibition remains to be fully elucidated *in vivo*. These mechanisms seem to involve: (i) direct HIV killing by lactic acid, H_2O_2, bacteriocins, and other inhibitory agents; (ii) maintenance of vaginal integrity and competition with pathogens for binding to vaginal epithelium; (iii) HIV neutralization by lectin molecules, which are present in lactobacilli surface, that bind virus glycoproteins and in this way preventing infection; (iv) and enhancement of the local host-immune defenses [Aldunate et al., 2013; Kaewsrichan et al., 2006; Klebanoff and Coombs, 1991; Olmsted et al., 2005; Petrova et al., 2013; Pretzer et al., 2005; Reid et al., 2011].

Generally, the low pH of the vaginal environment, caused mainly by lactic acid, is believed to be the main strategy to prevent bacterial and viral infections in the vaginal mucosa. For example, cell-free HIV-1is inactivated upon incubation in a solution with pH as low as 5.7 for 2 h. However, incubation in the same condition did not affect cell-associated viral infectivity [Martin et al., 1985; Ongradi et al., 1990]. Other studies showed that the acidic environment of the vagina may inhibit the activation of HIV target cells, thus reducing their susceptibility to HIV-1 infection. Monocytes, macrophages, and lymphocytes, were also found to lose the motility at a pH below 6.0 [Hill and Anderson, 1992; Olmsted et al., 2005]. Human cervico-vaginal mucus, obtained from donors with normal lactobacilli-dominated vaginal microbiota efficiently traps HIV by decreasing viral diffusion at low pH [Nunn et al., 2015]. Of interest, at pH 4, lactic acid, but not HCl, abolished the negative surface charge on HIV without lysing the viral envelope, thus may alter HIV surface protein structures and/or possibly inactivate the virus by disrupting the envelope membrane and exposing the capsid [Lai et al., 2009]. Taken together, these results support the idea that maintaining a low pH in the vaginal lumen by production of lactic acid is important to reduce HIV transmission.

The effect of H_2O_2 on the survival of different sexually transmitted viruses including HIV has not been extensively addressed. It was reported that the H_2O_2-producing strain *L. acidophilus*, was virucidal to HIV in cell line infection assay. The anti-HIV activity was not observed when *L. acidophilus* strain was treated for 15 min at 100 °C or when it was replaced by a strain unable to produce H_2O_2. Additionally, the virucidal effect was inhibited by catalase, but not by heat-inactivated catalase [Klebanoff and Coombs, 1991]. Besides these results, the effect of H_2O_2 *in vivo* has not been studied yet plus the vaginal tract is microaerophilic and iron-rich, which raises the question of whether lactobacilli produce enough H_2O_2 amounts to inhibit pathogens *in vivo.*

Molecules on the cell surface of the vaginal microbiota that directly interact with pathogens or host cells are postulated to play a role in the exclusion of bacterial and/or viral pathogens. Such interactions could be established via carbohydrate-binding proteins known as lectins, which interact specifically with carbohydrates on the surface of pathogens and are highly

specific for the ligand of interaction. Lectins on the cell surface of vaginal microorganisms could play a role in pathogen exclusion by competitively binding to the same glycans on the host surface, thereby blocking adhesion or by binding glycans on the pathogenic surfaces, thereby blocking virulence mechanisms such as adhesion and invasion. Some lectins, especially the ones highly specific for recognition of mannose (e.g. actinohivin and griffithsin) and *N*-acetylglucosamine residues, have been shown to possess activity against HIV by binding of the glycans on the viral envelope and thereby blocking the virus entry process. Some of these lectins can inhibit the infection of T cells by cell-free virions through binding to the HIV gp120 glycoprotein. They can also block the interaction between HIV and the macrophage mannose receptor, thereby preventing the infection of macrophages. Additionally, lectins can block the dendritic cell-directed transmission of the virus to uninfected T cells [Balzarini, 2007]. Nevertheless, the information on lectins encoded by *Lactobacillus* species and especially vaginal isolates is still limited.

Little is known about the relationship between the vaginal microbiota and the immune system. HIV infections are characterized by an increase of pro-inflammatory cytokines and pro-inflammatory responses. This has been linked with a disruption of the integrity of the vaginal mucosa and consequently further activation of HIV in infected people. Therefore, vaginal microbiota that can reduce a pro-inflammatory response could contribute to a decreased activation of HIV. For example, in vaginal epithelial multilayers treated with TLR agonists, a significant reduction of IL-6 and IL-8 expression after treatment with *L. crispatus* was observed. Additionally, *L. crispatus* and *L. jensenii* could induce a significant reduction of TNF secretion as well as some pro-inflammatory chemokines (MIP-1β and RANTES) [Rose et al., 2012]. Further studies need to address the immunomodulatory effect of lactobacilli as well.

1.4.4.3 Human *ex vivo* tissues to study HIV transmission and pathogenesis

Understanding the mechanisms of HIV-1 transmission requires knowledge of the functions and interactions of all immune cells and of the extracellular matrix. Although conventional isolated cell lines cultures or peripheral blood mononuclear cells have been useful in many

areas of HIV research, interpretations of the results of experiments with these cultures are limited by the fact that they do not reproduce the spatial distribution of cells and their native communication within the tissue cytoarchitecture.

The human *ex vivo* tissue system is important for the study of multiple aspects of HIV pathogenesis and consist in a raft culture, in which blocks of human tissue are cultured on collagen sponges at the air-liquid interface. This *ex vivo* model of tissue culture developed and optimized by Grivel and Margolis is based on the work of Joseph Leighton [Grivel and Margolis, 2009; Leighton, 1963] and includes cultures of tonsillar, cervico- vaginal, and rectosigmoid tissues. These tissues serve as the first gateway for HIV-1 sexual transmission, preserving the specific mucosal cell phenotypes therefore important for understanding HIV transmission and pathogenesis.

This *ex vivo* model has many advantages (Table 1). Upon inoculation *ex vivo*, human tissues support productive HIV infection without exogenous activation and stimulation, and retain tissue cytoarchitecture as well as the pattern of expression of key cell surface molecules relevant to HIV infection for around 2–3 weeks. Some of the tissue functions are preserved in *ex vivo*, including the ability to release a spectrum of cytokines similar to those released *in vivo*, and the ability of tissue challenged with recall antigens (tetanus or diphtheria toxoids) to respond by producing specific antibodies. *Ex vivo* tissues support HIV replication without the artificial stimulation that is necessary for productive HIV infection in isolated lymphocytes [Grivel and Margolis, 2009; Lisco et al., 2007]. On the other hand, the system of *ex vivo* tissues have some limitations in the study of infection of human pathogens (Table 1), mainly the difficulty of applying many modern investigative tools developed for isolated cells to these tissues. For example, although microscopy gives subcellular resolution in imaging of single cells, the problem of deep tissue penetration beyond a few hundred microns is only partially solved with two-photon excitation microscopy [Rubart, 2004].

Advantages	**Disadvantages**
• Preservation of tissue cytoarchitecture, including major lymphocyte subtypes and follicular- dendritic cell network. • *In vivo*-like spectrum of cytokine release. • Continuous expression of HIV coreceptors over 2 week in culture. • Support of HIV replication without exogenous stimulation or activation. • *In vivo* pattern of replication: HIV replicates in both activated and nonactivated cells. • Other tested human viruses, bacteria and parasites readily infect tissues, allowing the study of their pathogenesis and interactions. • Viral movement in the tissue can be followed in real time by confocal microscopy.	• Tissues start to deteriorate after 3 weeks in culture. • Difficulty in monitoring cells beyond the depth of confocal microscopy (unless cells are isolated for analysis). • The system does not reflect the effects of *in vivo* systemic factors. • Labor: multiple blocks of tissue are required for every experimental condition to overcome tissue heterogeneity. • Donor-to-donor variability

Table 1. Advantages and disadvantages of human tissue ex vivo model [Grivel and Margolis, 2009].

The *ex vivo* tissues have proved to be useful in studies of the effect of HIV-1 copathogens on HIV-1 replication [Grivel et al., 2001; Lisco et al., 2007; Vanpouille et al., 2007] as well as in pre-clinical drug testing [Andrei et al., 2011; Vanpouille et al., 2012]. These tissues *ex vivo* have been shown to support productive infection of the following viruses as well: herpes virus (HHV)-6, HHV-7, HCMV (HHV-5), HSV-2 (HHV-2), vaccinia virus and measles virus [Condack et al., 2007; Grivel et al., 2001; Lisco et al., 2007; Vanpouille et al., 2007]. Moreover, this *ex vivo* system have also been shown to support replication of the bacterium *Borrelia burgdorferi* [Duray et al., 2005] and of the parasite *Toxoplasma gondii* [Sassi et al., 2009]. Recently, these tissues *ex vivo* were also used to study the role of vaginal lactobacilli against HIV-1 [Nahui Palomino et al., 2017]. In summary, the explant system of cultured human tissues *ex vivo* permits the study of normal and

pathogenic processes, including the ones caused by human infectious agents, in the context of tissue cytoarchitecture under controlled laboratory conditions.

The technique of culturing human tissue *ex vivo* to study the pathogenesis of HIV and other infectious agents consists of the following steps, briefly: the tonsillar and mucosa layers from ecto- and endo-cervix (Figure 6) tissues are cut in blocks of 2 mm^3. Cervico-vaginal tissue blocks are infected with viral stock. After infection, tissue blocks are washed to eliminate the free virions then transferred at the liquid-air interface onto Gelfoam in a 12-well plate containing RPMI 1640 medium at 1 mL/well supplemented with 15% FBS, sodium pyruvate at 1 mM, non-essential amino acids at 1 mM, and antibiotics (gentamicin sulfate at 50 µg/mL, amphotericin B at 2.5 µg/mL). Instead, tonsillar tissue blocks are placed on collagen sponge gels in 6-well plate, and then tissue blocks are infected with viral stock, on top of each block. Cervico-vaginal and tonsillar tissue are incubated at 37°C for 12 days, with replacement of culture medium every 3 days.

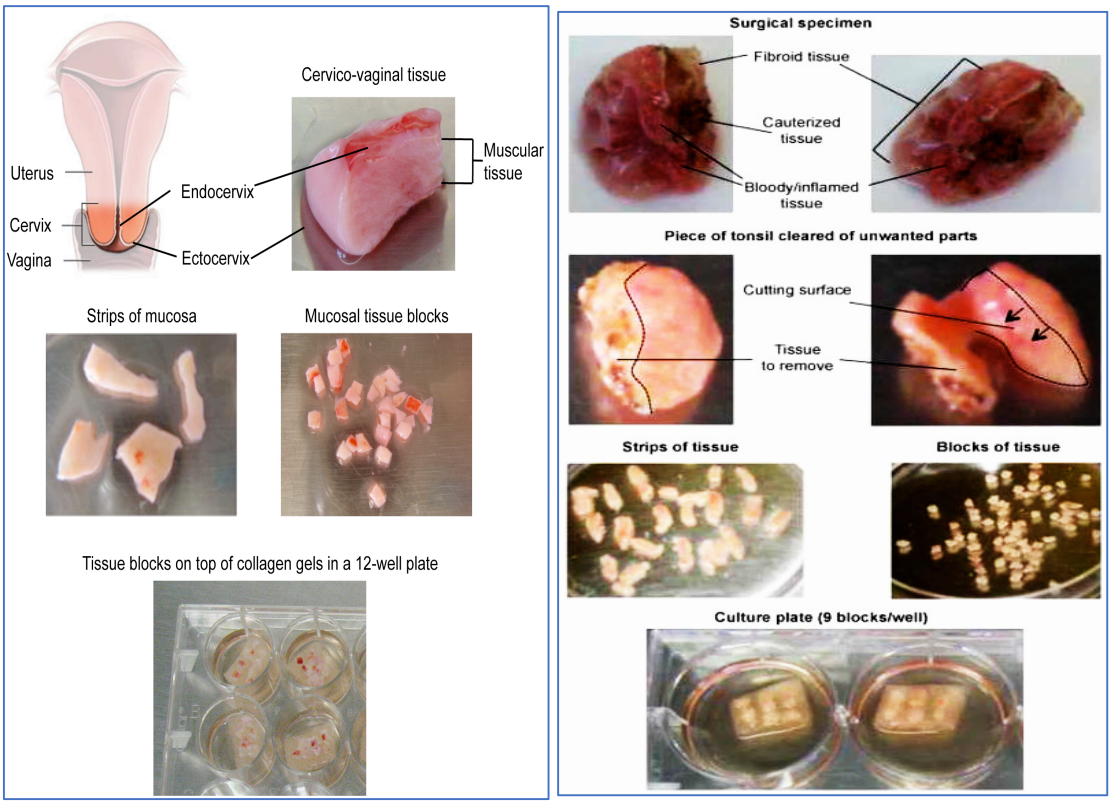

Figure 6. Preparation of cervico-vaginal and tonsillar tissues for histoculture [Introini et al., 2014].

2. AIM OF THE RESEARCH

The vaginal microbiota of healthy reproductive-age women is generally dominated by *Lactobacillus* species [Ravel et al., 2011]. The lactobacilli are considered "health promoting" microorganisms since they play an active role in maintaining a proper balance of the vaginal microbiota by preventing overgrowth of pathogenic and opportunistic microorganisms [O'Hanlon et al., 2013; Ronnqvist et al., 2006]. Indeed, lactobacilli play a key role in the prevention of numerous urogenital diseases, such as bacterial vaginosis, yeast infections, as well as sexually transmitted infections (STIs) [Cherpes et al., 2003; Martin et al., 1999; Taha et al., 1998].

Vulvovaginal candidiasis (VVC) is a common fungal infection compromising the quality of life of numerous women. *Candida* infection affects 70-75% of women at least once during their lives [Peters et al., 2014]. Many lactobacilli are known to inhibit the growth of *Candida* spp. but the mechanisms underlying antifungal activity are still not clearly understood. The development of VVC has been associated with either a low number of lactobacilli in the vagina or with the presence of H_2O_2-non-producing *Lactobacillus* species [Goffeng et al., 1997; Vitali et al., 2007].

Chlamydia trachomatis is the most common causative agent of bacterial STIs in the world, with more than 100 million new cases per year according to global estimates [Senior, 2012]. About 70% of infections caused by this pathogen remains asymptomatic and thereby left untreated, representing a relevant public health problem. If not adequately treated, *C. trachomatis* infection induces pelvic inflammation, cervicitis, endometritis, and salpingitis which could cause infertility and ectopic pregnancy [Malhotra et al., 2013]. Despite the importance of a healthy vaginal microbiota in preventing genital infections, only a few studies have focused on the protective effects of vaginal lactobacilli towards chlamydial infection [Gong et al., 2014; Mastromarino et al., 2014; Rizzo et al., 2015], and therefore the molecular mechanisms underlying the interactions between *Lactobacillus* and *C. trachomatis* in the vaginal environment have not yet been elucidated.

Moreover, the female genital tract mucosa is a portal of entry for several STI viruses, such as HIV. Today, women constitute more than half of all people living with HIV [CDC report

2017]. A *Lactobacillus*-dominated microbiota appears to be a biomarker for healthy vaginal communities, as changes in the vaginal microbiota, especially shifting away from *Lactobacillus* dominance, are associated with bacterial vaginosis and increased risk of acquisition of sexually transmitted infections, in particular HIV [Atashili et al., 2008; Gosmann et al., 2017; Mirmonsef and Spear, 2014; Nardis et al., 2013; Petrova et al., 2015; Taha et al., 1998]. Nevertheless, the exact mechanisms of HIV inhibition by vaginal lactobacilli remain to be fully clarified.

In this context, the aim of this study was to investigate the potential antagonistic effect of vaginal lactobacilli toward *Candida* spp., *C. trachomatis,* and HIV, which are among the infectious agents that most significantly affect women's health worldwide.
In particular, from vaginal swabs of healthy premenopausal women, we isolated seventeen *Lactobacillus* strains belonging to 3 species mainly represented in the human vaginal microbiota: *L. crispatus* (BC1-BC8), *L. gasseri* (BC9-BC14), and *L. vaginalis* (BC15 - BC17) [Parolin et al., 2015]. It was evaluated their capacity to produce antimicrobial compounds to individuate a possible antimicrobial mechanism of action i.e, production of lactic acid, hydrogen peroxide, butyrate. It was studied their fungistatic/fungicidal activity against numerous clinical isolates of *Candida* spp., and their capacity to interfere with yeast adhesion to HeLa cells [Parolin et al., 2015]. It has been evaluated the capacity of lactobacilli to inhibit the infectivity of *C. trachomatis* on HeLa cells [Nardini et al., 2016]. Moreover, we studied the metabolic profiles of lactobacilli supernatants by ^{1}H-NMR to identify active metabolites and to find correlations between metabolism of lactobacilli and anti-*Candida* or anti-*Chlamydia* effect [Nardini et al., 2016; Parolin et al., 2015]. The potential role of vaginal lactobacilli in the mechanisms of transmission and pathogenesis of HIV was studied in *ex vivo* human cervico-vaginal and tonsillar tissues [Nahui Palomino et al., 2017].

A major potential application of this research is the identification of active *Lactobacillus* strains to propose as probiotics for prophylaxis and/or adjuvant therapy for the different urogenital disturbances that strongly affect women's health.

3. MATERIALS AND METHODS

3.1 Isolation of vaginal lactobacilli and characterization of anti-*Candida* activity

3.1.1 Isolation and taxonomic characterization of vaginal lactobacilli

Fifteen pre-menopausal Caucasian women, aged between 18–45 years old, were recruited for the present study. The women were non-menstruating, with no symptoms of reproductive tract infection, and not receiving oral/local antimicrobial therapy within the previous 2 weeks. All volunteers provided a written informed consent in accordance with the Ethics Committee of the University of Bologna and the institutional review board approved the study (52/2014/U/Tess). Mid-vaginal secretions were self-collected by women with E-swabs (Copan, Brescia, Italy) and immediately processed for lactobacilli isolation. The specimens were coded to assure full anonymousness.

Lactobacillus clones were isolated onto de Man, Rogosa, and Sharpe (MRS) and Brain-Heart Infusion (BHI) agar plates (Difco, Detroit, MI), both supplemented with 0.05% L-cysteine. Plates were incubated anaerobically for 24 h at 37°C in anaerobic jars containing GasPak EZ (Becton Dickinson, Sparks, MD). Colonies with different morphologies yielding variable rods by microscope observation were selected for glycerol stock preparation. Thereafter, genomic DNA was extracted from lactobacilli using DNeasy Blood&Tissue Kit (Qiagen, Hilden, Germany) following the protocol "Pretreatment for Gram-positive bacteria". The extracted DNA was amplified with *Lactobacillus* genus-specific primers Lac1 (AGC AGT AGG GAA TCT TCC A) and Lac2 (ATT YCA CCG CTA CAC ATG) [Walter et al., 2001]. The positive isolates were taxonomically characterized to the species level by sequencing the 16S ribosomal RNA (rRNA) gene. Briefly, the complete 16S rRNA gene (1.5 kb) was amplified with the universal primers 27F (AGA GTT TGA TCM TGG CTC AG) and 1492R (TAC GGY TAC CTT GTT ACG ACT T) and then sequenced [Lane, 1991]. The obtained sequences were compared with the sequences available in the Ribosomal Database Project (RDP, http://rdp.cme.msu.edu/) in order to identify the *Lactobacillus* species [Cole et al., 2009]. The nucleotide sequences of the 16S rRNA genes of the *Lactobacillus* strains BC1 to BC17 have been deposited in

the DDBJ nucleotide sequence database (http://www.ddbj.nig.ac.jp/) under accession numbers AB976542 to AB976558.

3.1.2 Determination of lactobacilli hydrogen peroxide production

Lactobacillus strains were tested for their ability to produce H_2O_2 as described by Pendharkar et al. [Pendharkar et al., 2013] with slight modifications. Isolates were cultured onto MRS agar plate containing 0.25 mg/mL of 3,3', 5,5'-tetramethylbenzidine and 0.01 mg/mL of horseradish peroxidase in anaerobic condition for 72 h. Plates were exposed to air and based on the time required for the appearance of the blue color, isolates were scored as low (>20 min; score 1), medium (10–20 min; score 2), and high (<10 min; score 3) H_2O_2 producer strains. Isolates not producing blue coloration were scored as 0.

3.1.3 Preparation of lactobacilli fractions

Lactobacilli were cultured in MRS or BHI broth supplemented with 0.05% L-cysteine. Incubation was carried out in anaerobic jars supplemented with GasPak EZ overnight at 37 °C. The turbidity of lactobacilli cultures was measured spectrophotometrically, considering that an optical density (OD_{600}) of 0.4 corresponds to a cell concentration of 10^8 colony forming unit (CFU)/mL. Lactobacilli cultures, corresponding to 5×10^8 CFU/mL, were centrifuged at 5,000 × g for 10 min at 4 °C. Supernatants were filtered through a 0.2 μm membrane filter to obtain stock of *Lactobacillus*-conditioned medium (CM). *Lactobacillus*-cell pellet (CP) were washed and resuspended in sterile saline containing 0.05% L-cysteine to obtain a stock bacterial suspensions of 5×10^8 CFU/mL.

3.1.4 ^{1}H-NMR analysis

One mL of *Lactobacillus*-CM was added to 160 μL of a D_2O solution of 3-(trimethylsilyl)-propionic-2,2,3,3-d4 acid sodium salt (TSP) 6.25 mM set to pH 7.0 by means of a 100 mM phosphate buffer. ^{1}H-NMR spectra were recorded at 298 K with an AVANCE III spectrometer (Bruker, Milan, Italy) operating at a frequency of 600.13 MHz. To avoid the

presence of broad signals arising from slowly tumbling molecules, a T_2 filter of 400 echoes, separated by an echo time of 400 μs, was applied. The signals were assigned by comparing their chemical shift and multiplicity with Chenomx software data bank (Chenomx Inc., Canada, ver 8.02). To understand the correlation between the taxonomy/anti-*Candida* activity and metaboloma of lactobacilli, a Principal Component Analysis (PCA) was performed. The PCA algorithm calculates and sorts linear combinations of the original variables, so to highlight the data structure by means of a low number of orthogonal projections (Principal Components). The score-plot is the representation of the samples in the generated space and highlights the similarities and differences between the samples.

3.1.5 Evaluation of fungistatic/fungicidal activities of lactobacilli supernatants

All *Candida* strains used in the present study were isolated from routine diagnostic procedures at the Microbiology Laboratory of Sant'Orsola-Malpighi University Hospital of Bologna. In particular we used 4 isolates of *C. albicans*, and 5 isolates of non-*C. albicans*: *C. glabrata*, *C. krusei*, *C. tropicalis*, *C. parapsilosis,* and *C. lusitaniae*. *Candida* strains were grown aerobically in Sabouraud dextrose (SD) medium (Oxoid, Basingstoke, Hampshire, UK) at 35°C.

The fungistatic activity of *Lactobacillus*-CM was determined by broth microdilution test in accordance with the European Committee on Antimicrobial Susceptibility Testing guidelines (EUCAST, http://www.eucast.org/) with slight modifications. Briefly, from 24-h *Candida* cultures were prepared *Candida* stock suspensions at OD_{530} of 0.5 MacFarland with sterile water. *Candida* stock suspensions were diluted 1:10 with RPMI 1640 medium (EuroClone, Pero, Italy), buffered to pH 7 with 0.165 M morpholinepropanesulfonic acid and 2% glucose, in order to obtain a yeast suspension of $1–5 \times 10^5$ CFU/mL. Thereafter, each well of a flat-bottom microdilution plate was inoculated with 100 μL of yeast suspension, subsequently filled with 100 μL of *Lactobacillus*-CM, and incubated at 35°C for 24/48 h. A control well contained 100 μL of sterile MRS medium and 100 μL *Candida* suspension was performed as well. The results were obtained by measuring the absorbance at 450 nm. Anti-*Candida* activity of *Lactobacillus*-CM was determined by considering at least 50% of reduction in growth in comparison to the control. To determine a fungicidal

effect, 20 µL of samples from wells exhibiting less than 50% of growth were spotted onto SD agar plates and incubated at 35°C for 24/48 h. Fungicidal activity was defined as a reduction of at least 3 $\log_{10}$ from the starting inoculum [Pendharkar et al., 2013]. The fungistatic/fungicidal activity of metabolites (butyrate, orotate, pyroglutamate, and isoleucine), whose concentrations were significantly greater in the supernatant of lactobacilli strains with anti-*Candida* activity, was tested as well.

3.1.6 Adhesion assays

Lactobacillus adherence capacity to HeLa cells was evaluated in tubes containing sterile coverslips as previously reported [Mastromarino et al., 2002; Verdenelli et al., 2014] with slight modifications. Briefly, one mL of HeLa cell suspension, at a concentration of 5×10^4 cells/mL, was seeded onto each glass coverslip and incubated in Dulbecco's minimal essential medium (DMEM, EuroClone, Pero, Italy), supplemented with 10% foetal bovine serum (FBS), 1% L-glutamine, 100 IU/mL penicillin G, and 100 µg/mL streptomycin at 37°C in 5% CO_2. After 48 h, the cells were washed twice with PBS, added 900 µL of DMEM free antibiotics, and treated with 100 µL of *Lactobacillus*-CP (stock 5×10^8 bacteria/mL). The tubes were then incubated for 1 h at 37°C. Cell monolayers were washed several times in PBS, fixed with May-Grünwald, and stained with Giemsa. The results were obtained at light-microscopy (1000×) and HeLa cells were scored according to the number of lactobacilli attached, chosen 200 cells randomly. Each adherence assay was conducted in duplicate.

Three types of assays were performed to study the mechanisms of interference of vaginal *Lactobacillus* (CM or CP) with the adherence of *Candida* on HeLa cells: exclusion, competition, and displacement [Osset et al., 2001]. *C. albicans* 1 was chosen as model strain. *C. albicans* was cultured in BHI broth at 30°C for 18 h under constant shaking. Yeast cells were centrifuged at 5,000 × g for 10 min, washed, and resuspended in PBS to obtain a stock of 5×10^8 yeasts/mL. In the exclusion assay, 100 µL of *Lactobacillus*-CM or CP (from stock 5×10^8 bacteria/mL) were incubated for 1 h at 37°C on HeLa cells. Afterwards, 100 µL of *Candida* cells (stock of 5×10^8 yeasts/mL) were added and incubated for 1 h. In the competition assay, lactobacilli fractions and *Candida* were

inoculated simultaneously onto HeLa cells and incubated for 1 h at 37°C. In the displacement assay, *Candida* cells were inoculated onto HeLa cells for 1 h at 37°C. Successively, lactobacilli fractions were added and further incubated for 1 h. Yeast adhesion to HeLa cells was assessed by microscopy (400×) after Giemsa staining by counting the number of *Candida* cells attached to 200 randomly chosen HeLa cells. The results were expressed as the percentage of *C. albicans* adhered to each HeLa cell compared to the control without lactobacilli fractions (control value 100%). All experiments were conducted three times and in triplicate for each experimental condition.

3.1.7 Histone acetylation profile analysis

To study the capacity of lactobacilli to induce histone acetylation on *Candida*, *C. albicans* 1 was chosen as model strain. Log-phase yeast cells were inoculated at an OD_{600} of 0.5 in *Lactobacillus*-CM or MRS broth (negative control) and incubated at 30°C for 6 h. Sodium butyrate (20 mM) was used as positive control; and a culture of *S. aureus* in MRS was used as a representative Gram-positive organism. Histones were extracted from yeast cultures as described by Knapp *et al*. [Knapp et al., 2007] with slight modifications. A volume equivalent to 20–40 OD_{600} units of each culture was collected and subjected to nuclei isolation. Nuclei were washed for 15 min with wash buffer (10 mM Tris–HCl, pH 8, 75 mM NaCl, 30 mM Na-Butyrate, 0.5% NP-40, 1.0 mM PMSF, and 10 μg/mL each of protease inhibitors) in ice. Washes were repeated four times. Histones were extracted by incubating nuclei in H_2SO_4 0.4N for 1 h in ice, then precipitated overnight in acetone at -20°C. Equal amount of histones were loaded to a 15% acrylamide gel and separated by SDS-PAGE, then transferred to a nitrocellulose membrane and probed with anti-acetyl Lysine primary antibody (Merck Millipore, Darmstadt, Germany) and peroxidase-conjugated anti-mouse IgG secondary antibody (GE Healthcare, Milan, Italy). Peroxidase activity was detected by Westar XT system (Cyanagen, Bologna, Italy). Digital images and densitometric analyses were performed by using the GS-800 calibrated densitometer (Bio-Rad Laboratories, Milan, Italy). For each strain, histone acetylation profile was analyzed in triplicate.

3.1.8 Statistical analysis

Differences in the metabolome composition were assessed by means of a two-tailed unpaired Wilcoxon test, through the homonym function implemented in R computational software. Linear correlations between fungistatic/fungicidal activities and metabolome were assessed by means of ANOVA test. For the adhesion and interference assays data analysis were performed by using ANOVA test (GraphPad Prism version 5.02 for Windows, San Diego, CA). Results were expressed as mean ± Standard Error of the Mean (SEM). Differences were deemed significant for P values < 0.05 or highly significant for P values < 0.01.

3.2 Antagonistic effect of vaginal lactobacilli toward *Chlamydia trachomatis* infection

3.2.1 Preparation of *Chlamydia* elementary bodies (EBs)

C. trachomatis strain GO/86, serotype D, was used in this study [Finco et al., 2011; Marangoni et al., 2015]. This strain was isolated from urethral swab at the Microbiology Laboratory of Sant'Orsola-Malpighi University Hospital of Bologna. For the *Chlamydia* EBs preparation, confluent HeLa cells were infected with *Chlamydia* in DMEM medium supplemented with cycloheximide 1 μg/mL, which block cellular macromolecular synthesis and thus promote the intracytoplasmic development of *Chlamydia*, in constant agitation at 640 × g for 2 h to facilitate cell penetration, and then incubated at 37°C with 5% CO_2 for 48 h [Mastromarino et al., 2014]. HeLa cells were afterward detached and fragmented by sonication. Samples were centrifuged at 500 × g for 10 min at 4°C, and supernatants, which contain EBs, were further centrifuged at 40,000 × g at 4°C for 1 h. The resulting pellets, containing the purified EBs, were resuspended in sucrose-phosphate-glutamate 0.2 M, divided into small aliquots, and stored at -70°C.

3.2.2 *C. trachomatis* inhibition test

Chlamydia inhibition experiments were tested using three dilutions of *Lactobacillus*-CM (1:1, 1:10, and 1:100) and three different *Lactobacillus*-CP concentrations (2.5×10^8, 2.5×10^7, and 2.5×10^6 CFU/mL). All lactobacilli were cultured in MRS medium and their fractions were obtained as described earlier (see Materials and Methods section 3.1.3). *Lactobacillus*-CM and CP (100 µL, 10 µL, 1 µL of stock suspension) were mixed with 5×10^3 *Chlamydia* EBs and diluted to 200 µL with sterile PBS. pH values were measured in the final volume. The same amount of *Chlamydia* EBs was used as control after verifying the lack of effects exerted by MRS medium on EBs infectivity. Mixes were incubated for 7, 15, and 60 min at 37°C in 5% CO_2 atmosphere, afterward they were centrifuged at 20,000 × g for 10 min at 4°C. Supernatants were used to infect HeLa cells, grown to confluence in individual tubes containing sterile coverslips, in constant agitation at 640 × g for 2 h, and then incubated at 37°C with 5% CO_2 for 48 h. In order to evaluate the effect of both pH and organic/inorganic acids against *C. trachomatis* infectivity, inhibition experiments were also carried out with lactic acid and hydrochloric acid (HCl) solutions at different concentrations (10 mM and 50 mM) and pH values (pH 4 and 7). The anti-*Chlamydia* effect of orotic acid alone (30 µM) or in combination with lactic acid (10/50 mM, pH 4/7) was tested as well. Moreover, in order to address the importance of glucose depletion in the inhibition of *Chlamydia* infectivity, supernatants of *L. crispatus* BC1 and *L. gasseri* BC13 (dilution 1:1) were added with glucose 30 mM and tested in the *Chlamydia* inhibition experiments.

C. trachomatis infection was evaluated by direct immunofluorescence, using a monoclonal antibody against the chlamydial membrane lipopolysaccharide antigen conjugated with fluorescein (Meridian, Cincinnati, OH) [Marangoni et al., 2015]. The results were obtained by counting *Chlamydia* inclusion-forming units (IFU) under epi-fluorescence microscope (Eclipse E600, Nikon, Japan). The number of IFU was counted in 30 randomly chosen 200× microscopic fields.

3.2.3 Correlation of metaboloma and anti-*Chlamydia* activity

To investigate for correlations between anti-*Chlamydia* activity and metabolome of lactobacilli, a PCA model was built on the concentration changes of the identified molecules (see section 3.1.4), scaled to unit variance. For each molecule, the correlation between the concentration change and the loading value with the component of PCA describing a metabolome-activity link was calculated. When the correlation was higher than 0.6, a statistically significant difference between groups with high activity (H) and low activity (L) was searched by means of a Wilcoxon test, with an accepted Bonferroni-adjusted P value of 0.05.

3.2.4, Statistical analysis

All statistical analysis was performed by using R computational software, applying the non-parametric signed- or matched paired- Wilcoxon rank tests. 1- or 2-tailed tests were used as well. Differences were deemed significant for P values < 0.05. Spearman correlation was calculated by using GraphPad software Prism version 5.02.

3.3 Role of vaginal lactobacilli against HIV-1 replication in human tissues *ex vivo*

3.3.1 *Lactobacillus* culture conditions

Fifteen *Lactobacillus* strains (*L. crispatus* BC1, BC3–BC8; *L. gasseri* BC9–BC14; and *L. vaginalis* BC16, BC17) were cultured overnight in modified medium at 37°C in anaerobic conditions. This modified medium contained 75% RPMI 1640 medium (GibcoBRL, Carlsbad, CA), supplemented with 15% FBS, sodium pyruvate at 1 mM, non-essential amino acids at 1 mM, and 25% MRS broth supplemented with 0.05% L-cysteine. *Lactobacillus* fraction (CM or CP) were obtained as described in section 3.1.3.

3.3.2 *Ex vivo* tissue cultures and HIV-1 infection

Human cervico-vaginal tissue explants obtained from routine hysterectomy (National Disease Research Interchange, Philadelphia, PA) and tonsillar tissue (Children's National Medical Center, Washington, DC) were dissected and cultured as described in Grivel and Margolis [Grivel and Margolis, 2009] with slight modifications. Briefly, the tonsillar and mucosa layers from ecto- and endo- cervix tissues were cut in blocks of 2 mm^3. Eighteen cervico-vaginal tissue blocks were infected with 0.4 mL of viral stock HIV-1_{BaL} (120 ng/mL $p24_{gag}$ obtained from the Virology Quality Assurance Laboratory at Rush University, Chicago, IL) for 2.5 h at 37°C in agitation. After infection, tissue blocks were washed three times with PBS and transferred at the liquid-air interface onto Gelfoam (9 blocks/well) in a 12-well plate containing RPMI 1640 medium at 1 mL/well supplemented with 15% FBS, sodium pyruvate at 1 mM, non-essential amino acids at 1 mM, and antibiotics (gentamicin sulfate at 50 µg/mL, amphotericin B at 2.5 µg/mL). Twenty-seven tonsillar tissue blocks (nine blocks per well in 3 mL of RPMI 1640 medium supplemented as above) were placed on collagen sponge gels, and tissue blocks were infected with 7.5 µL of viral stock, on top of each block. Cervico-vaginal and tonsillar tissue were incubated at 37°C for 12 days, with replacement of culture medium every 3 days. 3TC (lamivudine at 1 µM) was used as a positive control for HIV-1 inhibition.

3.3.3 *Lactobacillus* colonization on tonsillar explants and evaluation of tissue cell depletion

Tonsillar tissues were colonized with fifteen vaginal *Lactobacillus* strains (twenty-seven blocks per condition) at a starting inoculum of 10^4 CFU/mL. At day 3 after inoculation with bacteria, all tissue blocks were collected and digested with collagenase IV (5 mg/mL; GibcoBRL) for 30 min with agitation in a Thermomixer at 900 rpm at 37ºC. Following digestion, tissue cells were filtered with 100 µm cell strainers (Corning) and washed with 50 mL of PBS. Cells were then resuspended in 1 mL of PBS and stained with 1 µL of live/dead Fixable Viability Dye eFluor 450 (EF 450, Invitrogen) for 15 min. After incubation, cells were washed and diluted in staining buffer (PBS, 1% normal mouse

serum, 1% normal goat serum, 1 mM EDTA) and stained with anti-CD3-APC for 20 min. After surface staining, cells were permeabilized with the Fix&Perm Cell Fixation and Cell Permeabilization Kit (Invitrogen) then stained for 20 min with anti-Bcl2-PE, a mitochondrial anti-apoptotic antigen. Data were acquired with a Novocyte flow cytometer (ACEA Biosciences, CA) equipped with 405, 488, and 640 nm laser lines using NovoExpress version 1.2.4 software (ACEA Biosciences) and analyzed using the same software.

3.3.4 HIV-1 infection of human tissues *ex vivo* treated with *Lactobacillus*-CM

Cervico-vaginal and tonsillar tissue blocks were cultured in *Lactobacillus*-CM from six *Lactobacillus* strains (*L. crispatus* BC3, BC5; *L. gasseri* BC12, BC13; and *L. vaginalis* BC16, BC17). Tissue blocks were pre-incubated with *Lactobacillus*-CM undiluted and diluted 1:5 with normal medium for 2 h before HIV-1 infection. After HIV-1 infection, tissue cultures were kept in the same medium (undiluted or diluted 1:5) for the next 3 days of culture, then the medium was replaced with complete RPMI medium every 3 days, and the culture was kept until day 12.

3.3.5 Virucidal effect

Virucidal experiments were carried out by treating HIV-1 with *Lactobacillus*-CP or *Lactobacillus*-CM. HIV-1 viral suspensions at 400 µL were mixed with 100 µL of *Lactobacillus*-CP (stock 5×10^8 CFU/mL), corresponding to a final concentration of 10^8 CFU/mL, or 100 µl of undiluted *Lactobacillus*-CM (corresponding to a final 1:5 dilution) or with 100 µL of normal medium (experimental control condition). Cultures under these three experimental conditions were then incubated for 60 min at 37°C and centrifuged at 4,000 × *g* for 10 min at 4°C. Supernatants were used to infect cervico-vaginal tissue, as described above (see section 3.3.2).

3.3.6 Lactic acid quantification, pH measurement of *Lactobacillus*-CM, and evaluation of their effect on HIV-1 replication

We quantified titers of lactate isomers D and L from overnight-cultured *Lactobacillus*-CM using a lactate quantification assay kit according to the manufacturer's instructions (BioAssay system, EFDLC-100 and EFLLC-100). Isomers D (3 mM), L (23 mM), and D + L (3 mM + 23 mM), corresponding to the average titers found in all undiluted *Lactobacillus*-CM, were tested for HIV-1 inhibition in human cervico-vaginal and lymphoid tissues. Isomers D and L at concentrations corresponding to those found in dilution 1:5 were also tested in lymphoid tissues. pH values in all *Lactobacillus*-CM, undiluted or diluted 1:5, were measured as well. Furthermore, in order to evaluate the effect of low pH on HIV-1 replication in tissues *ex vivo*, as measured in *Lactobacillus*-CM (undiluted average around pH 4 and diluted 1:5 up to pH 6.9), we evaluated HIV-1 infectivity in *ex vivo* tissue at pH 4 and pH 6.9, buffering the medium with HCl.

3.3.7 Evaluation of HIV-1 replication

HIV-1 replication on tissue was evaluated by measuring the levels of $p24_{gag}$ in tissue culture medium using a dynamic immunofluorescent cytometric bead assay as described by Biancotto et al., [Biancotto et al., 2009].

3.3.8 Statistical analysis

All statistical analyses were performed using ANOVA test GraphPad Prism version 7 (GraphPad Prism software Inc., San Diego, CA). Results were deemed significant for p values < 0.05.

4. RESULTS

4.1 Isolation of vaginal lactobacilli and characterization of anti-*Candida* activity

4.1.1 Taxonomy of vaginal lactobacilli and production of antimicrobial compounds

Seventeen *Lactobacillus* strains belonging to 3 species mainly represented in the human vaginal microbiota were isolated from vaginal swabs of 15 healthy premenopausal women. The *Lactobacillus* isolates were taxonomically identified to species level by sequencing the 16S rRNA gene: 8 isolates belong to *L. crispatus* (BC1-BC8), 6 isolates to *L. gasseri* (BC9-BC14), and 3 isolates to *L. vaginalis* (BC15-BC17) (Table 2).

Species	Strain	pH	H_2O_2 (score)	Lactate (mM)	Butyrate (mM)
L. crispatus	BC1	3.93	3	2.91	3.48×10^{-1}
L. crispatus	BC2	4.13	3	6.83	0.00
L. crispatus	BC3	4.21	1	9.45	0.00
L. crispatus	BC4	3.87	1	3.32	3.54×10^{-1}
L. crispatus	BC5	3.70	2	5.10	1.25×10^{-1}
L. crispatus	BC6	4.03	2	7.87	8.33×10^{-1}
L. crispatus	BC7	3.91	1	1.42	1.35×10^{-2}
L. crispatus	BC8	4.08	1	3.05	4.64×10^{-1}
L. gasseri	BC9	3.90	2	4.75	0.00
L. gasseri	BC10	4.54	3	9.40	0.00
L. gasseri	BC11	4.20	3	1.46	0.00
L. gasseri	BC12	4.17	3	9.47	0.00
L. gasseri	BC13	3.87	1	1.62	1.84×10^{-2}
L. gasseri	BC14	4.74	nd*	3.63	1.00×10^{-2}
L. vaginalis	BC15	3.95	1	4.74	6.42×10^{-1}
L. vaginalis	BC16	4.59	1	2.44	0.00
L. vaginalis	BC17	4.28	3	2.34	3.27×10^{-1}

Table 2. Characterization of the vaginal lactobacilli: taxonomy, pH of cultural supernatants, and production of antimicrobial compounds. *H_2O_2 production was scored as low (>20 min; score 1), medium (10–20 min; score 2), and high (<10 min; score 3). Lactate and butyrate were measured in Lactobacillus-CM by ^{1}H-NMR analysis.* ****nd: not determined.***

To characterize the antimicrobial properties of the vaginal lactobacilli isolated in this study, we measured the pH of *Lactobacillus*-CM, production of H_2O_2, lactate, and butyrate (Table 2). The pH of the lactobacilli supernatants was in the range 3.7–4.7, showing the ability of all lactobacilli strains to maintain an acidic environment. H_2O_2 was produced by the totality of the strains. The levels of H_2O_2 production did not seem related to a particular species. The strongest H_2O_2-producers (score 3) were *L. crispatus* (BC1, BC2), *L. gasseri* (BC10-BC12), and *L. vaginalis* BC17. *Lactobacillus gasseri* BC14 was not tested for H_2O_2 production since its incapacity of growing on MRS agar plates and in BHI agar plates tetramethylbenzidine and horseradish peroxidase precipitated as crystals. Lactate was produced by all isolates at concentrations ranging from 1.42 to 47.4 mM. The highest capacity to produce lactate was showed by *L. vaginalis* species, in particular *L. vaginalis* BC15. Butyrate was produced by 9 out of 17 lactobacilli at concentrations ranging from 1.00×10^{-2} to 8.33×10^{-1} mM. The highest levels of this metabolite were found in the supernatants of *L. crispatus* BC6 and *L. vaginali*s BC15. Production of butyrate appeared to be negligible in *L. gasseri* species and variable in the other two species.

4.1.2 Fungistatic and fungicidal activities of vaginal lactobacilli

The fungistatic and fungicidal activities of *Lactobacillus*-conditioned medium (CM) were evaluated against 4 clinical isolates of *C. albicans* and 5 species different than *C. albicans* (*C. tropicalis*, *C. krusei*, *C. parapsilosis*, *C. glabrata,* and *C. lusitaniae*) (Figure 7). In general, *Lactobacillus* strains were more active toward *C. albicans* isolates. On the other hand, none of the *Lactobacillus* strains showed activity against *C. krusei* and *C. parapsilosis*. The strains that showed the broadest spectrum of anti-*Candida* activity were *L. crispatus* (BC1, BC4, BC5) and *L. vaginalis* BC15, since they exerted fungicidal activity against all strains of *C. albicans* and *C. lusitaniae*. In addition, *L. crispatus* BC1 and *L. vaginalis* BC15 exhibited fungistatic activity towards *C. tropicalis* and *C. glabrata*, showing the broadest anti-*Candida* profile. *Lactobacillus crispatus* BC4 and BC5 were fungistatic towards eighter *C. tropicalis* or *C. glabrata*. A good spectrum of activity was also shown by *L. crispatus* BC7 that was fungicidal for all *C. albicans* isolates and fungistatic for *C. tropicalis* and *C. glabrata*. Among the remaining lactobacilli, exhibiting

an intermediate profile of antifungal activity, the most interesting were *L. crispatus* BC3 and BC6 (fungicidal against 3 species of *C. albicans* and *C. lusitaniae*), and *L. crispatus* BC2 (fungicidal against 2 species of *C. albicans* and *C. lusitaniae*). The less active strains were *L. gasseri* (BC10, BC11, BC14) and *L. vaginalis* BC16. *Lactobacillus vaginalis* BC16 exerted a fungistatic activity only towards *C. albicans* 1 while *L. gasseri* BC14 was fungistatic for *C. albicans* 1 and *C. glabrata*. *Lactobacillus gasseri* BC10 and BC11 showed no fungistatic/fungicidal activity towards any of *Candida* isolates.

In summary, the anti-*Candida* activity of *Lactobacillus*-CM was strongly associated with *L. crispatus* spp. being highly or moderately active against *Candida*. Conversely, poor anti-yeast activity was exhibited by *L. gasseri* spp. Notably, *L. vaginalis* spp. showed extremely variable profiles of antifungal activity, comprising one highly active strain, one with an intermediate spectrum, and one poorly active.

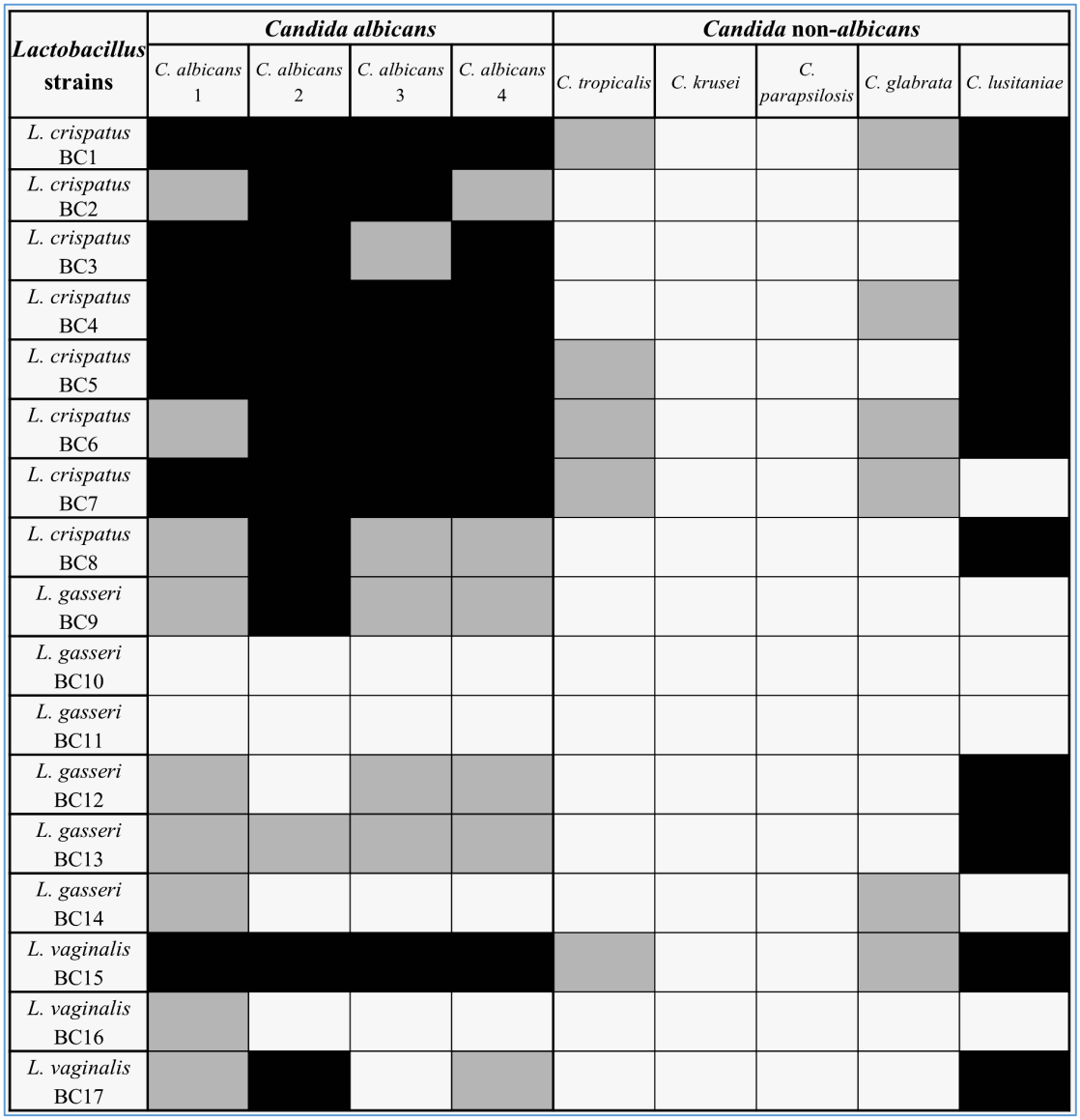

Figure 7. Fungistatic/fungicidal activity of Lactobacillus strains against Candida isolates. *The fungistatic activity of Lactobacillus-CM was determined by broth microdilution test (EUCAST, http://www.eucast.org/). Fungistatic activity of Lactobacillus-CM was determined by considering at least 50% of reduction in growth compared to the control. To determine a fungicidal effect, samples exhibiting less than 50% of growth were spotted onto SD agar plates. Fungicidal activity was defined as a reduction of at least 3 log_{10} from the starting inoculum. Fungicidal (black cells), fungistatic (grey cells), and no anti-fungal activity (white cells).*

4.1.3 Lactobacilli metabolome correlates with taxonomy and fungistatic/fungicidal activity

Lactobacillus-CM of each lactobacilli strain was analyzed by ^{1}H-NMR. We identified 40 molecules mainly belonging to the families of aminoacids, organic acids monosaccharides, ketones, and alcohols (Table 3).

	Molecule																			
Strain	**2-Hydroxybutyrate**	**Leucine**	**Isoleucine**	**Valine**	**Ethanol**	**Propylene glycol**	**Alanine**	**Valerate**	**Acetate**	**Methionine**	**Acetoin**	**Acetone**	**Pyruvate**	**Sarcosine**	**Aspartate**	**N.N-dimethylglycine**	**Creatine**	**Creatinine**	**Choline**	**sn-glycero-3-phosphocholine**
BC1	-0.66	0.86	0.41	0.55	0.08	0.01	1.01	-0.09	-29.21	0.12	0.03	0.64	0.90	0.15	-0.02	0.00	-0.19	-0.30	0.01	-0.20
BC2	-0.37	1.20	0.38	0.51	0.05	0.02	1.00	-0.07	-23.14	0.16	0.02	0.46	0.70	0.15	-0.01	0.00	-0.25	-0.29	0.02	-0.18
BC3	1.53	3.44	1.10	0.89	0.47	-0.01	0.83	-0.20	-17.86	0.22	0.12	0.03	0.77	0.11	-0.01	0.09	-0.35	-0.48	-0.01	-0.20
BC4	-0.72	0.57	0.23	0.35	0.15	0.00	0.61	0.10	-27.97	0.20	0.01	0.42	0.17	0.10	-0.03	0.00	-0.30	-0.33	0.04	-0.22
BC5	-0.27	1.28	0.24	0.33	0.00	0.01	0.65	0.12	-23.89	0.20	0.02	0.33	0.33	0.10	-0.03	0.00	-0.34	-0.37	0.04	-0.15
BC6	0.15	0.94	0.23	0.26	-0.02	0.03	0.03	0.42	-26.06	0.16	-0.01	0.01	0.31	0.12	-0.02	-0.01	-0.36	-0.42	-0.01	-0.18
BC7	1.31	3.40	0.74	0.63	0.49	0.00	1.00	-0.20	-32.95	0.07	0.09	1.80	0.15	0.08	-0.03	0.00	-0.31	-0.46	0.04	-0.17
BC8	2.10	3.35	0.91	0.84	0.21	0.03	0.40	0.48	-30.98	0.24	0.02	0.00	0.12	0.14	-0.03	0.00	-0.33	-0.40	-0.01	-0.22
BC9	-0.92	0.66	0.34	0.55	0.51	0.01	1.30	-0.27	-36.67	0.20	1.98	2.95	0.17	0.16	-0.02	0.00	-0.16	-0.35	0.03	-0.23
BC10	-0.78	0.74	0.52	0.57	0.13	0.02	0.90	0.04	-22.91	0.26	0.00	0.36	0.25	0.13	0.00	0.00	0.30	-0.08	0.00	-0.14
BC11	-0.48	1.34	0.63	0.69	0.01	0.02	0.86	0.06	-24.80	0.27	0.02	0.41	0.40	0.14	0.01	0.00	-0.36	-0.36	0.01	-0.21
BC12	-0.46	1.35	0.77	0.83	0.07	0.01	1.02	0.13	-32.12	0.28	0.06	0.84	0.42	0.14	-0.02	0.00	-0.34	-0.34	0.01	-0.24
BC13	-0.47	1.48	0.58	0.89	0.16	0.01	1.56	0.18	-30.40	0.32	0.01	0.53	0.19	0.14	-0.03	0.12	-0.27	-0.31	0.05	-0.20
BC15	0.96	0.50	0.43	0.07	10.10	0.14	-0.18	0.19	-5.22	-0.14	0.00	-0.06	0.29	0.02	0.02	0.04	-0.23	-0.37	-0.16	-0.18
BC16	1.67	1.96	0.83	0.69	36.18	0.33	1.33	0.07	-11.55	-0.06	-0.01	0.01	0.25	0.03	0.03	0.08	0.13	-0.03	-0.07	-0.21
BC17	2.00	2.93	0.95	0.76	43.74	0.22	0.31	0.46	-13.63	0.07	-0.01	-0.01	0.14	0.08	0.00	0.00	0.11	-0.12	-0.02	-0.15

	Molecule																			
Strain	**Fructose**	**Pyroglutamate**	**1,3-dihydroxyacetone**	**Lactose4.45**	**Glucose**	**Ribose**	**Uracil**	**2-deoxyuridine**	**Uridine**	**Adenosine**	**Inosine**	**Orotate**	**N-Acetyltyrosine**	**Tyrosine**	**Tryptophan**	**Phenylalanine**	**Cytidine**	**Formate**	**Lactate**	**Butyrate**
BC1	-0.13	-0.16	1.11	0.60	-28.54	0.14	-0.09	-0.07	-0.01	0.15	-0.11	0.03	-0.47	0.03	-0.15	-0.43	-0.06	-0.16	2.91	0.35
BC2	-0.07	0.25	0.79	0.73	-20.89	0.12	-0.06	-0.06	-0.02	0.11	-0.09	0.03	-0.05	0.41	-0.17	-0.04	-0.06	-0.17	6.83	0.00
BC3	-0.01	-0.49	0.96	0.34	-18.98	0.11	-0.05	-0.06	-0.01	0.07	-0.06	0.01	-0.68	0.40	-0.65	0.13	-0.06	-0.09	9.45	0.00
BC4	-0.02	-0.20	0.79	0.79	-24.02	0.08	-0.06	-0.05	-0.01	0.08	-0.09	0.03	-0.11	0.47	-0.02	0.17	-0.07	-0.17	3.32	0.35
BC5	-0.11	-0.43	0.76	0.76	-19.47	0.06	-0.05	-0.05	-0.01	0.05	-0.08	0.02	-0.54	0.11	-0.07	0.16	-0.06	-0.16	5.10	0.13
BC6	0.11	-0.63	0.56	0.27	-19.16	0.05	-0.07	-0.04	0.01	0.05	-0.06	0.02	-0.07	0.13	-0.20	0.13	-0.05	-0.16	7.87	0.83
BC7	0.13	-0.17	0.79	0.60	-23.64	0.09	-0.04	-0.06	-0.01	0.06	-0.09	0.02	-0.04	0.24	-0.12	-0.04	-0.07	-0.17	1.42	0.01
BC8	-0.02	-0.71	0.91	-2.11	-19.66	0.06	-0.09	-0.05	-0.01	0.05	-0.06	0.02	-0.13	0.23	-0.30	0.03	-0.05	-0.17	3.05	0.46
BC9	0.13	1.28	1.28	1.20	-26.73	0.00	-0.06	-0.03	-0.03	0.04	-0.04	0.01	-0.41	0.03	-0.18	-0.29	-0.06	-0.14	4.75	0.00
BC10	-0.02	-0.27	0.92	0.44	-6.11	0.04	0.05	-0.04	-0.02	0.01	-0.05	0.01	-0.17	0.63	-0.21	0.75	-0.04	-0.19	9.40	0.00
BC11	0.04	-0.23	1.39	0.52	-18.10	0.04	-0.01	-0.06	-0.03	0.03	-0.05	0.00	-0.21	0.62	-0.25	0.64	-0.06	-0.16	14.60	0.00
BC12	-0.02	-0.71	1.19	0.50	-21.40	0.06	0.04	-0.08	-0.02	0.06	-0.02	0.00	-0.15	0.53	-0.24	0.52	-0.07	-0.16	9.47	0.00
BC13	-0.03	-0.82	1.92	1.16	-28.83	0.01	-0.10	-0.04	-0.04	-0.01	-0.01	0.01	-0.59	0.70	-0.10	-0.18	-0.07	-0.13	1.62	0.02
BC15	-0.07	-0.60	0.37	-1.09	-26.41	0.03	-0.09	0.03	-0.03	0.00	-0.09	0.03	0.08	-0.22	-0.09	-1.30	-0.05	-0.06	47.40	0.64
BC16	-0.14	0.05	0.14	-0.30	-16.41	0.05	-0.08	-0.01	-0.01	-0.01	-0.04	0.00	-0.15	0.45	-0.60	-0.10	-0.03	0.15	24.40	0.00
BC17	-0.12	0.11	0.39	-2.08	-20.81	0.03	-0.09	-0.02	-0.02	-0.02	-0.07	0.01	-0.57	0.10	-0.28	0.18	-0.03	-0.09	23.40	0.33

Table 3. Metabolites identified by ^{1}H-NMR in Lactobacillus-CM. *Values are expressed as mmol/l. L. gasseri BC14 was not included in the metabolomics analysis because the metabolic profile of BHI supernatant could not be compared with the metabolic profiles of MRS supernatants.*

On the entire set of metabolites a Principal Component Analysis (PCA) and the distribution of *Lactobacillus* strains in relation to the pool of metabolites was performed (Figure 8). This multivariate analysis showed two interesting correlations: (i) metabolome *versus*

taxonomy (PC1, expl. var 28%) and (ii) metabolome *versus* fungistatic/fungicidal activity (PC2, expl. var 17.4%). Figure 8A shows the distribution of the three different species of lactobacilli (*L. crispatus*, *L. gasseri,* and *L. vaginalis*) in relation to the metaboloma. These correlations were best visualized by means of box blots representing the distribution of *Lactobacillus* species (Figure 8B). Figure 8C shows the relation between fungistatic/fungicidal activity and metabolome. In general, metabolic profiles varied according to the *Lactobacillus* taxonomy. In particular, metabolome of *L. vaginalis* significantly differed from those of *L. crispatus* and *L. gasseri* ($p < 0.05$). The highest metabolic heterogeneity was observed within *L. crispatus*, as demonstrated by the width of the corresponding boxplot (Figure 8B). Moreover, the fungistatic/fungicidal activities of lactobacilli were related to their metabolome. Strains with different anti-*Candida* activity were clearly separated in the vertical direction: the most active strains occupied the lower positions while the less active strains were placed in the upper areas of the two-dimensional space represented by the biplot (Figure 8C).

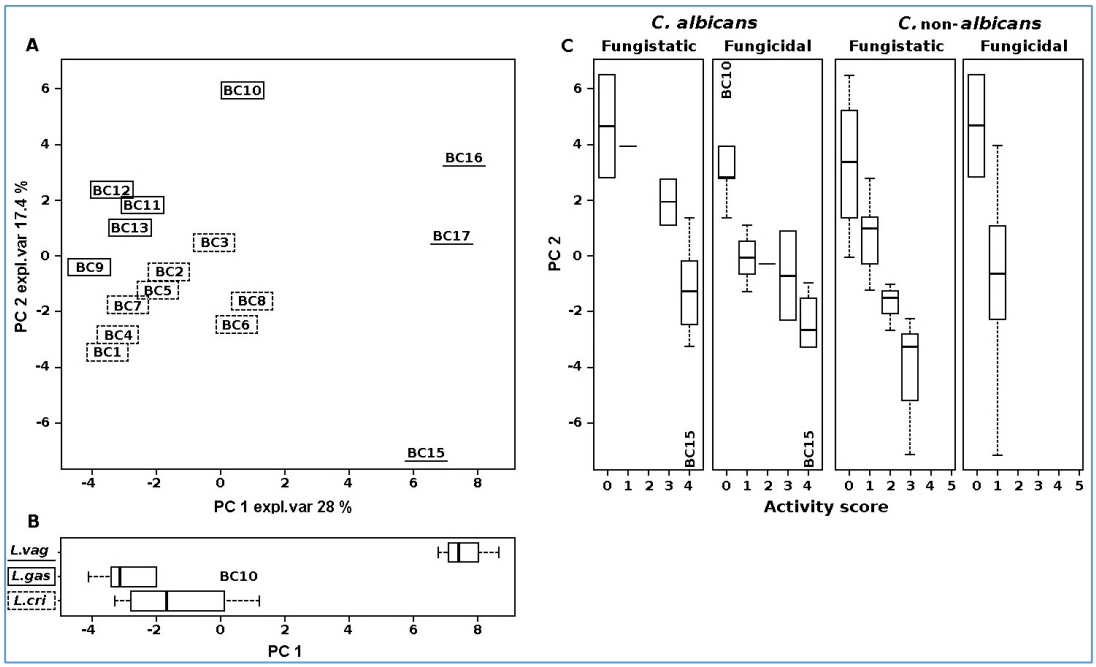

Figure 8. Lactobacilli metabolome correlation with taxonomy and fungistatic/fungicidal activity. (A) *Biplot of a PCA performed on the total metabolites identified by* ^{1}*H-NMR in Lactobacillus-CM. Expl. Var, explained variance.* ***(B)*** *Box plots representing the distribution of Lactobacillus species in relation to the metabolome. Lines within the boxes*

indicate the median values of the samples groups corresponding to L. crispatus, L. gasseri and L. vaginalis species. ***(C)*** *Box plots representing the distribution of fungistatic/fungicidal activity towards C. albicans and C. non-albicans in relation to the metabolome. Lines within the boxes indicate the median values of the samples groups corresponding to the different activity scores (0-4 for C. albicans; 0-5 for C. non-albicans). Each box represents the interquartile range (25–75th percentile). The bottom and top bars indicate the 10th and 90th percentiles, respectively. Outlier values are indicated (BC10 and BC15).*

Next, we searched which metabolites varied in relation to antifungal activity through Wilcoxon univariate statistical test. We identified 4 metabolites (butyrate, orotate, pyroglutamate, and isoleucine) whose concentrations significantly increased ($p < 0.05$) in *Lactobacillus*-CM of active strains. Fungistatic/fungicidal activities of these compounds (alone or in combination) were evaluated, but no anti-*Candida* activity was observed. However, we cannot exclude a synergistic action of these metabolites in the more complex cultural medium where other bacterial molecules may act as enhancers.

4.1.4 Vaginal lactobacilli interfere with *C. albicans* adhesion to HeLa cells

The adhesion of vaginal lactobacilli to epithelial tissue represents the first defense barrier to prevent undesirable microbial colonization [Donnarumma et al., 2014; Rizzo et al., 2013; Verdenelli et al., 2014]. Thus, all *Lactobacillus* strains were examined for their adherence ability to HeLa cells (Figure 9). Adherence capacity varied among the lactobacilli analyzed, in a range between 0.07 ± 0.03 and 17.68 ± 0.78 (mean ± SEM) bacteria/cell. In particular, *L. crispatus* (BC1, BC3) and *L. gasseri* BC8 were the most adhesive strains (> 10 bacteria/cell). *Lactobacillus crispatus* BC2 and *L. vaginalis* BC15 showed an intermediate adhesiveness (2–10 bacteria/cell). The remaining strains showed low adherence capacity (< 2 bacteria/cell). These data demonstrate that the adhesive properties are strain-specific rather than species-specific, varying considerably between strains of the same species.

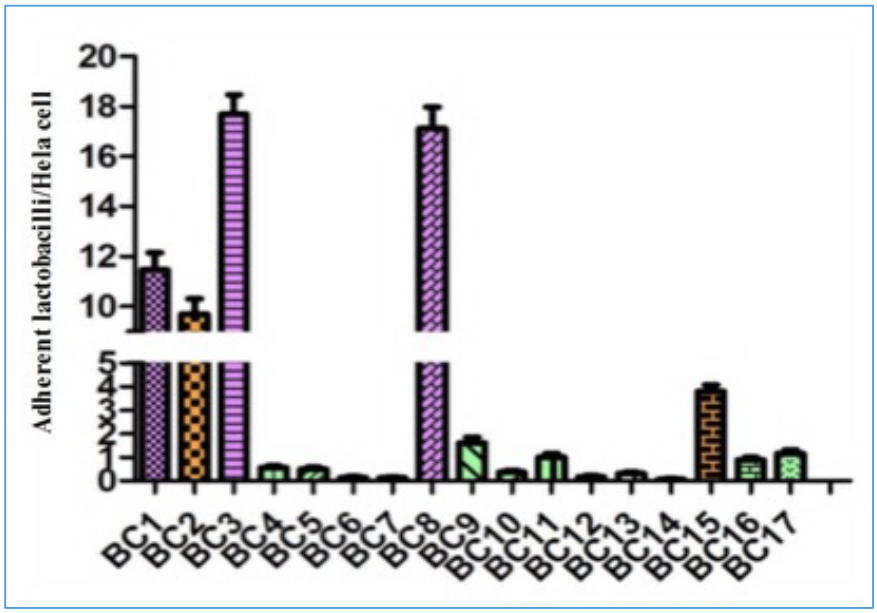

Figure 9. Vaginal lactobacilli adhesion capacity to HeLa cells. *Confluent HeLa cells were treated with Lactobacillus-CP at a concentration 5 × 10^8 bacteria/mL for 1 h at 37°C. Cell monolayers were washed, fixed with May-Grünwald, and stained with Giemsa. The results were obtained at light-microscopy (1000×) and HeLa cells were scored according to the number of lactobacilli attached, chosen 200 cells randomly. The results were expressed as average number of adherent bacteria per cell. Error bars represent SEM.*

To verify the antagonist effect of lactobacilli toward *C. albicans*, the influence of *Lactobacillus* (CM or CP) on the adhesion capacity of the yeast to HeLa cells was investigated (Figure 10). Three mechanisms of adhesion interference were examined: exclusion (Figure 10A), competition (Figure 10B), and displacement (Figure 10C). Ten lactobacilli strains out of seventeen: *L. crispatus* (BC1, BC2, BC5, BC7, BC8), *L. gasseri* (BC9, BC10, BC11, BC12), and *L. vaginalis* BC16 significantly reduced the adhesion of *C. albicans* through all three mechanisms. The inhibitory effect was exerted in some cases by *Lactobacillus*-CP and in other cases by *Lactobacillus*-CM. Particularly, *L. crispatus* BC2, *L. gasseri* BC10, and *L. gasseri* BC11 interfered *Candida* adhesion by both *Lactobacillus*-CP and CM, being these strains the most effective in terms of interference on pathogen adhesion. Only three strains did not exercise any effect on *C. albicans* adhesion: *L. gasseri* BC13, *L. gasseri* BC14, and *L. vaginalis* BC17. The remaining strains showed an intermediate effect on yeast adhesion through one or two of these mechanisms.

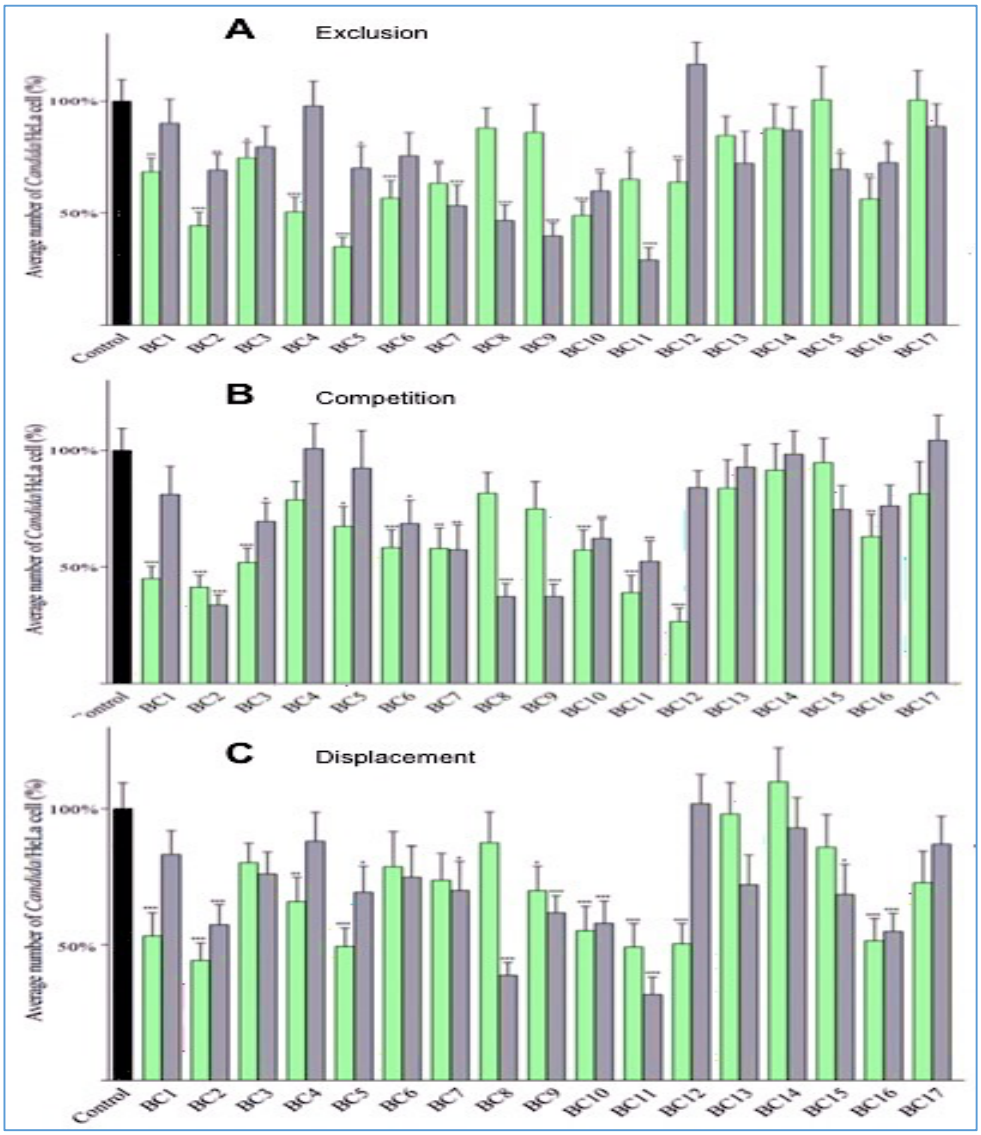

Figure 10. Interference of C. albicans adhesion to HeLa cells by vaginal lactobacilli. *Exclusion* ***(A)****, competition* ***(B)****, and displacement* ***(C)****. The results were expressed as percentages of adherent yeasts per HeLa cell and compared to the control (free of lactobacilli). Yeast adhesion to HeLa cells was assessed by microscopy (400×) after Giemsa staining by counting the number of Candida cells attached to 200 randomly chosen HeLa cells. The control value was taken as 100% of adhesion (black bars). Green and grey bars show the adhesion of C. albicans in presence of Lactobacillus-CP and CM, respectively. Statistical significance was determined at $p < 0.05$*, $p < 0.01$**, and $p < 0.001$***. Error bars represent SEM.*

4.1.5 Lactobacilli effects on *C. albicans* histone acetylation

Both the yeast histone deacetylase (HDAC) inhibition and the consequent histone hyper-acetylation, represent a novel mechanism by which *Candida* growth and adhesion to the host epithelium can be reduced [Simonetti et al., 2007; Smith, 2002]. Thus, we evaluated whether the anti-*Candida* activity and *Candida* adhesion interference exerted by the lactobacilli could be associated to this mechanism. Acetylation profiles of H2/H3 and H4 histones of *C. albicans* 1 were evaluated for all *Lactobacillus*-CM, except for *L. gasseri* BC14. We attributed hyper-acetylating ability of H2/H3 or H4 histones at least equal to those induced by sodium butyrate, used as positive control (Table 4). The majority of lactobacilli induced histone hyper-acetylation. Histones H2/H3 were hyper-acetylated by 12 strains and histone H4 by 11 strains. Both *L. gasseri* BC13 and *L. vaginalis* BC17 were the only strains that did not induce histone acetylation. Interestingly, these lactobacilli showed no fungicidal activity towards *C. albicans* 1 and also any inhibitory effect on the adhesion of the pathogen. These data suggest that the complete lack of inhibition of HDACs could compromise antifungal activity of lactobacilli.

Stimulus	Histone acetylation	
	H2/H3	H4
NaBu	+	+
S. aureus	-	-
L. crispatus BC1	-	+
L. crispatus BC2	+	-
L. crispatus BC3	+	-
L. crispatus BC4	+	+
L. crispatus BC5	+	-
L. crispatus BC6	+	+
L. crispatus BC7	+	+
L. crispatus BC8	+	+
L. gasseri BC9	+	+
L. gasseri BC10	+	+
L. gasseri BC11	-	+
L. gasseri BC12	+	+
L. gasseri BC13	-	-
L. vaginalis BC15	+	+
L. vaginalis BC16	+	+
L. vaginalis BC17	-	-

Table 4. Acetylation of C. albicans histones H2/H3 and H4 by Lactobacillus-CM. *Yeast cells were inoculated in Lactobacillus-CM and incubated at 30°C for 6 h. Histones were extracted from yeast cultures and histone acetylation profile was analyzed by western blot. Sodium butyrate (NaBu, 20 mM) was used as a positive control. (+) Acetylation ≥ NaBu. (-) Acetylation < NaBu.*

4.2 Antagonistic effect of vaginal lactobacilli toward *C. trachomatis* infection

4.2.1 Effects of *Lactobacillus*-CM on *C. trachomatis* infectivity

To investigate the potential antagonist role of vaginal lactobacilli against *C. trachomatis*, we evaluated the ability of seventeen *Lactobacillus* strains to abolish infectivity of *Chlamydia* EBs, before they interact with cellular host receptors.

Three different dilutions (1:1, 1:10, 1:100) of *Lactobacillus*-CM were tested at three different time points (7, 15, 60 min) (Figure 11). After the challenge with *Lactobacillus*-CM, the capacity of *C. trachomatis* EBs to infect HeLa cells was assessed by immunofluorescence.

At the highest supernatant concentration (dilution 1:1), the majority of *Lactobacillus* strains significantly reduced *C. trachomatis* EB infectivity on HeLa cells. Ten *Lactobacillus* strains: *L. crispatus* (BC1-BC8), *L. gasseri* BC9, and *L. vaginalis* BC15 completely abolished the infectivity of *Chlamydia* EBs at any time point. The supernatants of five *Lactobacillus* strains: *L. gasseri* (BC11-BC14) and *L. vaginalis* BC16 decreased *C. trachomatis* infectivity at any contact time, inhibiting completely after a long-term exposure (60 min). *Lactobacillus vaginalis* BC17 showed a moderate anti-*Chlamydia* activity at short contact time (7 min) and *L. gasseri* BC10 did not exert any inhibitory activity (Figure 11A). Supernatants of *L. crispatus* (BC2, BC6, BC7) diluted 1:10 were still capable to reduce significantly *C. trachomatis* infectivity at all three time points. *Lactobacillus crispatus* (BC1, BC3-BC5, BC8), *L. gasseri* (BC9, BC11-BC13), and *L. vaginalis* (BC16, BC17) retained the anti-*Chlamydia* activity at short time points (7 and/or 15 min). *Lactobacillus gasseri* (BC10, BC14) and *L. vaginalis* BC15 did not alter *Chlamydia* EBs infectivity (Figure 11B). At the lowest concentration (dilution 1:100), eleven *Lactobacillus* strains: *L. crispatus* (BC2-BC6, BC8), *L. gasseri* (BC9, BC11, BC13), and *L. vaginalis* (BC16, BC17) decreased *C. trachomatis* infectivity when applied for short contact times, while none of the *Lactobacillus* strains were effective after 60 minutes of exposure. *L. crispatus* (BC1, BC7) and *L. gasseri* BC12 supernatants did not exert any inhibitory activity against *C. trachomatis* EBs (Figure 11C).

In general, *L. crispatus* supernatants were the most active strains in counteracting *C. trachomatis* infectivity, as all of them abrogated *Chlamydia* inclusions at the highest concentration (1:1), and retained their effect at the intermediate (1:10) and lowest (1:100) concentrations, especially *L. crispatus* BC6 and BC8 strains. Six strains (BC2-BC6, BC8) out of eight belonging to *L. crispatus* species maintained a good activity even at 1:100 concentrations; indeed, they caused a significant reduction of *Chlamydia* infectivity for at least one time point. Among *L. gasseri* and *L. vaginalis* strains, heterogeneous activity profiles have been found, especially at the highest concentration: *L. gasseri* BC9 and *L. vaginalis* (BC15, BC16) supernatants were very effective in *C. trachomatis* inhibition, in contrast to *L. gasseri* BC10 and *L. vaginalis* BC17 which showed no *Chlamydia* inhibition.

The inhibitory activity of lactobacilli supernatants towards *C. trachomatis* was strictly concentration-dependent, being fifteen *Lactobacillus*-CM (out of seventeen) effective in reducing EBs infectivity at the highest concentration, whereas only eleven retained a certain efficacy after 1:100 dilution. Notably, at the highest concentration, lactobacilli culture supernatants were found to have pH values comprised in the range 3.71-5.28 (pH mean value 4.19±0.42). On the contrary, diluted lactobacilli supernatants showed pH values, in the range 4.3-7.15 (pH mean value 5.84±0.86) for the dilution 1:10, and in the range 6.76-7.31 (pH mean value 7.08±0.15) for the dilution 1:100. This finding indicated a strict link between acidity and the ability to inactivate *Chlamydia* EBs. Indeed, pH values of culture supernatants were positively correlated with *Chlamydia* IFU/field median values, showing a Spearman coefficient of 0.7486 (two-tailed $p = 9.7357 \times 10^{-29}$). Moreover, both at the intermediate and lower concentration *Lactobacillus*-CM exhibited higher efficacy when applied for short contact times (7 and/or 15 min).

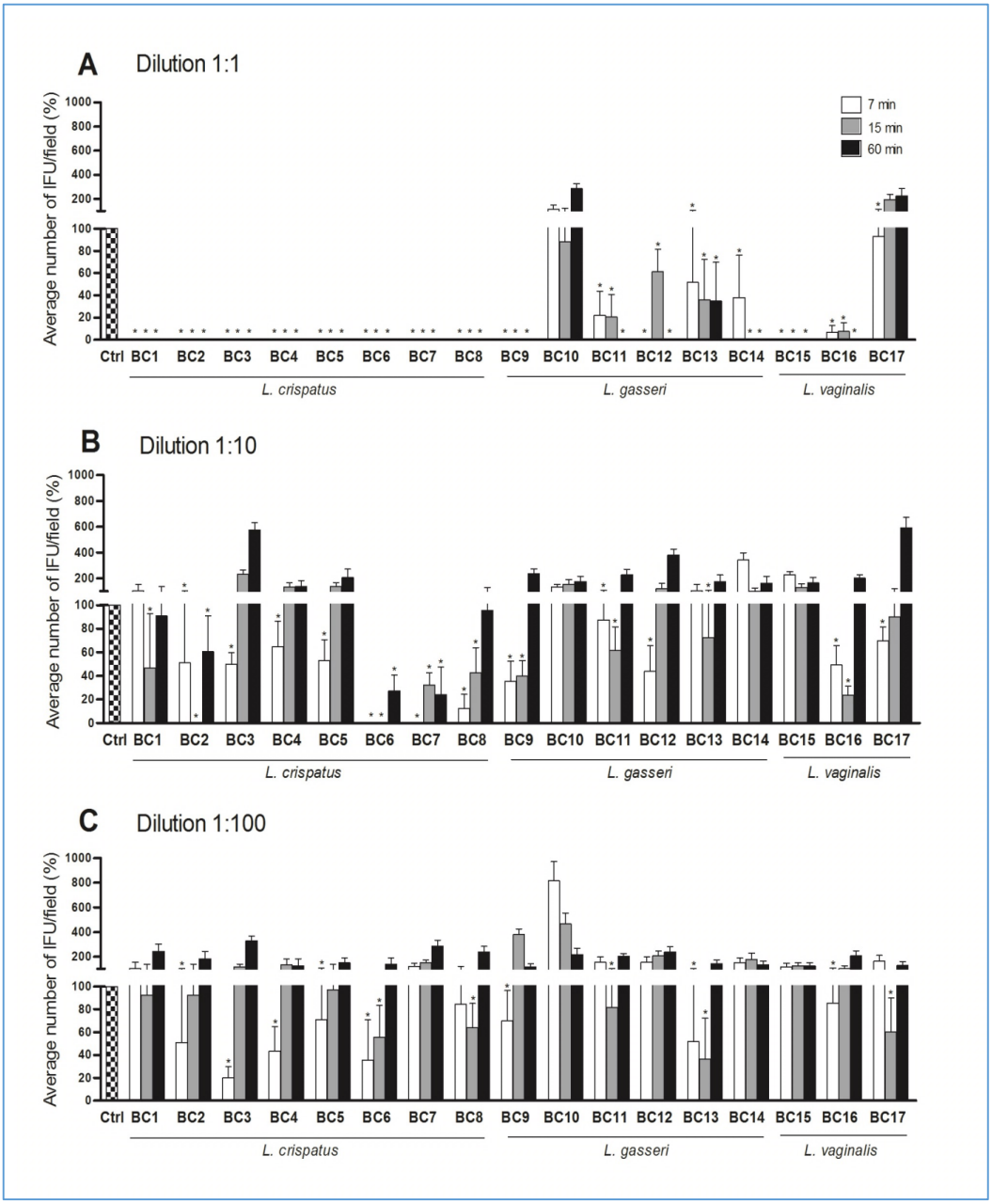

Figure 11. Effect of Lactobacillus-CM on C. trachomatis infectivity. *Experiments were performed with different dilutions of Lactobacillus-CM: 1:1* ***(A)****, 1:10* ***(B)*** *and 1:100* ***(C)****, and different time points: 7 minutes (white bars), 15 minutes (grey bars), and 60 minutes (black bars). Chlamydia trachomatis infectivity was expressed in terms of percentage of inclusions forming units (IFU)/field compared to control. The results were expressed in percentage compared with control, taken as 100% (dotted bars). Bars represent median values and error bars represent median absolute deviations. Statistical significance was calculated vs control. * $p < 0.05$.*

4.2.2 Effects of *Lactobacillus*-CP on *C. trachomatis* infectivity

Similarly to supernatants, *Lactobacillus*-cell pellets (CP) were tested at three different concentrations (2.5×10^8, 2.5×10^7, and 2.5×10^6 CFU/mL) and time points (7, 15, 60 min) (Figure 12).

At the concentration of 2.5×10^8 CFU/mL, five *Lactobacillus* strains: *L. crispatus* (BC1, BC3-BC5) and *L. gasseri* BC13 strongly reduced *C. trachomatis* infectivity at any time point. At short contact times (7 and/or 15 min) a significant inhibitory activity was observed for ten lactobacilli: *L. crispatus* (BC6-BC8), *L. gasseri* (BC9-BC12), and *L. vaginalis* (BC15-BC17). *Lactobacillus crispatus* BC2 and *L. gasseri* BC14 cells did not affect *C. trachomatis* EBs infectivity (Figure 12A). At the intermediate concentration of *Lactobacillus*-CP (2.5×10^7 CFU/mL), only *L. crispatus* BC3 retained a strong inhibitory activity at any time point. *Lactobacillus crispatus* (BC1, BC2, BC4, BC6, BC8), *L. gasseri* (BC9, BC11, BC13), and *L. vaginalis* BC15 significantly reduced *C. trachomatis* infectivity at short exposure times, while *L. crispatus* BC7 showed activity after 60 minutes of contact. No inhibitory effect was exerted by *L. crispatus* BC5, *L. gasseri* (BC10, BC12, BC14), and *L. vaginalis* (BC16, BC17) (Figure 12B). At the concentration of 2.5×10^6 CFU/mL, *L. crispatus* BC3 cells were still able to inhibit *C. trachomatis* infectivity at all contact times, and *L. crispatus* (BC1, BC2, BC4-BC8) and *L. gasseri* (BC9, BC13) were effective at short time points. Cells from *L. gasseri* (BC10-BC12, BC14) and *L. vaginalis* (BC15-BC17) did not show any inhibitory effect (Figure 12C).

In analogy with the results obtained using the lactobacilli supernatants, *L. crispatus* cells were the most effective in reducing *C. trachomatis* infectivity, exhibiting good inhibitory skills at the concentration of 2.5×10^8 CFU/mL, and being almost all active after dilution for at least one time point. *L. gasseri* and *L. vaginalis* cell pellets showed a more concentration-dependent activity, since their dilution caused the loss of *Chlamydia* inhibition for four *L. gasseri* strains (out of six) and for all *L. vaginalis* strains. Moreover, the challenge experiments with lactobacilli cells confirmed the major efficacy for short contact times (7 and/or 15 min), independently of the cell concentration.

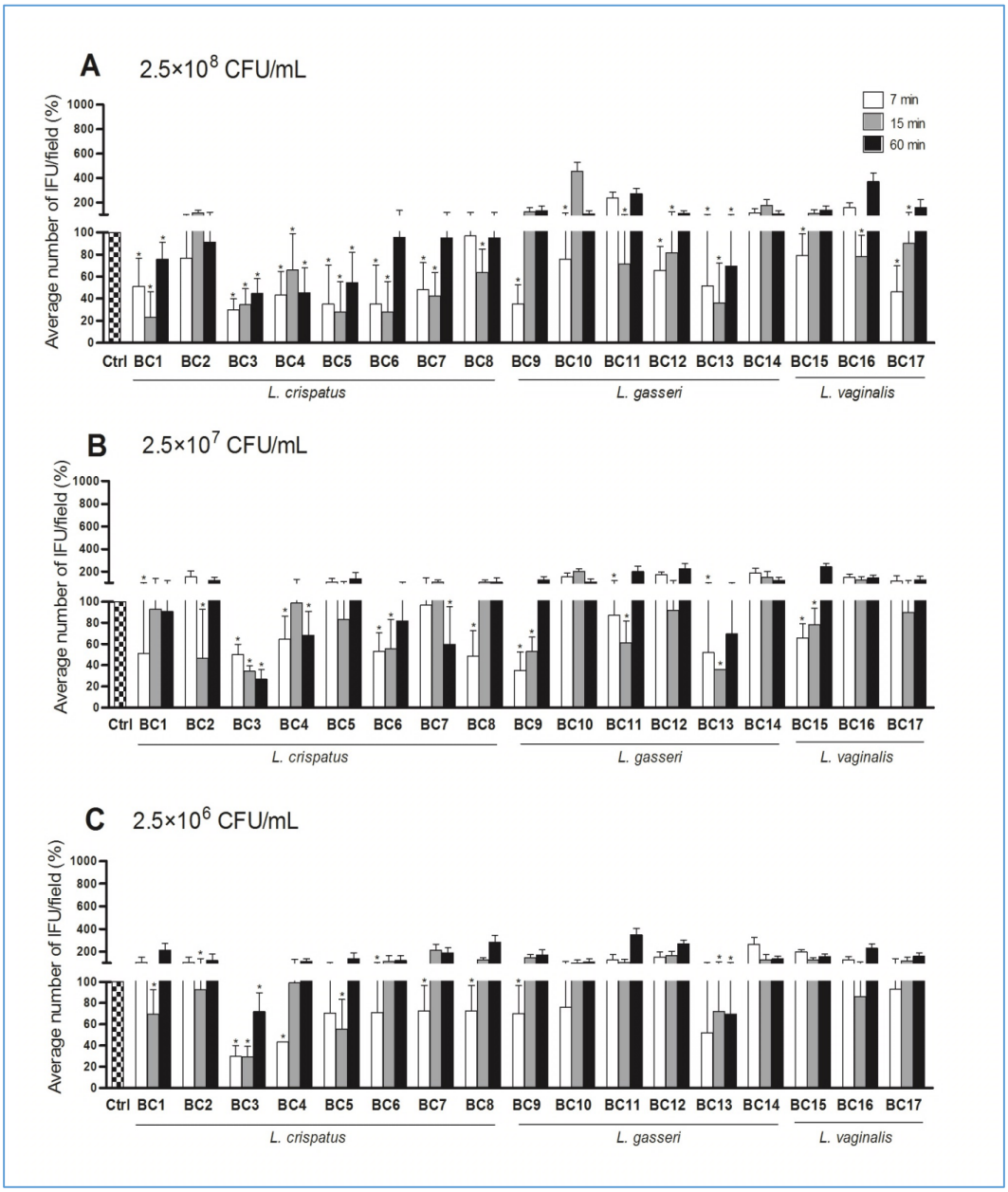

Figure 12. Effect of Lactobacillus-CP on C. trachomatis infectivity. *Experiments were performed at different concentrations of cell pellets: 2.5×10^8 CFU/mL **(A)**, 2.5×10^7 CFU/mL **(B)**, and 2.5×10^6 CFU/mL **(C)**, and different time points: 7 minutes (white bars), 15 minutes (grey bars) and 60 minutes (black bars). Chlamydia infectivity was evaluated as number of IFU/microscopic field. The results were expressed in percentage compared with control, taken as 100% (dotted bars). Bars represent median values, error bars represent median absolute deviations. Statistical significance was calculated vs control. * $p < 0.05$*

4.2.3 *C. trachomatis* inhibition by lactic acid and hydrochloric acid

The effect of lactic acid on *C. trachomatis* infectivity was evaluated after challenge of *Chlamydia* EBs for 7, 15, and 60 min. Two concentrations of lactic acid (10 mM, 50 mM), corresponding to the mean and higher titer registered by ^{1}H-NMR in *Lactobacillus*-CM (Table 3), were tested. Lactic acid solutions were buffered at two pH values (pH 4, pH 7), corresponding to the pH range measured in *Lactobacillus*-CM. The effect of HCl on *C. trachomatis* infectivity was evaluated in the same experimental conditions to compare the effects of lactic acid with an inorganic acid (Figure 13).

At pH 4, lactic acid was able to strongly inhibit EBs infectivity, both at 10 and 50 mM, and for all exposure times. In contrast, lactic acid lost its chlamydiacidal activity when buffered at pH 7, independently of contact time (Figure 13A). HCl did not interfere with *Chlamydia* infectivity, both at 10 mM or 50 mM, and at any pH value (Figure 13B). These results indicate that the presence of a high concentration of H^{+} ions is essential but not sufficient for the inhibition of *Chlamydia* EBs.

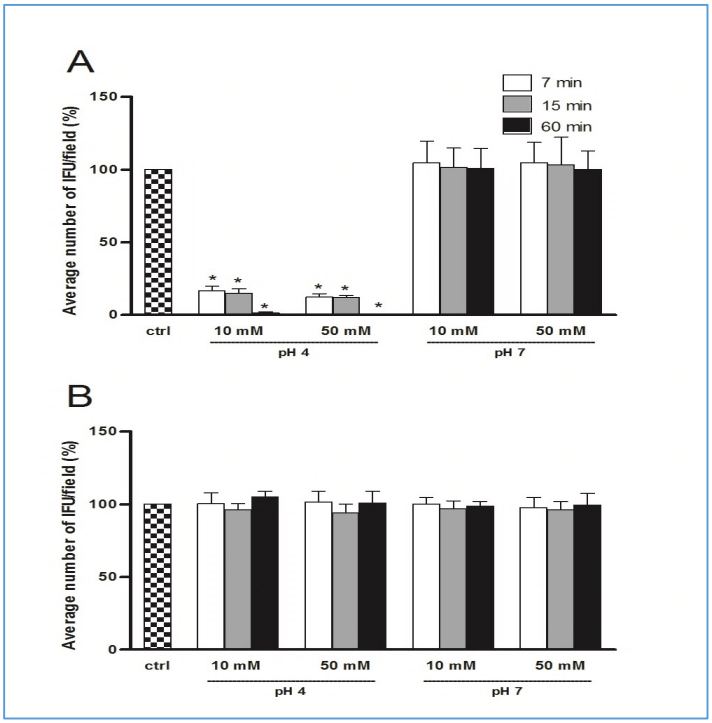

Figure 13. Effect of Lactic/HCl acid on Chlamydia infectivity. *Chlamydia infectivity was evaluated after challenging with lactic* ***(A)*** *or HCl* ***(B)*** *acid at different concentrations (10*

*mM, 50 mM), pH values (4, 7), and time points: 7 min (white bars), 15 min (grey bars), and 60 min (black bars). Infectivity was evaluated as number of Chlamydia IFU/microscopic field. The results were expressed in percentage compared with control (100%, dotted bars). Bars represent median values, error bars represent median absolute deviations. Statistical significance was calculated vs control. * $p < 0.05$.*

4.2.4 *Ranking of lactobacilli in relation to anti-Chlamydia activity*

With the aim to delineate a ranking of *Lactobacillus* strains on the basis of their capability to counteract *Chlamydia* infectivity, we approached a statistical analysis on the entire set of median values, considering any concentration and time point. As a first step, we wondered if *Lactobacillus*-CP and CM fractions were equally effective in reducing *Chlamydia* infectivity. We firstly compared all median data collected from *Chlamydia* EBs pre-incubated with CP fractions with the data of untreated EBs, by means of a non-parametric statistical test: the infectivity of *Chlamydia* EBs pre-incubated with *Lactobacillus*-CP was not significantly different from the infectivity of untreated EBs (p = 0.4245, 1-tailed Wilcoxon signed rank test). Similarly, we compared median data obtained with *Chlamydia* EBs pre-incubated with *Lactobacillus*-CM fractions to data of untreated EBs, and we ascertained that pre-incubation of EBs with CM significantly reduced *Chlamydia* infectivity (p = 0.0384). Indeed, comparing data obtained with CP-treated EBs to those obtained with CM-treated EBs we confirmed that anti-*Chlamydia* effect of *Lactobacillus*-CM were significantly different from that of the respective CP (p = 0.0043). Being *Lactobacillus*-CM the fraction capable of reducing *Chlamydia* infectivity, we classified lactobacilli only on the basis of the anti-*Chlamydia* activity. For each *Lactobacillus* strain, CM efficacy has been expressed as the odds between data collected with CM-treated EBs and untreated EBs (control, taken as 100%), by means of the 1-tailed Wilcoxon signed rank p-values. A low p-value indicates that medians obtained with *Chlamydia* EBs pre-incubated with *Lactobacillus*-CM are different from the control. Conversely, a high p-value denotes that medians obtained with *Chlamydia* EBs pre-treated with *Lactobacillus*-CM are similar to the control. Thus, we classified *Lactobacillus* strains into 3 groups in relation to the inhibitory activity (Figure 14). The first group (H, high

activity) consists of lactobacilli with *p*-values below 0.2; the second group (I, intermediate activity) consists of lactobacilli with *p*-values ranging between 0.2 and 0.6; the last group (L, low activity) comprises lactobacilli with *p*-values over 0.6. Group H counts 7 strains (BC1, BC2, BC4, BC6, BC7, BC8, BC13), group I comprises 5 strains (BC5, BC9, BC11, BC15, BC16), group L included 5 strains (BC3, BC10, BC12, BC14, BC17).

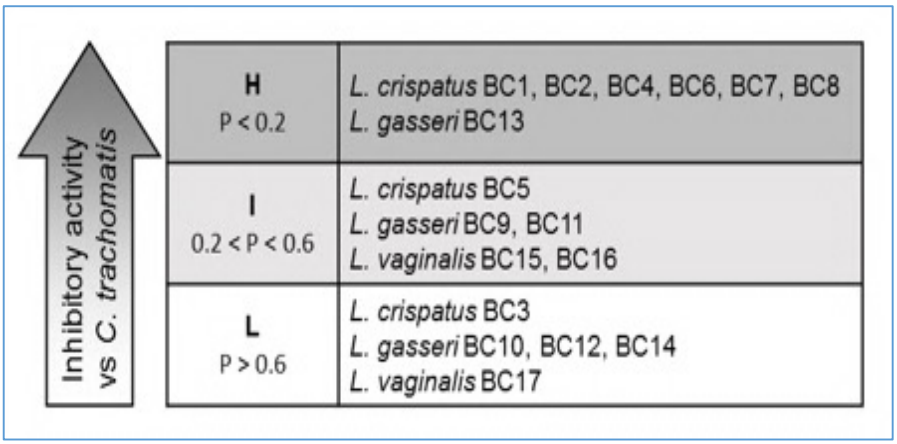

Figure 14. Ranking of lactobacilli in relation to anti-Chlamydia activity. *Lactobacillus strains were classified on the basis of the inhibitory activity of their conditioned medium (CM), expressed as the difference between CM-treated EBs and untreated EBs, by means of the 1-tailed Wilcoxon signed rank P-values. Group H comprises lactobacilli strains with p-values below 0.2, group I comprises of lactobacilli with p-values ranging between 0.2 and 0.6; group L comprises lactobacilli with p-values over 0.6.*

4.2.5 Identification of *Lactobacillus* metabolic profiles associated with anti-*Chlamydia* activity

All *Lactobacillus*-CM were analyzed by ^{1}H-NMR. We identified 40 metabolites mainly belonging to the families of aminoacids, organic acids monosaccharides, ketones, and alcohols (Table 3). A Principal Component Analysis (PCA) model was built on the concentrations of the identified molecules in order to search for correlations between anti-*Chlamydia* activity and metabolome of lactobacilli (Figure 15). In the biplot describing the distribution of *Lactobacillus* strains in relation to the pool of metabolites, Principal Component (PC) 1 and PC2 accounted for the 44.3% of the whole variance of the

investigated samples (Figure 15A). The first component PC1, accounting for the 25.8% of the total variance, was mainly influenced by the taxonomy of lactobacilli. The second component PC2, accounting for the 18.5% of the total variance, was mainly influenced by the activity of lactobacilli against *Chlamydia*. The correlation between metabolome and anti-*Chlamydia* activity was best visualized by means of box plots representing the distribution of the groups of lactobacilli (H, I, L) (Figure 15B). Strains with different anti-*Chlamydia* activity were clearly separated in the vertical direction: the most active strains occupied the lower positions while the less active strains were placed in the higher areas of the two-dimensional space. The highest metabolic homology was observed within the group of lactobacilli exerting high activity, as demonstrated by the lower height of the corresponding boxplot.

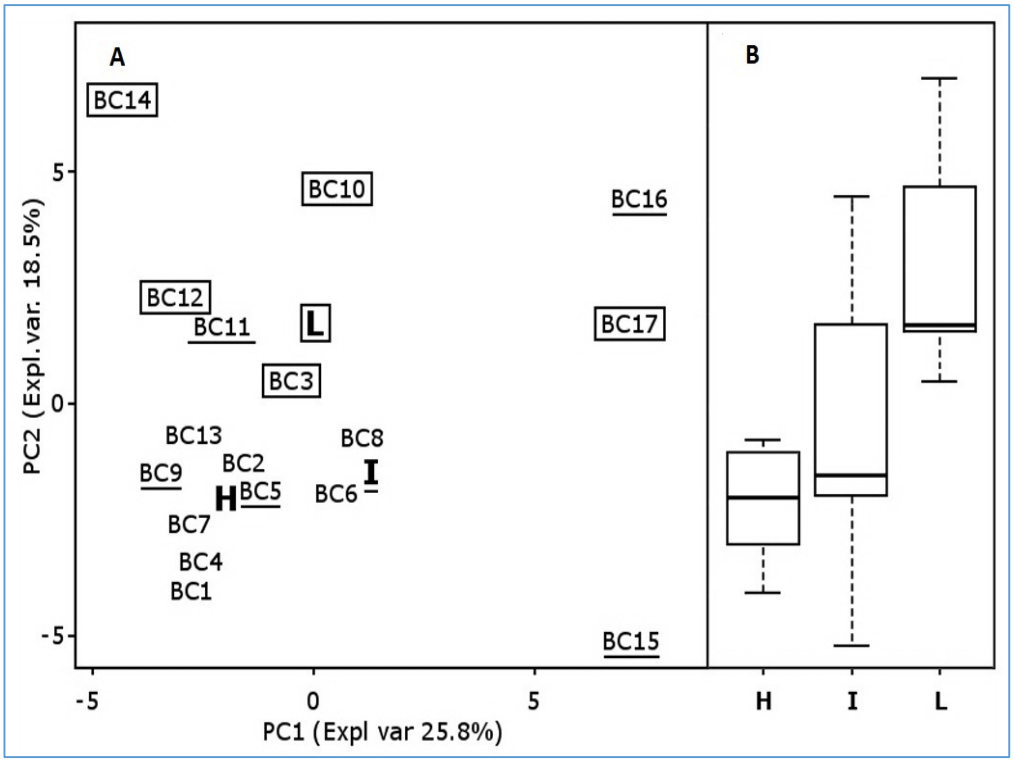

Figure 15. Correlation between Lactobacillus metabolome and inhibitory activity towards C. trachomatis. (A) *Score plot of Lactobacillus strains on PC1 and PC2 of a PCA model built on the total metabolites identified by ^{1}H-NMR in Lactobacillus-CM. High activity (H), intermediate activity (I), and low activity (L) indicate the median values of lactobacilli grouped according to anti-Chlamydia activity. Strains without marks belong*

to group H; underlined strains belong to group I; strains within rectangles belong to group L. Expl. Var, explained variance. ***(B)*** *Box plots representing the distribution of activity against Chlamydia in relation to the metabolome. Lines within the boxes indicate the median values of the samples groups corresponding to the different activity scores. Each box represents the interquartile range (25–75th percentile). The bottom and top bars indicate the 10th and 90th percentiles, respectively.*

We searched which metabolites varied in relation to anti-*Chlamydia* activity, according to the approach of De Filippis et al. [De Filippis et al., 2014]. Orotate was produced in greater concentrations by lactobacilli highly active (group H) while phenylalanine, isoleucine, valine, and tyrosine were produced in greater concentrations by lactobacilli less effective against *Chlamydia* (group L). Orotate and phenylalanine production was found to be statistically different between lactobacilli in groups H and L (Orotate, p = 0.005; Phenylalanine, p = 0.005; 2-tailed Wilcoxon signed rank test). Inhibitory activity of orotate, at the highest concentration found in *Lactobacillus*-CM (30 mM), was tested, but no significant reduction of *C. trachomatis* infectivity was observed. Orotic acid was also tested in association with lactic acid (10/50 mM; pH: 4/7), but this combination did not enhance the lactic acid effect.

Moreover, we observed that glucose was consumed at higher levels by lactobacilli belonging to group H while tryptophan was consumed more by lactobacilli of group L. Thus, to understand whether the competition for the carbonate source could be an additional mechanism of action towards *Chlamydia*, we added glucose (30 mM) to the supernatants of *L. crispatus* BC1 and *L. gasseri* BC13 (they consumed the highest amount of glucose within the H group). The addition of glucose to *L. crispatus* BC1 supernatant led to a significant increase (51 fold) of *C. trachomatis* infectivity at the shortest contact time (7 min), while no increase of infectivity was found at the time points 15 and 60 min. Similarly, *L. gasseri* BC13 supernatant enriched with glucose showed a reduction in anti-*Chlamydia* activity both after 7 minutes (infectivity increase: 8.7 fold) and 15 minutes (infectivity increase: 6.1 fold) of contact (Table 5). These data confirm the importance of the depletion of glucose as an additional mechanism for the inhibition of *C. trachomatis* EBs by vaginal lactobacilli, in particular for short contact times.

Strain	Contact time		
	7 min	15 min	60 min
L. crispatus BC1	51.2*	-	-
L. gasseri BC13	8.7*	6.1*	-

Table 5. Increase of C. trachomatis infectivity following the addition of glucose to supernatants of L. crispatus BC1 and L. gasseri BC13. *Dilutions 1:1 of Lactobacillus-CM were tested. Increase was calculated as ratio between the infectivity of the supernatant added with glucose 30 mM and the infectivity of the corresponding not enriched supernatant. Significant increases were indicated with an asterisk ($p < 0.05$, 1-tailed Wilcoxon matched paired rank test). (-) No variation.*

4.3 Role of vaginal lactobacilli against HIV-1 replication in human tissues *ex vivo*

4.3.1 *Lactobacillus* colonization of tissue explants

Fifteen *Lactobacillus* strains belonging to *L. vaginalis*, *L. gasseri,* and *L. crispatus* were evaluated for their capacity to colonize *ex vivo* tissue blocks. All lactobacilli colonized tissue explants with similar kinetics, reaching maximum colonization (approximately $1 \times 10^{8.5}$ CFU/mL) after three days of culture, and then plateaued for the entire 12 days of culture duration (Figure 16A). Cell depletion in tissue explants by lactobacilli was evaluated 3 days after bacterial inoculation (Figure 16B). Colonization of explants with six out of fifteen *Lactobacillus* strains, *L. crispatus* (BC3, BC5), *L. gasseri* (BC12, BC13), and *L. vaginalis* (BC16, BC17), did not result in cell depletion, as compared with control (Figure 16B lower panel, BC5 representative of this group). In contrast, the colonization of tissue blocks by the remaining nine *Lactobacillus* strains, *L. crispatus* (BC1, BC4, BC6, BC7, BC8) and *L. gasseri* (BC9, BC10, BC11, BC14), resulted in a loss of T cells ($CD3^+$) as well as an increase in the expression of the apoptotic marker Bcl 2 (data not shown). The losses of $CD3^+$ cells were not characteristics of particular species of *Lactobacillus,* as some strains of *L. crispatus*, *L. gasseri*, and *L. vaginalis* induced cell depletion while others did not. *Lactobacillus* strains that induced $CD3^+$ cell depletion were not used in further experiments.

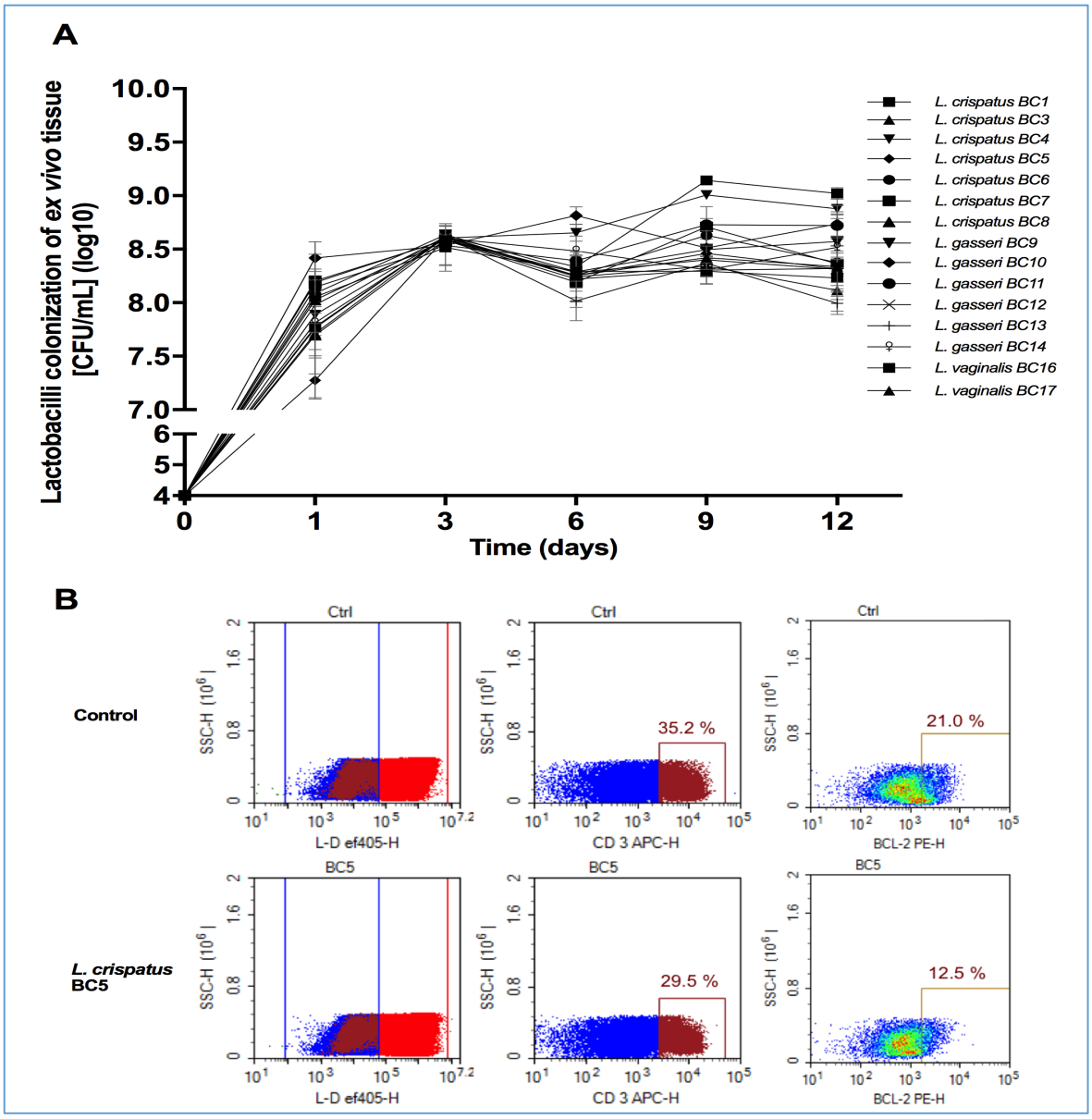

Figure 16. Lactobacillus colonization of ex vivo tissue. ***(A)*** *Tonsillar tissues were colonized with fifteen vaginal Lactobacillus strains: L. crispatus (BC1, BC3–BC8), L. gasseri (BC9-BC14), and L. vaginalis (BC16, BC17), at a starting inoculum of 1 ×10^4 CFU/mL and cultured for 12 days. Lactobacillus colonization was evaluated every 3 days spectrofometrically by measuring OD_{600}. Bars represent mean ± SD from tissues of three donors.* ***(B)*** *We evaluated tissue cell depletion induced by Lactobacillus colonization of ex vivo tissues 3 days after bacterial inoculation using flow cytometry. Panels (from left to right) represent live/dead staining, $CD3^+$ expression in live cells, and Bcl-2 expression in $CD3^+$ cells in control (upper row) and L. crispatus BC5–colonized tissue (lower row).*

4.3.2 *Lactobacillus*-CM inhibits HIV-1 replication

To investigate the effects of metabolites secreted by lactobacilli on HIV-1 replication in *ex vivo* tissues, tissue blocks were pre-incubated with *Lactobacillus*-CM of *L. crispatus* (BC3, BC5), *L. gasseri* (BC12, BC13), and *L. vaginalis* (BC16, BC17), infected with HIV-1, and cultured as described in Materials and Methods. In both cervico-vaginal and lymphoid tissue blocks all undiluted *Lactobacillus*-CM suppressed replication of HIV-1 compared with the control by 91.9±4.3% and 98.3±1.4% (*L. crispatus* BC3, $p < 0.0001$, $n = 5$), 95.9±4.8% and 97.7±1.8% (*L. crispatus* BC5, $p < 0.0001$, $n = 5$), 93.5±3.6% and 98.2±1.9% (*L. gasseri* BC12, $p < 0.0001$, $n = 5$), 91.9±1.5% and 98.3±2.3% (*L. gasseri* BC13, $p < 0.0001$, $n = 5$), 95.9±5.0% and 98.1±2.9% (*L. vaginalis* BC16, $p < 0.0001$, $n = 5$), 85.8±11.7% and 95.0±6.5% (*L. vaginalis* BC17, $p < 0.0001$, $n = 5$), respectively (Figure 17). All *Lactobacillus*-CM had an inhibitory effect on HIV-1 replication even when diluted 5-fold. Depending on the *Lactobacillus* strain, inhibition of HIV-1 replication by such diluted medium was ranging from 44.3±31.7% (*L. crispatus* BC3, $p = 0.0038$, $n = 5$) to 77.3±7.3% (*L. vaginalis* BC16, $p < 0.0001$, $n = 5$) in cervico-vaginal tissue (Figure 17B) and from 55.5±13.2% (*L. vaginalis* BC17, $p < 0.0001$, $n = 5$) to 93.1±5.2% (*L. crispatus* BC5, $p = 0.0001$, $n = 5$) in tonsillar tissue (Figure 17D).

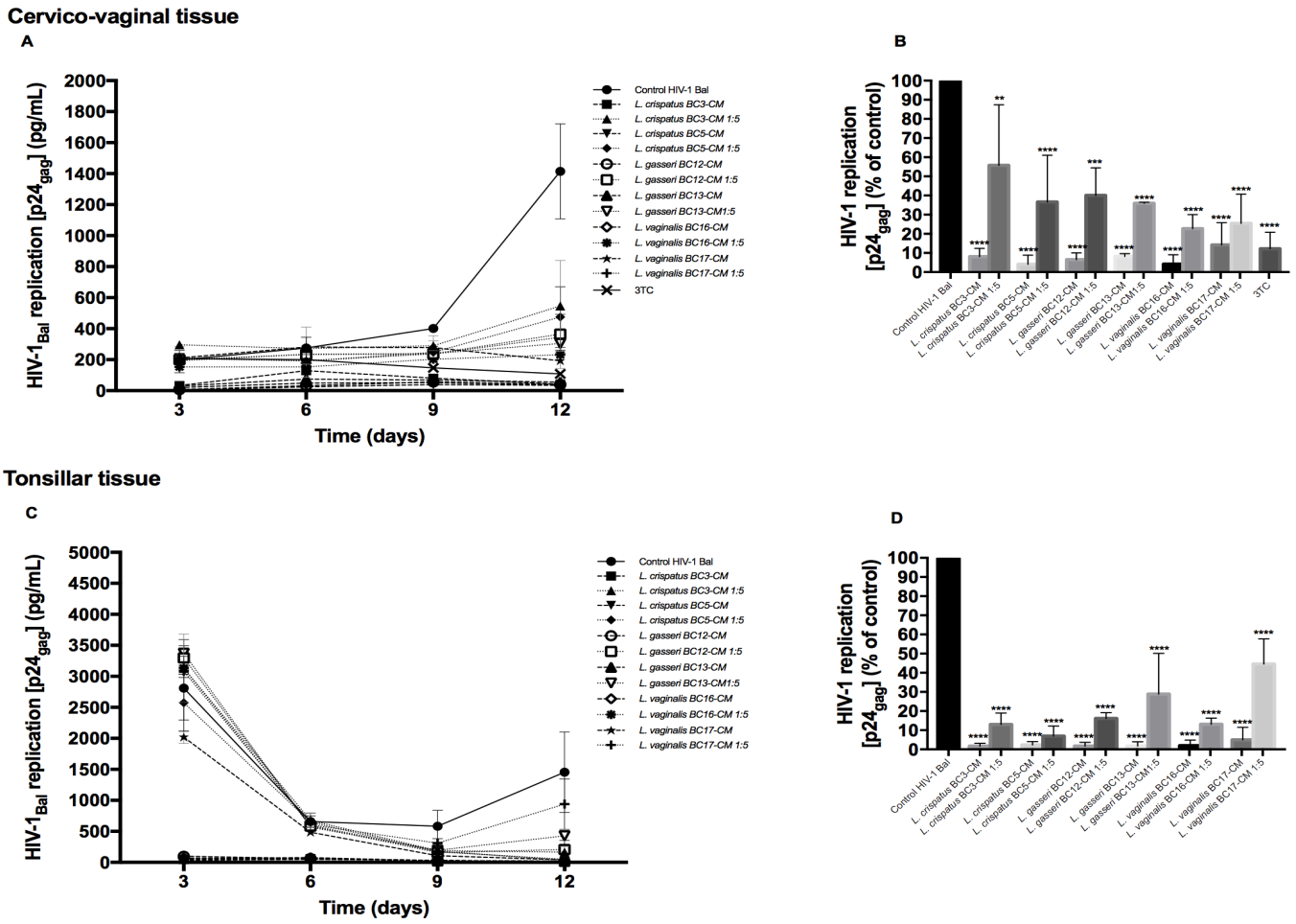

Figure 17. HIV-1 infection of human tissue ex vivo treated with Lactobacillus-CM. *Cervico-vaginal* ***(A, B)*** *and tonsillar* ***(C, D)*** *tissue blocks were pre-incubated with undiluted or diluted 1:5 Lactobacillus-CM from six strains: L. crispatus (BC3, BC5), L. gasseri (BC12, BC13), and L. vaginalis (BC16, BC17). Tissue cultures were infected with HIV-1 and kept in the Lactobacillus-CM for 3 days. At day 3, the Lactobacillus-CM was removed and cultures were kept in regular medium until day 12 post-infection.* ***(A, C)*** *Kinetics of HIV-1 replication in tissues was obtained by measuring the levels of $p24_{gag}$ in tissue culture medium.* ***(B, D)*** *Replication of HIV-1 in Lactobacillus-treated tissues expressed as percentages of HIV-1 replication compared to the control (black bars). Bars represent mean±SD from five tissue donors. Asterisks indicate statistical significance vs. control, obtained by one-way ANOVA multiple comparisons (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001). 3TC (lamivudine, 10 μM), a powerful HIV-1 inhibitor, used as a positive control.*

4.3.3 Effects of lactic acid and pH on HIV-1 replication

To investigate whether lactic acid produced by lactobacilli is responsible for HIV-1 inhibition, we measured the concentrations of lactic acid isomers D and L in *Lactobacillus*-CM (Table 6).

***Lactobacillus* strains**	**[D-Lactate]**		**[L-Lactate]**		**pH**	
	mM	mM/ 10^8 bacteria	mM	mM/ 10^8 bacteria	*Lactobacillus*-CM	*Lactobacillus*-CM 1:5
L. crispatus BC3	3.1	0.4	15.1	2.2	4.0	6.6
L. crispatus BC5	3.1	0.3	24.0	2.4	3.8	6.4
L. gasseri BC12	3.6	0.4	24.7	2.8	3.8	6.3
L. gasseri BC13	3.2	0.3	22.2	2.0	3.8	6.4
L. vaginalis BC16	2.7	0.3	22.4	2.2	3.8	6.4
L. vaginalis BC17	1.8	0.4	9.0	1.9	4.6	6.9

Table 6. Lactic acid isomers D and L and pH in Lactobacillus-CM. *Titers of lactate isomers D and L from overnight-cultured Lactobacillus-CM using a lactate quantification assay kit. pH values in all Lactobacillus-CM, undiluted or diluted 1:5, were measured as well.*

Depending on the strain, the concentrations of lactic acid isomers D and L ranged from 1.8 to 3.6 mM and from 9.0 to 24.7 mM, respectively. *L. gasseri* BC12 was the strain that produced the highest concentrations of both isomers, while *L. crispatus* BC17 was the strain that produced the lowest concentrations. Next, we tested the effects of lactic acid isomers at the concentration found in undiluted *Lactobacillus*-CM or CM diluted 1:5 on HIV-1 replication in tissues *ex vivo* (Figure 18). As shown in Figures 18A and C, lactic acid isomers D (3 mM), L (23 mM), and D + L (3; 23 mM) significantly reduced HIV-1 replication in both cervico-vaginal and tonsillar tissues. We found that D lactate (3 mM) inhibited HIV-1 replication by 48.2±6.2% in cervico-vaginal ($p = 0.0004$, $n = 3$) and by 57.6±33.2% in tonsillar ($p = 0.0125$, $n = 3$) tissue cultures, while L lactacte (23 mM) suppressed HIV-1 replication by 94.3±5.5% ($p < 0.0001$, $n = 3$) and by 99.3±21.9% ($p < 0.0001$, $n = 3$) in cervico-vaginal and tonsillar tissues, respectively. The mixture of D + L

lactate suppressed HIV-1 replication by 92.1±7.7% in cervico-vaginal and by 94.4±30.4% in tonsillar tissue ($p < 0.0001$, $n = 3$) (Figures 18B and D). Afterward, we evaluated the effect of lactic acid isomers at the concentrations found in 5-fold-diluted *Lactobacillus*-CM on HIV-1 replication in lymphoid tissue (Figures 18C and D). We found that isomer D did not inhibit HIV-1 replication while isomer L and the mixture of isomers D + L significantly reduced HIV-1 replication in tonsillar tissues by 67.8±0.8% (isomer L, $p = 0.0033$, $n = 3$) and by 56.5±9.5% (isomers D + L, $p = 0.0142$, $n = 3$) (Figure 18D).

Furthermore, we evaluated whether the effect of lactobacilli on HIV-1 replication is due to the acidic pH of the *Lactobacillus*-CM. As shown in Table 5, pH values of undiluted *Lactobacillus*-CM ranged from 3.8 to 4.6 and of 5-fold-diluted *Lactobacillus*-CM from 6.3 to 6.9. To mimic the effect of pH on HIV-1 replication, we acidified the culture medium of human cervico-vaginal and tonsillar tissues with HCl. In the culture medium buffered to pH 4, HIV-1 replication was reduced in both cervico-vaginal (90.1±0.1%, $p < 0.0001$, $n = 3$) and tonsillar tissue (88.0±17.5%, $p = 0.0003$, $n = 3$) compared with control tissue blocks cultured in regular medium (Figures 18B and D). No statistically significant inhibition of HIV-1 replication in cervico-vaginal or tonsillar tissues was observed when the culture medium was buffered to pH 6.9 (21.6±18.8%, $p = 0.1492$, $n = 2$ and 14.28±10.19%, $p = 0.9$, $n = 3$, respectively).

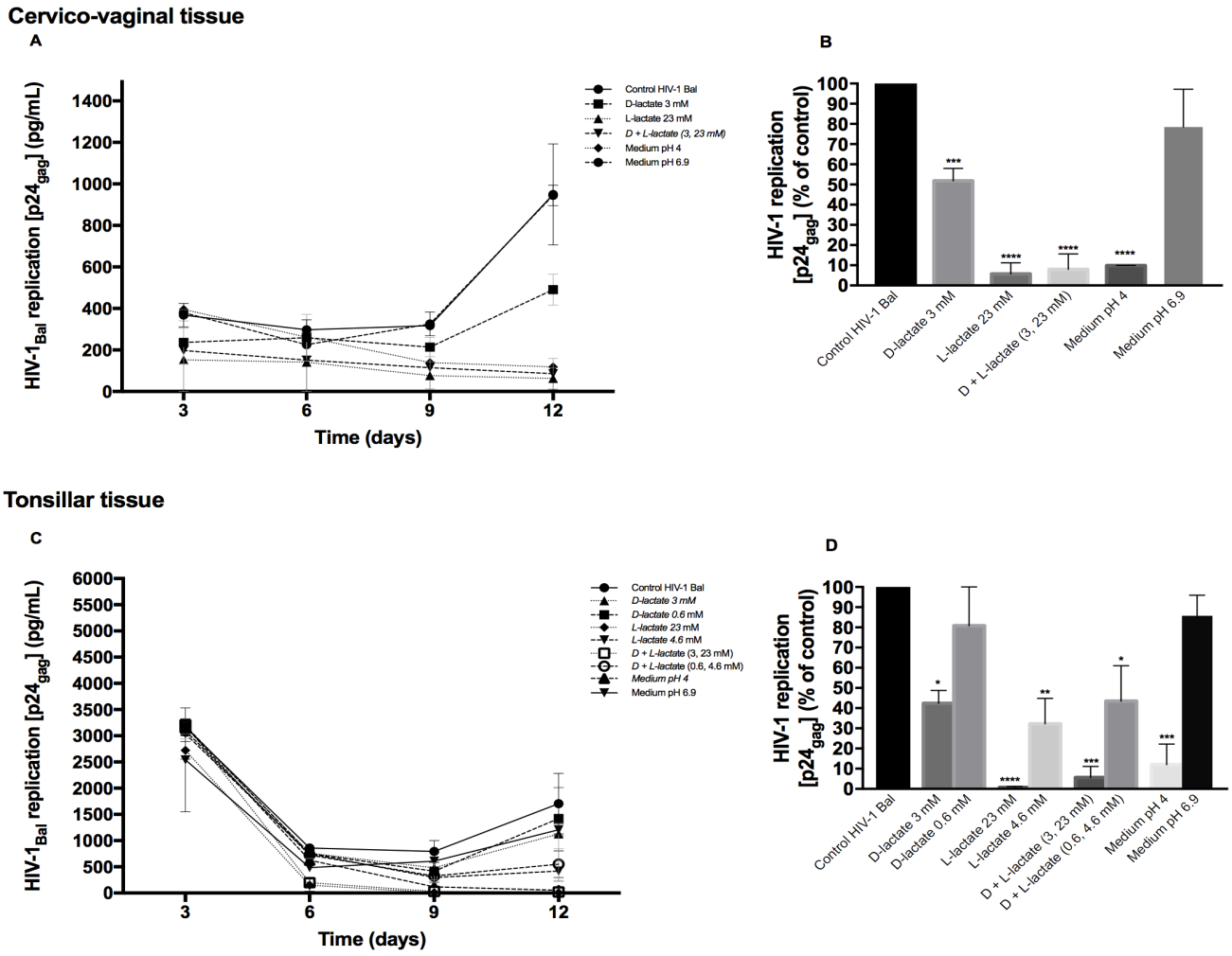

Figure 18. Effect of lactic acid isomers and of pH on HIV-1 replication. *The effects of lactate isomers D and L on HIV-1 replication were tested in cervico-vaginal* ***(A, B)*** *and tonsillar* ***(C, D)*** *tissues. Isomers D (3 mM), L (23 mM), and D + L (3 mM + 23 mM), corresponding to the average titers found in all undiluted Lactobacillus-CM were tested. We evaluated the effect of acidic pH on HIV-1 infectivity in ex vivo tissues by buffering the culture medium at pH 4 and pH 6.9 using HCl.* ***(A, C)*** *We evaluated the kinetics of HIV-1 replication in tissues by measuring the levels of* $p24_{gag}$ *in culture medium.* ***(B, D)*** *Replication of HIV-1 in Lactobacillus-treated tissues was expressed as percentage of HIV-1 replication in untreated control (black bars). Statistical significance vs. control is presented. Bars represent mean ± SD from tissues of three to five donors. Asterisks indicate statistical significance by one-way ANOVA multiple comparison (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).*

4.3.4 Virucidal capacity of *Lactobacillus*-CM against HIV-1

In order to understand whether *Lactobacillus*-CM can suppress HIV-1 infectivity before interaction with tissues, we pre-incubated HIV-1 for 1 h with *Lactobacillus*-CM (diluted 1:5) and tested HIV-1 infectivity in cervico-vaginal tissues *ex vivo*. We studied the virucidal capacities of *Lactobacillus*-CM of six strains: *L. crispatus* (BC3, BC5), *L. gasseri* (BC12, BC13), and *L. vaginalis* (BC16, BC17). As shown in Figure 19A, HIV-1 replication was reduced when cervico-vaginal tissues were infected with HIV-1 pre-treated with *Lactobacillus*-CM from *L. crispatus* BC3 (47.7±7.0%, $p = 0.005$, $n = 5$), *L. crispatus* BC5 (60.9±13.8%, $p = 0.0005$, $n = 5$), *L. gasseri* BC12 (64.0±11.4%, $p < 0.0001$, $n = 5$), and *L. vaginalis* BC16 (57.4±8.1%, $p = 0.003$, $n = 5$) (Figure 19B). No statistically significant inhibition was observed due to *Lactobacillus*-CM from *L. gasseri* BC13 (28.6±6.6%, $p = 0.13$, $n = 5$) and *L. vaginalis* BC17 (31.1±24.8%, $p = 0.06$, $n = 5$).

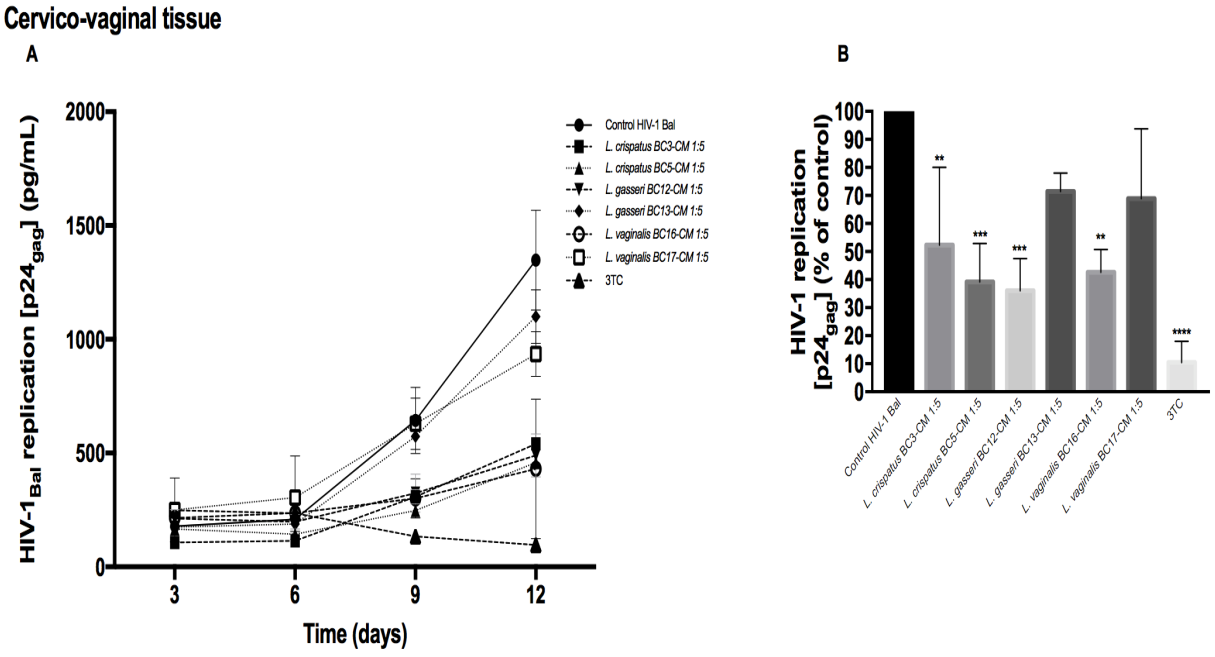

Figure 19. Virucidal effect of Lactobacillus-CM against HIV-1. *Virucidal capacities of six strains: L. crispatus (BC3, BC5), L. gasseri (BC12, BC13), and L. vaginalis (BC16, BC17) are presented. HIV-1 preparation was pretreated with Lactobacillus-CM diluted 1:5 for 1 h, and HIV-1 infectivity was tested in cervico-vaginal tissues ex vivo.* ***(A)*** *Kinetics*

of HIV-1 replication by measuring the levels of $p24_{gag}$ in tissue culture medium. ***(B)*** *Replication of HIV-1 in Lactobacillus-treated tissues expressed as percentages of HIV-1 replication in untreated control (black bars). Bars represent mean ± SD from tissues of five patients. Asterisks indicate statistical significance by one-way ANOVA multiple comparison (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001). 3TC used as a positive control.*

4.3.5 Virucidal capacity of *Lactobacillus* cells against HIV-1

Similar to *Lactobacillus*-CM, the virucidal capacities of *Lactobacillus*-CP were studied. HIV-1 was first incubated with *Lactobacillus*-CP, and after bacteria have been washed off, the infectivity of HIV-1 was tested in cervico-vaginal tissues *ex vivo*. As shown in Figure 20A, tissue infection with HIV-1 pre-incubated with *Lactobacillus*-CP for 1 h resulted in inhibition of HIV-1 replication by 64.7±14.9% for *L. crispatus* BC5 ($p < 0.0001$, $n = 5$), by 39.3±18.4% for *L. gasseri* BC12 ($p = 0.0124$, $n = 5$), and by 59.8±13.2% for *L. vaginalis* BC17 ($p = 0.0002$, $n = 5$). No statistically significant inhibition was observed with *L. crispatus* BC3 (19.4±18.5%, $p = 0.46$, $n = 5$), *L. gasseri* BC13 (16.1±27.9%, $p = 0.64$, $n = 5$), and *L. vaginalis* BC16 (9.0±15.6%, $p = 0.96$, $n = 5$) (Figure 20B).

Thereafter, to investigate whether this suppression of HIV-1 replication of cervico-vaginal tissue by *L. crispatus* BC5, *L. gasseri* BC12, and *L. vaginalis* BC17 in the above-described experiments was due to viral binding to *Lactobacillus* cells, we measured the concentration of $p24_{gag}$ on *Lactobacillus*-CP after bacteria were separated by centrifugation. We found that these three strains adsorbed 36.2±21.5%, 29.6±25.6%, and 39.2±9.6% of HIV-1, respectively, as evaluated from measurements of $p24_{gag}$ (Figure 20C). In the CP of the remaining strains (*L. crispatus* BC3, *L. gasseri* BC13, and *L. vaginalis* BC16) the $p24_{gag}$ was less than 10% of the original HIV-1 preparation (data not shown).

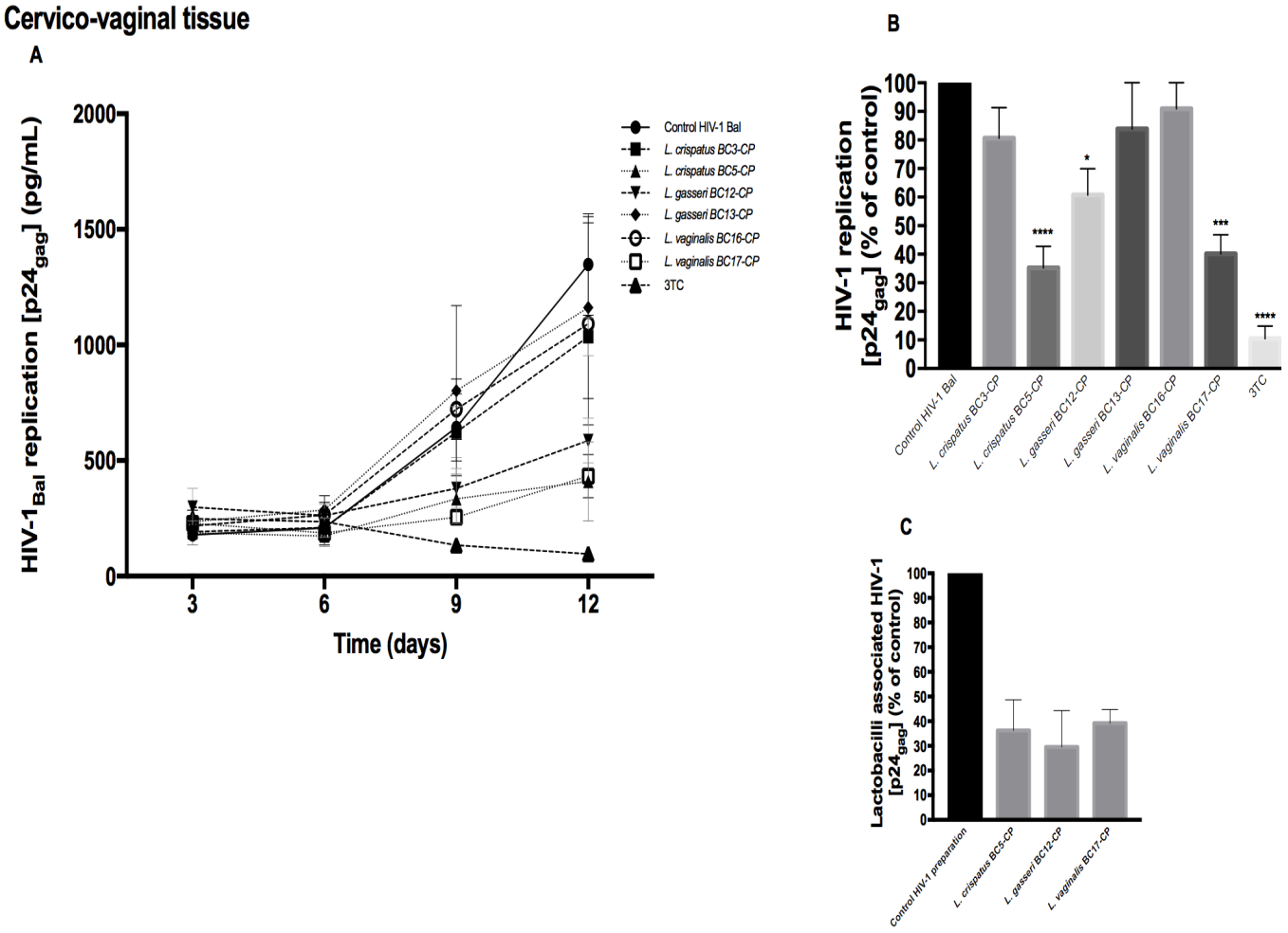

Figure 20. Virucidal effect of Lactobacillus-CPs against HIV-1. *Virucidal capacity of six strains, L. crispatus (BC3, BC5), L. gasseri (BC12, BC13), and L. vaginalis (BC16, BC17) is presented. HIV-1 was pre-treated with Lactobacillus-CP at 1 ×10^8 CFU/mL, and HIV-1 infectivity was then tested in cervico-vaginal tissues ex vivo.* ***(A)*** *We evaluated HIV-1 replication kinetics in tissues by measuring $p24_{gag}$ in culture medium.* ***(B)*** *Replication of HIV-1 in Lactobacillus-treated tissues was expressed as percentage of HIV-1 replication in untreated control (black bars).* ***(C)*** *Fractions of $p24_{gag}$ associated with CP after incubation with HIV-1 are presented. Statistical significance vs. control was calculated. Bars represent mean ± SD from five patients. Asterisks indicate statistical significance by one-way ANOVA multiple comparison (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001). 3TC (lamivudine) at 10 μM we used in our study as a positive control.*

5. DISCUSSION AND CONCLUSIONS

The vaginal mucosa is normally colonized by a vast number of microorganisms collectively referred to as the vaginal microbiota, which normally coexist with the host in a tightly regulated manner [Petrova et al., 2015]. Healthy women of reproductive age are generally dominated by *Lactobacillus* species [Hyman et al., 2005; Pavlova et al., 2002; Ravel et al., 2011]. A *Lactobacillus*-dominated microbiota appears to be a biomarker for a healthy vaginal communities, as changes in the vaginal microbiota, especially shifting away from *Lactobacillus* dominance, are associated with bacterial vaginosis [Allsworth and Peipert, 2011; Cherpes et al., 2003], vulvo-vaginal candidiasis (VVC) [Goffeng et al., 1997; Vitali et al., 2007], and increased risks of acquisition of sexually transmitted infections, including *Chlamydia trachomatis* [Brotman et al., 2010; Martius et al., 1988; Wiesenfeld et al., 2003] and human immunodeficiency virus (HIV) [Atashili et al., 2008; Gosmann et al., 2017; Mirmonsef and Spear; Nardis et al., 2013; Petrova et al., 2015; Taha et al., 1998].

The aim of the present study was to isolate vaginal lactobacilli from healthy women, to characterize them at a molecular level and to evaluate their antagonistic effect toward *Candida* spp., *C. trachomatis,* and HIV, which are among the infectious agents that most significantly affect women's health worldwide.

In this context, from vaginal swabs of healthy premenopausal woman we isolated seventeen *Lactobacillus* strains belonging to the 3 species highly represented in the human vaginal microbiota: *L. crispatus* (BC1-BC8), *L. gasseri* (BC9-BC14), and *L. vaginalis* (BC15 -BC17) [Parolin et al., 2015]. The isolation of *L. crispatus* has been strongly associated with a normal vaginal microbiota and absence of vaginal dysbiosis [Antonio et al., 1999]. Longitudinal studies have also shown that *L. crispatus* promotes stability of the vaginal microbiota [Petrova et al., 2015; Verstraelen et al., 2009], and seems to have a role in the restoration of the vaginal communities, and in the maintenance of remission from bacterial vaginosis, following antibiotic treatment [Cruciani et al., 2015]. Despite the high incidence of *L. iners* in the human vaginal microbiota, as detected by culture-independent

molecular studies [Ravel et al., 2011; van de Wijgert et al., 2014], we did not obtain isolates belonging to this species probably due to its stringent nutritional requirements and very low oxygen tolerance [Falsen et al., 1999]. On the other hand, as our goal was to identify health-promoting lactobacilli, *L. iners* was of little interest given its close correlation with vaginal dysbiosis [Macklaim et al., 2013].

In view of potential application of the isolated *Lactobacillus* strains as vaginal probiotics, we sought to characterize their capacities to produce antimicrobial compounds to individuate a possible antimicrobial mechanism of action. H_2O_2 and lactate are classically associated with the antimicrobial properties of *Lactobacillus* genus [Aroutcheva et al., 2001]. All strains produced H_2O_2 in agreement with the assumption that the vaginal microbiota of healthy women is dominated by H_2O_2-producing lactobacilli [Pendharkar et al., 2013]. Also, lactate was produced by all lactobacilli, while butyrate was produced only by certain strains.

The fungistatic and fungicidal activities of the vaginal lactobacilli were evaluated against of *C. albicans* and *C.* non-*albicans* strains, in the perspective to develop successful vaginal probiotics for VVC management [De Seta et al., 2014], considering that the high incidence and associated healthcare costs of VVC together with the growing problem of antibiotic resistance highlight the necessity for the development of new effective agents for the prevention and therapy of this gynaecological infection [Coste et al., 2007; Falagas et al., 2006]. Compared to previous studies focused on the antifungal activity of lactobacilli [Abramov et al., 2014; Kohler et al., 2012], our work has the additional value examining *Lactobacillus* isolates from vaginal source against numerous clinical isolates of *Candida* species. Therefore, the results obtained in this work provide important information about the real applicability of vaginal lactobacilli in the prevention and treatment of VVC. The broadest spectrum of activity was observed for *L. crispatus* (BC1, BC4, BC5) and *L. vaginalis* BC15, exhibiting fungicidal activity against all isolates of *C. albicans* and *C. lusitaniae*. Among these strains, *L. crispatus* BC1 and *L. vaginalis* BC15 exhibited the best anti-*Candida* profile covering also *C. tropicalis* and *C. glabrata*.

Next, we evaluated the interference of lactobacilli with pathogens adherence to human cells, considering this aspect of a major importance for the *in vitro* evaluation of probiotic

properties [FAO/WHO, 2006]. We observed that most of *Lactobacillus* strains significantly reduced *C. albicans* adhesion to HeLa cells through multiple mechanism including exclusion, competition, and displacement. *Lactobacillus crispatus* BC2 and *L. gasseri* (BC10, BC11) appeared to be the most active strains in reducing pathogen adhesion, as their effects were mediated by both *Lactobacillus*-cell pellet (CP) and *Lactobacillus*-conditioned medium (CM). Interestingly, the same bacteria were not the most adhesive strains suggesting that the inhibitory effects are not merely due to steric encumbrance and/or saturation of the adhesion sites, but rather to a reduction of the adherence of the pathogen itself and/or to modifications of the epithelial cells surface. Additionally, the same lactobacilli were not the best performing strains in terms of fungistatic/fungicidal activity. These findings suggest that lactobacilli isolated from healthy vagina can exert their protective role against *Candida* infection utilizing one strategy (either inhibition of growth or adhesion) rather than through the combination of two complementary mechanisms.

To interpret the differences in fungistatic/fungicidal capacities of the vaginal lactobacilli, through a metabolic key, we studied by ^{1}H-NMR the metabolome of *Lactobacillus*-CM and we looked for correlations with taxonomy and activity score. The strong correlation between metabolic profile and taxonomy highlighted the inter-specific variability of bacterial metabolism. Metabolic variance was also related to antifungal activity, confirming the excellent antifungal profile of most *L. crispatus* strains and *L. vaginalis* BC15. These data highlight the potential of metabolomics to measure the taxonomic distance between different *Lactobacillus* strains and predict their anti-*Candida* activity. Although metabolomics has been applied to evaluate the impact of probiotics on the host organism [Bisanz et al., 2014], to our knowledge this is the first study employing a metabolomic approach to investigate the antimicrobial activity of health-promoting bacteria, representing a new idea for future researches.

Since butyrate is a known histone deacetylase (HDAC) inhibitor [Nguyen et al., 2011], we hypothesize that it may enhance the anti-*Candida* activity of lactobacilli through histone hyper-acetylation. Inhibition of HDACs can influence fungal growth and adherence to host cells [Nguyen et al., 2011; Simonetti et al., 2007]. We observed that histone hyper-acetylation was a predominant capacity of most vaginal lactobacilli. Interestingly, the

strains that did not trigger histone acetylation (*L. gasseri* BC13 and *L. vaginalis* BC17) were the same ones that did not exercise any fungicidal activity or interference on *Candida* adhesion, suggesting that inhibition of HDACs could support antifungal activity of vaginal lactobacilli.

This study also provides experimental evidence for inhibition of *C. trachomatis* infection by vaginal lactobacilli. *Chlamydia trachomatis* is the most common cause of bacterial sexually transmitted infections in the world [Miller et al., 2004; Senior, 2012]. It has been hypothesized that *Lactobacillus* species play a critical protective role in the vaginal habitat by producing lactic acid, which maintain an acidic environment inhibiting sexually transmitted pathogens. Gong et al. suggested that acidic pH is fully responsible for chlamydiacidal activity of lactobacilli [Gong et al., 2014].

Our study showed that *Lactobacillus* strains exert a strong inhibitory effect on *Chlamydia* infectivity mainly through metabolites secreted out of the cell, in a concentration-dependent manner. We observed that the factor "concentration" is inversely correlated to the pH of the culture medium. Indeed, all *Lactobacillus*-CM were characterized by low pH, while diluted *Lactobacillus*-CM showed higher pH.

The factor "contact time" also seems to play a role in the inhibitory activity against *Chlamydia*. Lactobacilli, especially *Lactobacillus* cells, were more effective in inhibiting *Chlamydia* EBs infectivity at short contact times. This inhibition may be generated by a rapid and dynamic modification of *Chlamydia* EBs membrane; we assume that this modification could revert for longer exposure times. These findings suggest that lactobacilli could exert their protective role against *Chlamydia* in the early steps of the infection, probably due to inactivation of EBs before they can colonize and infect the epithelial host cells; these results are in accord to results previously described [Mastromarino et al., 2014].

Using a statistical approach, we classify *Lactobacillus* strains according to their anti-*Chlamydia* activity into three groups: high, intermediate, and low activity. The best anti-*Chlamydia* profile was shown by strains belonging to *L. crispatus* species. Conversely, *L. gasseri* and *L. vaginalis* showed a heterogeneous spectrum of activity. It has been reported that *L. crispatus* inhibit *in vitro* the growth of uropathogens and block their adhesion to

vaginal epithelial cells [Osset et al., 2001]. *Lactobacillus crispatus* was also shown to reduce the adhesion of *Neisseria gonorrhoeae* [Vielfort et al., 2008] and *C. trachomatis* [Mastromarino et al., 2014] to HeLa cells by competitive exclusion. Moreover, it was demonstrated the efficacy of *L. crispatus* to limit the inflammatory reaction in *C. trachomatis*-infected HeLa cells and macrophages [Rizzo et al., 2015].

Directly linked to the presence of lactobacilli, the production of lactic acid is accepted as a hallmark beneficial activity of the vaginal microbiota. Lactic acid has been associated with pathogen exclusion and its concentration could also be an important biomarker of vaginal health, although the current evidence is still mainly based on *in vitro* studies. Lactic acid is able to inactivate a wide range of reproductive tract pathogens, including *C. trachomatis* [Gong et al., 2014] and HIV-1 [Aldunate et al., 2013]. We investigated whether the anti-*Chlamydia* activity of lactic acid is merely associated with the pH, or other mechanisms may be involved. Lactic acid, at the concentrations found in the *Lactobacillus*-CM, strongly inhibit EBs infectivity only at acid pH for all exposure times, differently from HCl that did not show any activity in the same experimental conditions. These results indicate that a high concentration of H^+ ions is necessary but not sufficient to inhibit *Chlamydia* EBs. Therefore, the presence of lactic acid in an acidic environment seems to be crucial for this activity. It remains to elucidate the specific mechanisms by which lactate moiety inactivates chlamydial EBs. Notably, the inhibitory activity exerted by most of *Lactobacillus*-CM, characterized by similar lactate concentrations and pH values, was higher than that exerted by lactic acid solution, suggesting that other metabolites present in lactobacilli supernatants could determine a synergistic effect. Because *L. crispatus* strains were found to be the most active in counteracting *Chlamydia* infection, we assume that the effect of lactic acid may be enhanced by the pool of metabolites especially produced by this species.

Given the importance of the metabolic component in determining the inhibition of *Chlamydia*, we studied the metabolic profiles of lactobacilli supernatants by ^{1}H-NMR to identify active metabolites and to find correlations between metabolism of lactobacilli and anti-*Chlamydia* effect. Metabolic variance was strictly correlated with the *Chlamydia* inhibitory activity, confirming the excellent anti-chlamydial profile of the majority of *L. crispatus* strains. Interestingly, the metabolomic analysis highlighted the increased

consumption of glucose by the strains highly active against *Chlamydia*. We demonstrated that glucose depletion represents an additional mechanism of action for lactobacilli antagonism toward *Chlamydia*. It has been recently reported that *C. trachomatis* EBs have considerable metabolic and biosynthetic activity and utilize glucose as an energy source [Omsland et al., 2012] to fuel the developmental transition of EB to RB [Saka et al., 2011]. Consumption of glucose and production of organic acids are metabolically interrelated and represent defensive strategies implemented by vaginal lactobacilli against the attack of pathogens, such as *Chlamydia*. Notably, an increase of glucose concentration in the vaginal fluids collected from women affected by bacterial vaginosis was reported [Vitali et al., 2015]. These findings can be interpreted by assuming that the lack of lactobacilli, mainly those strains with high efficiencies of glucose fermentation, is associated with a greater availability of glucose in the vaginal environment. We hypothesize that the availability of glucose, in turn, could promote growth of undesirable microorganisms, including bacteria responsible for bacterial vaginosis and chlamydiae.

We also studied the role of vaginal lactobacilli in HIV pathogeneses and transmission. The vaginal microbiota seems to protect against HIV directly, by production of antiviral compounds (lactic acid, hydrogen peroxide, bacteriocins, and lectin molecules), or indirectly, stimulating immune responses or inhibiting colonization of microorganisms that cause bacterial vaginosis [Petrova et al., 2015; Petrova et al., 2013].

Here, we investigated some of these mechanisms by studying the effects of vaginal lactobacilli on HIV-1 in the context of human cervico-vaginal and tonsillar tissues *ex vivo* [Introini et al., 2014; Merbah et al., 2011; Saba et al., 2010]. These human tissue cultures offer major advantages over single-cell cultures, as they retain general tissue cytoarchitecture and important functional aspects of cell–cell interactions [Grivel and Margolis]. Therefore, they remain a model of choice to study host–pathogen interactions (reviewed in Grivel and Margolis, 2009). These *ex vivo* tissues have proved to be useful in studies of the effect of HIV-1 copathogens on HIV-1 replication [Grivel et al., 2001; Lisco et al., 2007; Vanpouille et al., 2007] as well as in pre-clinical drug testing [Andrei et al., 2011; Vanpouille et al., 2012].

To address the effects of lactobacilli on HIV-1 infection in the context of human tissues, we first colonized them *ex vivo* with different strains of *Lactobacillus*. We found that all lactobacilli colonized and grew in human tissues *ex vivo* to densities comparable with those observed in vaginal specimens [Aleshkin et al., 2011; Antonio et al., 2009]. Tissue colonization with some of the tested bacterial strains resulted in the depletion of T cells. Although this phenomenon maybe relevant to the protection against HIV-1 *in vivo*, we focused our study on six strains of *Lactobacillus* (*L. crispatus* BC3, BC5; *L. gasseri* BC12, BC13; and *L. vaginalis* BC16, BC17) that did not deplete cells in tissue. We found that all these lactobacilli efficiently suppressed HIV-1 replication in human tissues *ex vivo,* and we investigated the mechanisms of this phenomenon.

First, we investigated whether lactobacilli release suppressive factors that inhibit HIV-1 replication in human tissues ex *vivo*. We found that they do indeed release factors that suppress HIV-1 replication, since the CM inhibited HIV-1 replication in human cervico-vaginal and tonsillar tissues.

Although such a medium may contain multiple inhibitory factors, we first focused on two of them, pH and lactic acid, whose roles in suppressing HIV infection were suggested earlier [Aldunate et al., 2013; Martin et al., 1985; O'Connor et al., 1995; Ongradi et al., 1990]. Depending on the bacterial strain, the pH of *Lactobacillus*-CM varied from 3.8 to 4.6. We adjusted the pH of the tissue culture to these pH values, and in agreement with earlier studies [Martin et al., 1985; Ongradi et al., 1990; Ravel et al., 2011] we found that this acidification may be directly responsible for HIV-1 inhibition. Low pH (<4.5) is typical for the vaginal communities *in vivo* that are dominated by *Lactobacillus* species in healthy women [Boskey et al., 1999; Fox et al., 1973; O'Hanlon et al., 2013]. However, during vaginal intercourse, vaginal fluid is diluted by HIV-containing semen, resulting in neutral pH [Tevi-Benissan et al., 1997]. Also, in the presence of vaginal dysbiosis (i.e., bacterial vaginosis), vaginal pH increases [Onderdonk et al., 2016]. Therefore, in our experiments we diluted CM with normal media, resulting in a pH between 6.3 and 6.9; this diluted CM was still inhibitory for HIV-1 replication in human tissue *ex vivo.* Control experiments with pH 6.9 demonstrated no HIV-1 suppression, suggesting that other factors beyond lowered pH may also be important for HIV-1 inhibition, at least for some of the lactobacilli.

One such factor considered in the literature is the major *Lactobacillus* metabolite lactic acid [O'Hanlon et al., 2011, 2013]. The importance of this metabolite is evidenced by the fact that in our experiments we observed a correlation between the capacity of supernatant of lactobacilli to inhibit HIV-1 replication and the capacity of lactobacilli to produce lactic acid. Therefore, we investigated the effect of lactic acid isomers D and L on HIV-1 infection. We found that the addition of these isomers to tissue culture medium at concentrations that corresponded to their amounts released by lactobacilli resulted in HIV-1 inhibition. In our work, the racemic lactic acid in *Lactobacillus*-CM ranged from 10.8 to 28.3 mM and thus was not higher than the physiological level, reported to be around 110 mM [O'Hanlon et al., 2013]. The protective effect of lactic acid in our *ex vivo* tissue system is in agreement with the work of Nunn et al., who reported that a high concentration of lactic acid in cervico-vaginal mucus plays an important role in protection against HIV-1 and other sexually transmitted infections [Nunn et al., 2015]. We found that the L isomer rather than the D isomer was predominantly responsible for HIV-1 inhibition. These results indicated that lactic acid, in particular its L isomer, inhibited HIV-1 replication, independently from lowering the pH.

Next, we investigated whether *Lactobacillus* could have a direct virucidal effect on HIV-1. To answer this question, we incubated an HIV-1 preparation in *Lactobacillus*-CM and then tested HIV-1 infectivity in human tissue culture. We found that HIV-1 infectivity in cervico-vaginal tissue was significantly reduced. We previously reported similar findings when testing the effect of *Lactobacillus*-CM on *C. trachomatis* [Nardini et al., 2016]. Finally, we investigated whether direct interactions with lactobacilli themselves may affect HIV-1. We found that a significant fraction of virions are adsorbed on bacteria. These virucidal effects of lactobacilli may be relevant to the inhibition of HIV-1 transmission *in vivo*.

In general, the level of HIV-1 suppression may depend on the superimposition of multiple mechanisms, different for each *Lactobacillus* strain. These mechanisms include change of pH, production of lactic acid, HIV adsorption on the surface of lactobacilli, etc. However, lactic acid produced by lactobacilli in the context of human tissues *ex vivo* seems to be a major cause of HIV-1 inhibition.

Several molecular mechanisms by which this metabolite may affect HIV-1 have been suggested. It was reported that lactic acid could disrupt cellular membranes [Alakomi et al., 2000], acidify cytosol [Russell and Diez-Gonzalez, 1998], unfold proteins [Tang et al., 2003], and inhibit enzymatic activity [McWilliam Leitch and Stewart, 2002]. Any of these reported effects of lactic acid might be sufficient to suppress HIV infection, e.g., by destroying the viral envelope, unfolding gp120, and/or inhibiting HIV enzymes involved in the HIV cycle [Aldunate et al., 2013].

In conclusion, we identify vaginal lactobacilli active against *Candida*, *C. trachomatis,* and HIV-1 [Nahui Palomino et al., 2017; Nardini et al., 2016; Parolin et al., 2015]. We characterize the mechanisms of action underlying antagonism toward these pathogens. We have identified strains with a good spectrum of fungistatic/fungicidal activity (*L. crispatus* BC1 and *L. vaginalis* BC15) that may be associated with strains particularly active in reducing the adhesion of *Candida* (*L. crispatus* BC2, *L. gasseri* BC10, *L. gasseri* BC11). We demonstrate the ability of different *Lactobacillus* strains of vaginal origin to inactivate *C. trachomatis* through the production of extracellular metabolites in an acidic environment. We found that mostly species of *L. crispatus* inhibit *C. trachomatis* infectivity, stressing once again the importance of this species for the vaginal health. Finally, we found that lactobacilli inhibit HIV-1 replication in human tissue *ex vivo* by multiple mechanisms.

Extrapolated to *in vivo*, our results may explain why the presence of normal vaginal microbiota, which include multiple species of *Lactobacillus,* is associated with a decreased risk of bacterial vaginosis, vulvo-vaginal candidiasis, and decreased risks of acquisition of sexually transmitted infections, including *C. trachomatis* and HIV.

The positive effects of vaginal lactobacilli on the health of the female genital tract are generating increasing interest in the perspective of their use in probiotic formulations for the prophylaxis and therapy of several vaginal disturbances [Burton et al., 2003; Donders et al., 2010; Reid et al., 2001]. The application that follows is the combination of strains exerting different modes of action to obtain a probiotic blend with enhanced therapeutic

properties. The choice of different species is also an added advantage as it ensures a wider expression of metabolic functions.

Further studies are necessary for a thorough understanding of the antagonistic mechanisms of vaginal lactobacilli against pathogens, i.e. study if lactobacilli release extracellular vesicles/exosomes (EVs) rather than just soluble bioactive molecules (organic acids, bacteriocins, H_2O_2, etc.). Nowadays, EVs are becoming more and more studied in various aspects of the biomedicine. EVs are produced by all domains of life. These EVs contain varied cargo, including nucleic acids, toxins, lipoproteins and enzymes, and have important roles in microbial physiology and pathogenesis.

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