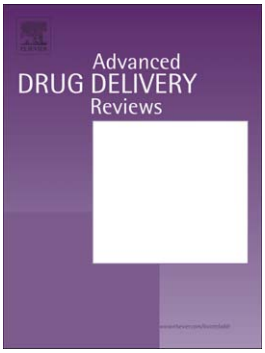

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PROFILES AND TECHNOLOGICAL REQUIREMENTS OF UROGENITAL PROBIOTICS

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ABSTRACT

Probiotics, defined as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host, are considered a valid and novel alternative for the prevention and treatment of female urogenital tract infections. Lactobacilli, the predominant microorganisms of the healthy human vaginal microbiome, can be included as active pharmaceutical ingredients in probiotics products. Several requirements must be considered or criteria fulfilled during the development of a probiotic product or formula for the female urogenital tract. This review deals with the main selection criteria for urogenital probiotic microorganisms: host specificity, potential beneficial properties, functional specifications, technological characteristics and clinical trials used to test their effect on certain physiological and pathological conditions. Further studies are required to complement the current knowledge and support the clinical applications of probiotics in the urogenital tract. This therapy will allow the restoration of the ecological equilibrium of the urogenital tract microbiome as well as the recovery of the sexual and reproductive health of women.

Keywords: Probiotics. Pharmabiotics. Female urogenital tract. Urogenital tract infections. Potential beneficial properties. Technological characteristics. Clinical assays.

Abbreviations

API: Active pharmaceutical ingredients
AV: Aerobic vaginitis
BV: Bacterial vaginosis
CFU: Colony forming units
CRL: Centro de Referencia para Lactobacilos Culture Collection
CVF: Cervicovaginal fluid
EFSA: European Food Safety Authority
FGM: Food Grade Microorganisms
FGT: Female genital tract
GRAS: Generally Regarded As Safe
HM: Human microbiome
ISAPP: International Scientific Association for Probiotics and Prebiotics
LAB: Lactic acid bacteria
PAMP: Molecular patterns associated with pathogens

QPS: Qualified Presumption of Safety

TLR: Toll-like receptors

UGTI: Urogenital tract infections

UTI: Urinary tract infections

VVC: Vulvovaginal candidiasis

1. Introduction

Human beings are colonized by a diverse and complex collection of microbes, contributing all of them to host nutrition, development of the immune system, response to pathogens and mucosal cell differentiation and proliferation. The knowledge of these communities and their gene contents has been referred collectively as the **human microbiome (HM),** supported by a NIH-funded project consortium [1,2]. The human microbiome is a complex system of many microbial communities inhabiting a diversity of environmental niches throughout the human body. Until recently, technological limitations precluded the global characterization of the human microbiome in terms of composition, diversity and dynamics. Massive parallel sequencing and other high throughput approaches have offered novel ways to explore and examine the microbiota from different human body cavities that includes eukaryotes, archae, bacteria and viruses. The sequences of more than 1000 bacterial genomes are now available and it is interesting to remark that bacteria numbers within an individual are estimated higher than number of human cells by an order of magnitude [1-3].

The increase in microbiota-related research has provided important advances toward the identity of specific microorganisms and microbial groups or microbial molecules, their functions and relationships between healthy-unhealthy status, being essential to the overall health of the host by performing relevant physiological functions, protection against pathogens and driven the development of the immune system during neonatal life. Additional projects are investigating the association of specific components and dynamics of the microbiome with a variety of disease conditions. HM project encountered an estimated 81-99% of the genera, enzyme families and community configurations occupied by the healthy western microbiome [1,2]. Studies on this HM have revealed that healthy individuals differ remarkably in the microbes that occupy gut, skin and vagina. But the microbial genera are highly dependent on the colonization of microorganisms carried out after the newborn delivery, on the prevalent environmental conditions and on different host factors that are modified through the time. There is some remarkable similarities in the bacterial species present in people with different ethnicity [3,4].

The microbiome colonizing the human body provides the host a huge coding and metabolic activities as will be described later [1,2,5-8]. Among this microbiota there are health-promoting indigenous species that are commonly consumed as live supplements [9].

Lactic acid bacteria (LAB) and some related genera were isolated from almost all the mucosa and human tracts. Referred specifically to the genus *Lactobacillus*, there are around 202 different described species up to present (http://www.bacterio.net/lactobacillus.htlm) and more than 100 with their chromosomal DNA sequenced. Their genomes have sizes varying from 1.8 to 3.3 MB, and their G+C content range from 33 to 51%. In such a way, a *Lactobacillus* core genome has been described, constituted by 383 sets of orthologous genes, designed as *Lactobacillus* core genome [5,10,11].

2. Vaginal microbiome. Ecological and functional aspects

Vaginal microbiota forms a mutually beneficial relationship with their host and has a major impact on health and disease. In the vaginal microbiome, lactobacilli constitute the dominant proportion (80%) of bacteria inhabiting the healthy women's vagina [1,2,5-7,12,13]. Some LAB strains have found to be endogenous from healthy human vagina, where there is a rather stable microbiota [14-16]. LAB members are consistently detected in healthy vaginal microbiota of different ethnic groups and/or women living in different geographical locations [7,17-22]. Four main species were identified: *Lactobacillus crispatus, Lactobacillus iners, Lactobacillus jensenii* and *Lactobacillus gasseri*, along with other lactobacilli at lesser extent, as *Lactobacillus acidophilus, Lactobacillus ruminis, Lactobacillus rhamnosus* and *Lactobacillus vaginalis* [7,15]. Our understanding of the vaginal microbial community composition and structure has significantly broadened as a result of studies using cultivation-independent methods based on the analysis of 16S ribosomal RNA (rRNA) gene sequences [6,10,11,15,23,24].

The high abundance of LAB is strongly associated with a healthy vagina, whereas a low abundance of LAB is more prevalent in women with a pathological condition [6-9,13,14,16,19,25-27]. Eventhough the four species indicated above are predominantly detected in human vagina, co-dominance between LAB is not very frequent [1,7,15]. In asymptomatic, otherwise healthy women, several kinds of vaginal microbiota exist, the majority often dominated by species of *Lactobacillus*, while others are composed of a diverse array of anaerobic microorganisms [7,8,13,15,21,27]. Ravel et al. [7] characterized the vaginal microbiome of asymptomatic, sexually active women who represented four ethnic groups (white, black, Hispanic, and Asian). The vaginal bacterial communities were classified according to community composition in five

major groups. Communities in group I were dominated by *L. crispatus*, whereas groups II, III and V were dominated by *L. gasseri*, *L. iners* and *L. jensenii*, respectively. Group IV was highly heterogeneous and had higher proportions of strictly anaerobic bacteria, including *Prevotella*, *Dialister*, *Atopobium*, *Gardnerella*, *Megasphaera*, *Peptoniphilus*, *Sneathia*, *Eggerthella*, *Aerococcus*, *Finegoldia*, and *Mobiluncus*. The proportions of each community group varied among the four ethnic groups. Communities with high Nugent scores (criterion used to diagnose bacterial vaginosis) were most often associated with communities in group IV, but were also observed in communities belonging to other groups.

Most of the *Lactobacillus* species described above were related to the healthy vagina, but some other authors suggested that *L. iners* was frequently isolated from non-healthy subjects [24]. Molecular-based and culture-based techniques used in combination have indicated that in the absence of lactobacilli, normality can be maintained by more fastidious lactic acid producing bacteria [15]. The dominant *Lactobacillus* species may differ racially or geographically, but the principle of numerical dominance persists [6,7,17,18,20-22,27], indicating that the LAB may be adapted to the vagina and possess characteristics enabling them to thrive in that environment [28].

The temporal dynamics of vaginal communities are poorly known because few studies have been done in which the same individuals are frequently sampled and variation in community composition assessed over time using cultivation-independent methods [24,26,29,30]. Fredricks [31] suggested that the vaginal microbiota can be highly dynamic, with dramatic shifts in bacterial composition and concentrations in response to numerous endogenous and exogenous factors. Ravel et al. [7] proposed different hypothesis that could explain the variation of vaginal community composition over time: 1) “dynamic equilibrium hypothesis”, in which the composition of a community is comparatively invariant over time and exists in a single dynamic equilibrium; 2) “community space hypothesis”, in which each community can and does occupy any position in community space over time and throughout a woman’s lifetime; 3) “alternative equilibrium states hypothesis”, wherein a woman’s community can change over time, but the number of alternative states are limited in number and governed by unknown factors, and 4) “community resilience hypothesis”, in which the composition and structure of a vaginal community can change to a transitional state in response to disturbance, but the resistance and resilience of a community determine the

extent and duration of a change. Further studies are required to understand the dynamics of vaginal communities.

On the other hand, there was an inverse correlation between *Lactobacillus* ratio and dryness and an increased bacterial diversity in women experiencing moderate to severe vaginal dryness, as indicated by Hummelen et al. [32]. In healthy participants, *L. iners* and *L. crispatus* were generally the most abundant species, countering the long-held view that lactobacilli are absent or depleted in menopause. Vaginal dryness and atrophy were associated with down-regulation of human genes involved in maintenance of epithelial structure and barrier function, while those associated with inflammation were up-regulated consistent with the adverse clinical presentation [32].

3. Urogenital tract infections (UGTI) and uses of urogenital probiotics

3.1. UGTI

The urogenital microbiome composition is influenced by genetics, hormone levels, hygiene, disease and use of contraceptives and antibiotics, among others factors [14,16,19,27,33,34]. The genetic polymorphism of each person is defined as small changes in the DNA sequence of a gene occurring between individuals that influence the production of high or low levels of anti- or pro-inflammatory factors [35,36]. The genetic polymorphism varies among the different ethnic groups and influences the urogenital microbial composition and the susceptibility to UGTI [37-40]. The imbalance of the female urogenital ecosystem, which can be caused by different external or internal factors, negatively affects the protective, indigenous microbiota and favors the income or the overgrowth of pathogenic or potentially pathogens causing UGTI [14,19,36].

The UGTI, their costs and frequencies are detailed in depth in other reviews of this special issue. Briefly, sexual transmitted infections and others infections affecting the reproductive tract (in sexually active, pregnant or post-menopausal women) are of main importance and with a high frequency at all the social and economic level human groups [14,19,22,24,41-45]. Single urinary tract infections (UTI) episodes are very common, mainly in adult women, in which there is a 50-fold predominance compared with adult men [46-52]. Recurrent UTI are also frequent, occurring in up to one-third of women after the first episode [53]. The major types of UGTI are the acute and recurrent bacterial and fungal infections, and certain anatomical characteristics as well as hormonal effects make women more susceptible to them [42]. Bacterial vaginosis (BV),

vulvovaginal candidiasis (VVC) and infections of urethra, bladder, ureter, kidney or cervix affect 300 million women per year worldwide; in USA alone, UTI resulted in an annual health-care costs of about U$1.6 billion [45].

The UGTI are associated to high morbidity and mortality of mothers, fetuses and the newborns. As example, preterm birth was recently related to a multiplicity of infections [43,54,55], in such a way that while bacterial ascent from the vaginal tract was recognized as the primary cause of intrauterine infection, the microbiomes of the other tracts were shown to be involved by means of hematogenous spread [56]. Most of the UGTI are treated with a different set of traditional antimicrobial drugs [47,49,51,57]. Eventhough these therapies have improved the quality of the life of patients, they have produced a long list of inconvenient, as adverse effects, limitations of use in pregnant women, high costs and recurrence rates, increase of resistant and multiresistant microbial strains and the horizontal genetic resistance transference evidenced more frequently [57-60].

3.2. Probiotics

The description of the main LAB species isolated from the vaginal tract of women around the world and their differences between healthy and non-healthy patients (as mentioned in item 2) supports the idea of the rebalancing the urogenital microbiome with some specific and well characterized *Lactobacillus* strains. Moreover, the long list of disadvantages associated to the conventional antimicrobial drug uses are important reasons to search for novel and alternative treatment options for UGTI, such as probiotics.

Probiotics are defined as "***live microorganisms that, when administered in adequate amounts, confer a health benefit on the host***" (microbial, viable and beneficial cells to health) [61,62]. The microbial genera more frequently used as probiotics are included into the LAB and related groups of microorganisms, such as *Lactobacillus,* some species of *Streptococcus* and *Bifidobacterium*. These microorganisms are considered as GRAS (Generally Regarded as Safe), many of them are also designed as Food Grade Microorganisms (FGM) and included in the Qualified Presumption of Safety (QPS) approach by the European Food Safety Authority (EFSA) for their use and safety in foods [63].

On the other hand, the term **prebiotic** includes ***"a selectively fermented ingredient that produces specific changes, consumption or activity of the***

gastrointestinal microbiota that confers benefits on host health" [64,65], whereas synergistic combinations of probiotics and prebiotics are called symbiotics. Among the carbohydrates qualified as prebiotics, fructo-oligosaccharides and gluco-oligosaccharides are of main interest [65]. Despite the prebiotics definition refers to gastrointestinal use, this molecules are also used in vaginal products (e.g., in EcoVag® vaginal capsules, Bifodan A/S, Denmark) [66]. In the urogenital tract, the prebiotics could stimulate the growth of a limited number of potentially health promoting endogenous microorganisms, thus modulating the composition of the natural ecosystem [67]. Through *in vitro* assays, Rousseau et al. [67] have shown that the prebiotics affected the growth of vaginal lactobacilli, but inhibited some specific pathogens.

There was a high emergence of papers published during the last 30 years, together with the constitution of scientific associations related with this subject, as the International Scientific Association for Probiotics and Prebiotics (ISAPP) and the participation of some international non-government associations, as Food and Agriculture Organization of the United Nations (FAO), World Health Organization (WHO), EFSA, National Food Commission (CONAL) in Argentina (http://www.alimentosargentinos.gov.ar/contenido/marco/CAA/capitulospdf/Capitulo_XVII.pdf) that are working on the redefinition or in the correct use of some specific terms and the legal requirements for probiotics/prebiotics in each country or region [61-63,65,68].

The term pharmabiotic has arisen related to the probiotic concept, and it includes ***"live or dead microorganisms, and some other microbial components or metabolites able to interact beneficially with the host"*** [69]. On the other hand, some other active compounds such as vitamins, peptides or proteins, hormones, growth factors, vegetal extracts or phytoderivatives could be included in formulations to complement the probiotic effect [70-73].

The **probiogenomic** meaning genomic-based studies is beginning to provide insights into how probiotic bacteria sense and adapt to each tract environment [74]. This approach helps in the elucidation and understanding of the molecular basis of probiotic effects. The term **metagenomic** was also lately included to mean the carriage of metabolic pathways that are stable among individuals, and ethnic/racial background proved to be one of the strongest associations of both pathways and microbes with clinical metadata [23,75].

3.3. Clinical studies of urogenital probiotics

Supported by the concepts described before and by the tendency to apply preventive politics around the world, the use of probiotics for urogenital applications has increased in the last years. One of the requirements for the design of a vaginal probiotic product or formula is to perform clinical assays to determine its safety (absence of adverse effects), efficacy and effectiveness in the clinical application proposed [61,62,64,65].

Up to date, numerous clinical studies have been performed to evaluate the effects of probiotic or potential probiotic microorganisms on the urogenital tract of women under different physiological conditions: fertile, non-pregnant women (Table 1), pregnant women (Table 2) and post-menopausal women (Table 3). Moreover, several pathological conditions were considered, as shown in Tables 1-3. In this review, most of the available clinical trials that were published between 2009 and 2014 were included, because a deep analysis of trials published between 1991 and 2008 was previously reported [76].

In reproductive age, non pregnant women (Table 1), the use of probiotic or potential probiotic strains was evaluated mainly in three ways: 1) as a simultaneous treatment to antimicrobial drugs employed to treat BV and VVC, 2) as a subsequent treatment to antimicrobial drugs used to treat and/or prevent BV, CVV and UTI, and 3) as a single therapy to improve the vaginal ecosystem status and to treat and/or prevent BV, CVV, aerobic vaginitis (AV) and trichomoniasis [66,77-94]. In most of the studies, commercial probiotic products or new potential probiotic formulations were vaginally administered. Oral probiotic administration is considered an alternative route, believing that microorganisms can ascend to the vaginal tract after their excretion from the rectum [12]. Regardless of the administration method and of the strain assayed (referred as active pharmaceutical ingredient (API)), most of the clinical studies reported promising results by application of probiotics in the urogenital tract, such as higher cure rates and lower recurrences mainly in patients with BV, restoration of vaginal ecosystem balance, colonization of probiotic microorganism (in those cases where it was determined) and absence of serious adverse effects.

With respect to probiotic uses in pregnant women (Table 2), which was mainly carried out by oral administration, clinical trials have evidenced positive results on the modulation of the vaginal microbiota (an increase of beneficial microorganism populations and a decrease of pathogenic microorganisms) [95-98]. The prophylaxis and

treatment of BV are of main relevance in pregnant women, because they have potential implications in preventing preterm birth associated to this pathology. Recent results suggest that the dominance of only *L. iners* in early pregnancy of healthy women might be associated with preterm delivery [43]. Oral probiotics were not effective to prevent spontaneous preterm delivery associated with BV [99], while the addition of vaginal probiotic to ampicillin prolonged the latency period in patients with preterm premature rupture of membranes (PPROM) remote remote from term [100].

Some clinical assays were carried-out in post-menopausal women in whom the menopause was natural or after a surgery (Table 3) [101-105]. Treatments with vaginal probiotics were proved effective to prevent recurrent BV and to positively modify the vaginal microbiome in small groups of patients [102,103], but oral probiotics failed to prevent recurrent urinary tract infections [101]. On the other hand, the local application of *Lactobacillus acidophilus* KS400 combined with estriol caused resolution or improvement of vaginal atrophy and normalization of the vaginal ecosystem in post-menopausal women, while no adverse events were reported [104].

In conclusion, certain probiotic or potential probiotic strains, alone, combined or supplemented to antimicrobial drugs, were effective for prevention and treatment of BV. However, there is insufficient clinical evidence on the efficacy of probiotics for the treatment of UTI, VVC and other urogenital infections and for the prevention of preterm birth and its complications. More research in well-designed randomized controlled trials with larger patient size is needed.

4. Profile of beneficial microorganisms and probiotic products

When a probiotic/pharmabiotic product for the urogenital tract is going to be designed, several specific criteria should be considered (Figure 1). Some of the selection criteria, mainly those highly related with the demonstration of the mechanisms of action of specific strains in both *in vitro* and *in vivo* experimental models, will be described in the following items.

4.1. Host specificity

The microbial species show the phenomena of species-specificity and host-specificity, evidenced by different scientists from a long time ago [106]. Some results

revealed that strains have distinctly evolved and have distinct genetic signatures between different human or animal hosts [8,10,15,75,107,108].

In this way, those strains isolated from the same host and mucosal environment where they will be applied are microorganisms with higher possibilities to colonize, survive and maintain in a specific site, and to exert then a specific physiological effect on the host. Based on the host-specificity and on the predominance of the vaginal LAB, the isolation of lactobacilli from vaginal tract of mice was performed, in order to study them in a specific experimental animal model [109,110]. Different vaginal lactobacilli were isolated from healthy women, to develop novel probiotic/pharmabiotic formula for the restoration of the human urogenital microbiome [21,111-116]. The phenotypic and genetic identification was performed by applying different methodologies that allowed the correct classification of *Lactobacillus* species [111-118].

4.2. LAB functional genomic. Specific genes

A variety of functional genomics approaches have been reported in the last years relating LAB associated to different sites of the human body [1,2,10,11,15]. In the case of some vaginal LAB strains, studies on specific genes [24,28,115,119] and the whole-genome sequencing [113,114,120,121] were recently available.

In the vaginal tract, each microbial species may harbor genes that relate to unique adaptation signatures and allow their persistence and colonization regardless of the presence of other LAB members [15,28]. Several genomes of vaginal LAB species evaluated were significantly smaller and had significantly lower G+C content than those of the non-vaginal species, suggesting a loss of non-essential genes towards a vaginal adaptation [9,15,28]. No protein families were found to be specific to the vaginal species analyzed, but some were either over- or under-represented relative to non-vaginal species. Within the vaginal species, each genome coded for species-specific protein families [28]. As example, Macklaim et al. [114] described the whole-genome sequence of vaginal *L. iners* AB-1, showing an organism depleted of many metabolic pathways and that could have lost a high amount of genes. Moreover, *L. iners* seems to have undergone the horizontal acquisition of genes for survival in the vaginal environment, such as an iron-sulfur cluster assembly system, and several unique σ factors to regulate gene transcription in this fluctuating ecological niche. *L. iners* AB-1 showed be equipped with predicted fibronectin (Fn)-binding adhesins. Subsequent

phenotypic studies evidenced the ability of *L iners* AB-1 to bind immobilized Fn, which can be a mechanism of vaginal persistence of the microorganism [122].

Genome sequence of *Lactobacillus pentosus* KCA1, a vaginal isolate from a healthy premenopausal Nigerian woman, allowed an improved understanding of metabolic systems within lactobacilli and suggested the metabolic versatility of the microorganism assayed [120]. Their results evidenced: a) loci encoding additional putative mannose phosphotransferase systems, b) clusters of genes for utilization of hydantoin, isopropylmalate, malonate, rhamnosides, and genes for assimilation of polyglycans, c) loci encoding putative phage defense systems including clustered regularly interspaced short palindromic repeats (CRISPRs), abortive infection (Abi) systems and toxin-antitoxin systems (TA), d) a putative cluster of genes for biosynthesis of a cyclic bacteriocin precursor, among other genetic features, which support the specific versatility.

The draft genome of human vaginal *L. plantarum* CMPG5300 was recently reported, and a further detailed genome analysis could aid in identifying the factors implicated in adhesive properties of the strain [121]. In mutants of *L. plantarum* CMPG5300, the relevant role of sortase SrtA on auto-aggregation and adhesion abilities was evidenced [115]. Sortase SrtA is an enzyme that covalently anchors sortase-dependent proteins (SDPs) to the cell surface, which could promote bacterial adhesion to host cells in the vaginal niche.

In several potential probiotic vaginal *L. gasseri*, *L. reuteri* and *L. rhmanosus* strains, the presence of genes encoding sortases, pilin subunits and surface proteins, which could be involved in adhesion and colonization, were evaluated [119]. In *L. reuteri* CRL (Centro de Referencia para Lactobacilos Culture Collection) 1324 and CRL 1327, the genes encoding three adhesion proteins (mapA: mucus adhesion promoting protein, mubI: mucus-binding proteins, and cmbA: mucus- binding protein A) were identified. In *L. rhamnosus* CRL 1332, pilus-encoding genes were detected. In three *L. rhamnosus* strains assayed, two genes encoding for other surface proteins related to adhesion and biofilm formation were detected.

In summary, the functional genomic has a relevant role in the selection process for potential probiotic microorganisms as it enables *in silico* the analysis of key genotypes involved in the mechanisms of probiotic activity [123,124]. Moreover, integrated genomic techniques could allow an increased knowledge on niche-related microbial characteristics, which contribute to colonization and to complex, beneficial

interaction between probiotic strains and their host [114,123]. Anyway, the genomic and experimental evidence supporting the use of a specific strain in probiotic applications must be demonstrated by clinical trials [61-63,123].

4.3. Beneficial properties of probiotics for the urogenital tract

The beneficial roles of LAB in preserving a healthy vagina, preventing the infectivity and/or proliferation of pathogenic bacteria include the maintenance of acidic vaginal pH (lactic acid production), the protection from infections by products of endogenous bacteria as bacteriocins, hydrogen peroxide, and also by signaling to the host through activation of local innate and acquired immunity [76,125].

4.3.1. Adhesion properties

The female genital tract (FGT) consists of two different types of mucosal surfaces [126]. The lower genital tract (ectocervix and vagina) is a type II mucosal surface, with a stratified epithelium. In contrast, the upper female genital tract (oviducts, ovaries, uterus, and endocervix) consists on a type I mucosal surface, being monolayered as most of the epithelia. It was long believed that the upper FGT was sterile, but recent studies have shown that there is a constant exposure to antigenic material (mainly commensal bacteria), which is transported to the uterine lumen by peristaltic waves [127]. The layer of mucus bound the epithelia is formed from a continuous gel matrix composed primarily of complex glycoproteins that acts as a barrier to protect the host from harmful antigens and promote luminal motility [128].

The layer of mucus is the first physical barrier to host-cell interaction by bacteria. Adhesion to this mucus is therefore the first step required for probiotic or beneficial organisms to interact with host cells and elicit any particular response [129]. Once administered, the bacteria should be adhered to the mucosal surface, either mucus or to the eukaryotic cell as a first step in the permanence or colonization of the host. A wide variety of specific and non-specific adhesins, such as proteins, lipoteichoic acid, S layers and polysaccharides of the bacterial surface can be involved in specific and non-specific adhesion to host cells [130]. The structures of eukaryotic host cells, e.g., the double lipid layer-cell membrane, the mucus components (glycolipids) and the connective or basal membrane constituents (collagen, fibronectin, laminin, proteoglucanes) are also involved in colonization [119,122].

Martín et al. [131] evidenced that there is a strong interaction between vaginal *Lactobacillus salivarius* Lv72 with glycosaminoglycans present on the surface of HeLa epithelial vaginal cell line, mediated by a soluble binding protein on the ABC transporter system. These data suggest that glycosaminoglycans play a fundamental role in attachment of mutualistic bacteria to the epithelium that lines the cavities where the normal microbiota thrives, OppA being a bacterial adhesin involved in the process [131]. In some vaginal or intestinal lactobacilli, a collagen A-binding precursor and aggregation-promoting factor-like proteins were suggested to participate on adhesion to Caco-2 and HeLa cells respectively; while a gliceraldehyde-3 phosphate dehydrogenase could explain the good adhesion of some strains to mucin [132].

McMillan et al. [122] have shown that *L. iners* AB-1 bonded to immobilized fibronectin, and treating the bacterial cells with protease rendered the binding abilities to adhere to fibronectin to no functional, indicating the protein nature of the adhesion. In *L. crispatus* 2029, a vaginal strain isolated from fertile, healthy women and selected as a probiotic candidate, external protein surface layers (S layers) that completely surrounds the cells were described. S layers of *L. crispatus* 2029 were responsible for its adhesion on the surface of cervicovaginal epithelial cells [133]. A probiotic *L. rhamnosus* strain (Lcr35), which shows antimicrobial activity against some vaginal-associated pathogens, adhered *in vitro* to epithelial cells from the endocervix, ectocervix and vagina [134].

In human vaginal lactobacilli isolated from Argentinean women, the *in vitro* study of several surface characteristics, including adhesion to human scrapped vaginal epithelial cells and to mucin, was carried out [111,119,135]. These assays allowed the selection of several potential probiotic strains with higher possibilities to colonize the vaginal mucosa.

4.3.2. Aggregation and biofilm formation

Aggregation promoting factors are secreted proteins that have been associated with a diverse number of functional roles in lactobacilli, including self-aggregation, bridging of conjugal pairs, co-aggregation with other commensal or pathogenic bacteria, and maintenance of cell shape [136,137]. Bacterial self-aggregation is an interesting phenomenon that can promote adhesion to host cells and displacement of pathogens. Malik et al. [115] reported that a human vaginal isolate, *L. plantarum* strain CMPG5300, showed high auto-aggregative and adhesive capacity. The auto-

aggregating and adhesive phenotype of *L. plantarum* strain CMPG5300 was related to a sortase SrtA present on the surface of this strain, as mentioned above.

Co-aggregation is another suggested mechanism through which lactobacilli can exert their probiotic effect to create a hostile micro-environment around a pathogen [136,137]. It might enable to form a probiotic barrier that prevents colonization by pathogenic bacteria. Ekmekci et al. [138] have characterized the co-aggregation ability of vaginal lactobacilli with uropathogenic *E.coli*. Younes et al. [136] indicated that lactobacilli displaying strong adhesion forces resulted in significantly large co-aggregates with virulent toxic shock syndrome toxin 1-producing *Staphylococcus aureus* strains. With antimicrobial options fading, it therewith becomes increasingly important to identify lactobacilli that bind strongly with pathogens. The auto-aggregation ability of vaginal strains of different *Lactobacillus* species (i.e. *L. gasseri*, *L. salivarius*, *L. rhamnosus* and *L. reuteri*) and co-aggregation with some specific urogenital pathogens (i.e. *Streptococcus agalactiae*, *S. aureus* and *Candida albicans*) was assayed, defining that some specific protein structures were related with this phenomenon [119,139,140].

Several surface properties, such as adhesion, auto- and co-aggregation, could be related with the biofilm formation by different microorganisms on mucosal surfaces [137]. McMillan et al. [141] indicated that urogenital probiotic *L. rhamnosus* GR-1 caused a marked decrease in cell density and increased cell death of biofilms formed by pathogens associated to BV. Thus, probiotic lactobacilli could interfere with an aberrant vaginal microbiota through the eradication of pathogenic biofilms. This biofilm formation, which could promote the colonization and persistence of beneficial strains in the vaginal tract, was evaluated in several vaginal *Lactobacillus* strains [112,115,119]. Martín et al. [112] reported that the ability to generate a biofilm was observed in most of the 45 strains of vaginal lactobacilli, which were isolated from healthy women, and mainly in three *L. jensenii* isolates. Leccese Terraf et al. [119,142] demonstrated that some potential probiotic *L. reuteri* and *L. rhamnosus* strains formed biofilm in culture media without Tween 80. On the other hand, Malik et al. [115] observed that *L. plantarum* CMPG5300 showed an exceptionally high biofilm-forming capacity compared to other vaginal *Lactobacillus* strains, and that its mutant in the *srtA* gene lost the ability to form biofilm under the assayed conditions..

4.3.3. Enzymatic activities and production of biosurfactants

Referred to enzymatic activities, vaginal lactobacilli can be affected by enzymes present in the healthy or non-healthy urogenital tract [143]. Probiotic LAB should be resistant to enzymes that could lead to their inactivation, such as prolidase and sialidase that are relevant enzymes in the pathogenesis of BV [144].On the other hand, Spear et al. [143] reported that several *Lactobacillus* isolates did not grow in glycogen, but have shown to grow in glycogen-breakdown products, including maltose, maltotriose, maltopentose, maltodextrins and glycogen treated with salivary α-amylase. A temperature-dependent glycogen-degrading activity was detected in genital fluids that correlates with levels of α-amylase. Treatment of glycogen with genital fluids resulted in production of maltose, maltotriose, and maltotetraose, the major products of α-amylase digestion. These studies indicated that human α-amylase is present in the female lower genital tract and helped to elucidate how epithelial glycogen can support *Lactobacillus* colonization in the genital tract [143].

Matrix metalloproteinase (MMP-8) is an enzyme known to alter the integrity of the cervix [145]. Witkin et al. [145] indicated that concentrations of vaginal extracellular matrix metalloproteinase inducer (EMMPRIN) were influenced by members of the vaginal microbial community and concentrations of D- or L-lactic acid isomers in vaginal secretions. Elevated levels of D-lactic acid and the ratio of D- to L-lactic acid influenced EMMPRIN concentrations as well as MMP-8 levels. Thus, isomers of lactic acid may function as signaling molecules that alter host gene expression and influence risk of infection-related preterm birth.

There are some other products released by LAB, as biosurfactants, which are amphyphylic compounds with a high surface and emulsified activity that is accumulated on the interphase of two fluid phases with different degree of polarity and H bonds, decreasing thus the interfacial and surface tension [146]. Different properties have been related with biosurfactant production by microorganisms, including the increased solubility of non-soluble water compounds (hydrophobic), union and adhesion to heavy metals, cellular adhesion and aggregation, quorum sensing and biofilm formation, and antimicrobial and antiadhesive properties against urogenital pathogens [147,148].

4.3.4. Production of antagonistic substances

The antimicrobial substances produced by LAB include mainly organic acids, hydrogen peroxide and bacteriocins [76].

Lactic acid. Lactic acid at sufficiently acidic pH is a potent microbicide, and lactic acid produced by vaginal lactobacilli may help to protect against reproductive tract infections [149]. O'Hanlon et al. [149] demonstrated that women with vaginal microbiota dominated by lactobacilli had significantly more lactic acid-mediated protection against infections than commonly believed. Lactic acid is one of the main products of the fermentative pathway of lactobacilli. The acidification of the vagina comes from a nearly racemic mixture of D and L-isomers [145]. This lactic acid in physiological conditions inactivated BV-associated bacteria without affecting vaginal lactobacilli [149]. L-lactic acid showed a more potent HIV inactivation [150] or inhibition of *Neisseria gonorrhea* growth in anaerobic conditions [151]. The lactic acid levels have influenced on the extracellular matrix metalloproteinase inducer, as signaling molecules that alter the host gene expression [145]. Free glycogen in genital fluid is also associated with a genital microbiota dominated by *Lactobacillus*, as cited before, suggesting that glycogen is important for maintaining genital health. Treatments aimed at increasing genital free glycogen might impact *Lactobacillus* colonization, as suggested by Mirmonsef et al. [152].

Hydrogen peroxide. H_2O_2-producing *Lactobacillus* strains have been related some years ago to a healthy vaginal ecosystem, because H_2O_2 produced by vaginal lactobacilli was believed to protect against infection at that time [153].

H_2O_2-producing lactobacilli inactivated pathogens *in vitro* in protein-free salt solution [154]. However, cervicovaginal fluid (CVF) and semen had significant H_2O_2-blocking activity. Given the H_2O_2-blocking activity of CVF and semen, it is implausible that H_2O_2-production by vaginal lactobacilli is a significant mechanism of *in vivo* protection. Physiological concentrations of H_2O_2 produced no detectable inactivation of either BV-associated bacteria or vaginal lactobacilli [149]. Moreover, at very high concentrations, H_2O_2 was more toxic to vaginal lactobacilli than to BV-associated bacteria. On the basis of these *in vitro* observations, these authors concluded that lactic acid, not H_2O_2, is likely to suppress BV-associated bacteria *in vivo*.

The results of Graver and Wade [151] have shown that there was no evidence of a specific mechanism of *N. gonorrhoea* inhibition other than acid production by vaginal lactobacilli, while hydrogen peroxide was not produced. The acidification potential of

vaginal lactobacilli under anaerobic conditions may be their most important characteristic conferring protection against *N. gonorrhoeae* infection.

On the other side, Cadieux et al. [155] have shown that compounds secreted by lactobacilli likely protected the urogenital tract from uropathogenic *Escherichia coli* (UPEC) colonization and infection by inhibiting growth, inducing stress and down-regulating proteins critical for host attachment. In a dose- and pH- dependent manner, lactic acid, hydrogen peroxide and *Lactobacillus* supernatants all strongly inhibited UPEC C1212 growth and increased the promoter activity of outer membrane proteins (OMPs) A and X, two porins normally up-regulated in response to UPEC membrane stress. Lactic acid and the culture supernatants also down-regulated the promoter activity of the major subunits of type 1 and P fimbriae, critical adherence factors within the urogenital tract. The results of Atassi and Servin [156] indicated that in the presence of lactic acid at a concentration present in *Lactobacillus* supernatants, hydrogen peroxide displayed enhanced killing activity. Collectively, these results demonstrated for hydrogen peroxide-producing *Lactobacillus* strains that the main metabolites of *Lactobacillus* (lactic acid and hydrogen peroxide) act co-operatively to kill enteric, vaginosis-associated and uropathogenic pathogens.

Bacteriocins and bacteriocin-like substances. There is an increased interest in the development of treatments using antimicrobial derived primarily from *Lactobacillus*, such as lactic acid described previously and ribosomally produced antimicrobial peptides (bacteriocins) [157]. These substances effectively inhibit pathogenic bacteria, are safe and do not possess any threat to healthy vaginal microbiota [73,116,157-163]**.** Different bacteriocins have been published from strains isolated from the vaginal tract. Lactocin 160, produced by a vaginal *L. rhamnosus* [158] and a subtilosin, produced by a *Bacillus amyloliquefaciens* strain, were active against bacterial vaginosis associated pathogens, combined with glycerol monolaurate, lauric arginate, Y-poly-L-lysine, lauramide arginine ethyl ester [159,161]. The molecular mechanisms of action of lactocin 160 was evaluated, revealing that this compound targets the cytoplasmic membrane of *G. vaginalis*, causing the efflux of ATP molecules and dissipation of the proton motive force [158]. Zinc lactate and sapindin act synergistically with lactocin 160 against this pathogen and therefore have a potential to be successfully used as the components of the multiple-hurdle antimicrobial formulation for the treatment of BV [160].

Lactobacilli isolated from the human vaginal tract in Tucumán, Argentina, were screened for the production of antagonistic substances, lactic acid, hydrogen peroxide and bacteriocins against a wide variety of pathogenic microorganisms [164,165]. In those studies, *L. salivarius* CRL 1328 produced salivaricin CRL 1328, the first bacteriocin described from a vaginal isolate, showing antimicrobial properties against urogenital pathogens [166]. The complete biosynthetic cluster of salivaricin CRL 1328 was identified, and this bacteriocin was characterized as a class IIb two-peptide component [167]. Salivaricin CRL 1328 was shown to dissipate membrane potential and the transmembrane proton gradient of pathogenic *Enterococcus faecalis* MP97 cells [167].

Sabia et al. [116] have described a bacteriocin-like substance produced by *L. fermentum* CS57 from vaginal origin. Also, *Pediococcus pentosaceum* SB83, with bacteriocinogenic activity against *Listeria monocytogenes* and able to survive in simulated vaginal fluid, was indicated as a safe strain for probiotic vaginal use [162]. Stoyancheva et al. [163] have shown the presence of gasserin A operon in some of the vaginal strains with bactericidal effect.

Referred to *in vivo* effect of bacteriocins, nisin was studied as a anticonceptive agent and administered in high doses to animal experimental models without production of adverse effects [168]. *In vivo* and *in vitro* assays indicated that lactocin 160 did not produce irritation on the vaginal epithelia [169]. However, *in vivo* studies on bacteriocins that could be effective in the prevention of infections on the urogenital tract have not yet been reported, but only in some other tracts [170].

4.3.5. Competition by nutrients

The competition by nutrients provides a selective advantage for those microorganisms that can use them first and selectively, for their own growth and survival [76,171]. The main evidence of this phenomenon at the urogenital tract is the preferential use of glycogen, or some of other metabolic products release to this environment, by the endogenous vaginal LAB, as cited before [143].

4.3.6. Inhibition of pathogen colonization: animal experimental model

Different animal experimental models has been set up to evaluate the probiotic effect at the urogenital tract and the protection against pathogens, either to go further into the mechanisms of action or to explain the preventive/protective effect [172,173].

The most frequent animal used was mice, but also rabbit [157,169]) or macaque was used trying to simulate the situations of the human tracts [174].

Some years ago, a murine experimental model was setup to evaluate colonization in the urinary tract of a murine selected *L. fermentum* strain, after its inoculation (included in agarose beads) into the urethra of female mice [109]. Reduction of uropathogenic *E. coli* viable cells was observed after a prophylactic or therapeutic protocol with the *L. fermentum* strain assayed [175]. Hormonal treatment with estradiol and lactobacilli has shown to protect animals against challenge with *E. coli* [110]. In mice treated with amoxicillin, which is known to promote colonization of *E. coli* and reduce adherence of the normal vaginal microbiota, *L. fermentum* showed an inhibitory effect against UTI. Lymphocytic proliferation at the submucosal level was observed in *E. coli*-challenged animals while no histological modifications were associated with *L. fermentum* [176].

On the other hand, colonization of mouse vagina by inoculation of human vaginal potentially probiotic lactobacilli was also assayed. Different rates of colonization were obtained depending on the particular strain tested [177,178]. At cytological or histological levels, lactobacilli did not cause adverse effects in the murine vaginal tract. The intravaginal administration of *L. salivarius* CRL 1328 and *L. gasseri* CRL 1263 did not affect the amounts of granulocytes and macrophages present in vaginal washings, evaluated by flow cytometry [140]. Zárate et al. [179] demonstrated that, while the inoculation of *S. aureus* produced a remarkable inflammatory response and structural alterations in the vaginal mucosa, they decreased significantly when the mice were protected with *L. paracasei* CRL 1289. Recently, the preventive effect of *L. reuteri* CRL 1324 against vaginal challenge with *Streptocccus agalactiae* was demonstrated in the experimental murine model [140].

4.3.7. Immune system modulation

The female genital tract is equipped to deal with a variety of foreign substances including spermatozoa, a fetus that is immunologically distinct from its mother, and a wide array of pathogens. These pathogens include viruses, bacteria, fungi and parasites [14,108,126,180,181]. To add to this complexity, the various parts of the female genital tract are influenced by sex hormones during menstruation [182]. All of these components act in concert to optimize the conditions for reproduction and a successful pregnancy [108,126,180-184]. The female genital tract is protected against these various

invaders by two inter-related mechanisms: the innate and the adaptive immune systems. The intricate interplay between the two arms of the immune system, plus their interaction with the genital tract bacterial microbiota and the host epithelial cells, are ultimately responsible for the health of the lower female genital tract [181-184].

There are several layers of innate protection in the lower female genital tract. The epithelial cells covering the length of the female genital tract act as the first line of defense and a physical barrier by inhibiting the passage of pathogens and their associated particles [126,181]. It has been suggested that there is a site-specific mucosal immune system in the female upper genital tract that differs from that described in the gastrointestinal and respiratory tracts [185,186]. Furthermore, the immune system in the upper genital tract differs from that of the lower genital tract [14, 180-186]. The putative immune system in the upper genital tract appears to contribute to the maintenance of an aseptic milieu; that is, this immune system inhibits the growth of microorganisms that sporadically colonize this region [181,183].

In contrast, the lower genital tract is constantly exposed to microorganisms, including species of commensal as well as pathogenic organisms [184]. The mucosal components of the lower genital tracts have adapted to a dynamic, nonsterile environment challenged by a variety of antigenic/inflammatory stimuli associated with sexual intercourse and endogenous vaginal microbiota [184,187]. Clearly, it is essential that these mucosal tissues develop mechanisms for selectively respond to pathogens, while simultaneously avoid chronic inflammation due to immune responses to commensal microorganisms.

Innate immune system. The innate immune system in the female genital tract is highly complex and multifactorial. Mucosal epithelial cells, fibroblasts, lymphocytes, macrophages and dendritic cells associated with the female genital tract have evolved a unique mechanism for the recognition of pathogens [187]. These cells express a variety of Toll-like receptors (TLRs), allowing them to recognize the different repertoire of a wide range of molecular patterns associated with pathogens (PAMPs) [187,188]. TLRs recognize conserved PAMPs synthesized by microorganisms including bacteria, fungi, parasites, and viruses as well as endogenous ligands associated with cell damage. It is likely that TLR distribution in the female genital tract reflects an immunological tolerance of commensal organisms in the lower portions of the tract (i.e., vagina, ectocervix, and to some extent, the endocervix), as well as an intolerance of commensal microbiome in the upper portion of the tract (i.e., the endometrium and fallopian tubes)

[184**].** The mucosal surface of the upper portion of the female genital tract is generally considered a sterile site, in part due to the cervical mucus, which filters bacteria and other debris. However, this barrier can readily be crossed by a variety of infectious agents, typically leading to endometritis and salpingitis [181,182]. Thus, it is essential that the upper genital tract epithelium has the capacity to recognize and respond to ascending pathogens while simultaneously avoiding a state of unnecessary inflammation that might disrupt the epithelial barrier. The sequelae of such inflammation in the upper genital tract would be highly detrimental to the defense and reproductive functions of the mucosal surface [184**].** If the luminal epithelial barrier is broken by acute inflammation, damaged epithelial cells initiate and coordinate the inflammatory response, alerting adjacent epithelium and underlying immune cells of the potential danger posed by various microorganisms [184**].**

Vaginal epithelial cells produce several compounds with anti-microbial activity, some of them identified as antimicrobial peptides (AMP) synthesized and secreted also by different cell types related with the innate immunity and defensins that are microbicidal *per se.* They also produce mucus, which covers the internal surface of the vagina and cervix and serves to trap infectious agents [129,154]. The epithelial cells further help protect against pathogens by expressing several receptors as cited, including a number of TLRs, MD-2, as well as major histocompatibility complex (MHC) molecules [181,182,189]. These molecules help recognize process and initiate cellular immune responses to obliterate pathogens. When activated, epithelial cells can also produce a variety of cytokines and chemokines such as TNFα, G-CSF, GM-CSF, IL-6 and IL-8, to help recruit immune cells, induce their differentiation/activation and develop successful immune responses, indicating thus how the epithelial function is related also with the immune system [181].

If the protection afforded by the epithelial barrier is compromised, however, pathogens encounter a second layer of innate defense consisting of specialized immune cells and their products [180,181]. These cells, which include macrophages, dendritic cells, neutrophils, and natural killer cells, are dispersed throughout the female genital tract, surveying that environment [181].

Innate immunity is critical for controlling the first stages of infection, but the activity of dendritic cells (pDCs) and Natural Killer (NK) cells may not be sufficient for complete microbial clearance and therefore, the activation of adaptive immunity is fundamental for full protection, as DCs are key players in connecting the innate and

adaptive immunity [190-193]. In the FGT, as described for other mucosal sites, macrophages and DCs are the principal antigen presenting cells (APCs). Under normal conditions, the main populations found in the FGT mucosa are Langerhans cells located within the epithelium and submucosal DCs located beneath the epithelium [193,194]]

In the steady state, Langerhans cells and submucosal DCs are highly phagocytic and express several pattern recognition receptors (PRRs) that can recognize a wide array of microorganisms. After pathogen recognition through PRRs, DCs and Langerhans cells undergo a programmed maturation and migrate to the draining lymph nodes to prime naive T and B cells. Both cells are tolerogenic in the absence of pathogens [193]. In case of infection, or in an inflammatory state, other blood-derived populations of APCs such as pDCs and monocyte-derived DCs can also be found in the FGT mucosa after pathogen challenge. The current paradigm of immune induction to infectious agents at body surfaces covered by squamous epithelium such as the vagina is that Langerhans cells encounter pathogens within the epithelium, take up antigens from pathogens, and migrate to the draining lymph nodes to prime naive T cells [184**].** Therefore, some observations suggest that Langerhans cells provide critical antimicrobial defense functions and suggest that treatments to augment their activity may be useful therapeutic tools [193].

Specific or adaptative immune response. Then, an antigen-specific attack is launched by the adaptive arm of the immune system, consisting of T and B cells, after the microbial antigens have been recognized, processed and presented by antigen presenting cells [181,190,191]. The cross-talk between the innate and the adaptive immune systems, as well as the important role that T and B cells play in conferring long-term immunity in the genital tract, have been discussed previously [181,183,195].

The humoral immune compartment of the human genital tract exhibits features which are unique and functionally different from other compartments of the mucosal immune system [181,190,191,194]. The main immunoglobulin (Ig) isotype found in the lumen of the upper FGT is IgA as in other mucosal tissues [185]. This is not the case for vagina and ectocervix, where the main Ig isotype present in cervicovaginal secretions is IgG [195,196]. These differences are related to the transcytosis and the presence or absence of the corresponding Fc receptors, which allow the transport of the respective Ig across epithelial cells [181]. To add complexity to the humoral immune system in the FGT, it was reported that hormonally mediated variations modulate the expression of

receptors on epithelial cells involved in Ig transport and profoundly influence Ig levels in the vaginal fluid [183].

Although neutralizing antibodies are protective against infections with many microorganisms, induction of T cell mediated immunity, particularly antigen-specific CD4+ Th1 cells, is critical for full protection in specific infections [181]. Th1 cell-mediated immunity is indispensable for the destruction of intracellular pathogens and is driven primarily by T lymphocytes [197]. T cells are located in the stroma of the vagina, cervix and uterus both below the epithelium and also dispersed within epithelial cells where they are known as intraepithelial lymphocytes [181].

CD4+ T cells assist CD8+ T cells in their migration to vaginal mucosa by secreting recruiting cytokines and they are the main producers of IFN-γ, a pleiotropic cytokine, and exert its/their? powerful effect by several mechanisms [197]. On the other hand, CD8+ cytotoxic effector T cells recognize virus infected cells through peptide-bound MHC class I molecules expressed on their surface, inducing apoptosis through perforin- and granzyme-mediated cytolysis or inducing apoptosis infected cells Fas-ligand [198]. Thus, a CD4+ and CD8+ balance is needed for controlling infection, being CD4+T cells more important at the earlier stages of infection and CD8+T cells becoming more important later [199].

More recently, it has been described that Th17 are present at mucosal surfaces and are thought to play a role in maintenance of immune homeostasis discriminating autochthonous microbiota from pathogens [200]. They have been involved in responses to fungi and bacteria at mucosal sites, and only very recently they have been studied in the context of different type of infections [201].

Vaginal lactobacilli and immune system: There are specific studies performed with some strains that have shown strain-related effects on the innate or specific immune system, and also dealing with the signalling, that will be indicated as follows:

Lactobacilli help to a healthy vagina by producing several factors including lactic acid, as assessed by Mirmonsef et al. [184] who described the dramatic effects of TLR ligands and short chain fatty acids produced by bacteria microbiota on immune function.

L. crispatus 2029 induced NF-kB activation in epithelial cells and did not induce expression of innate immune mediators IL-8, IL-1B, IL-1a and TNF-a. It inhibited IL-8 in epithelial cells and increased production of anti-inflammatory cytokine IL-8 [133]. This specific strain, a H_20_2 producer, presented antagonistic spectrum and a S-layer

responsible for its adhesion on the surface of cervicovaginal epithelial cells and on type IV collagen (a major molecular component of epithelial cell extracellular matrix), did not cause toxicity, epithelial damage and apoptosis, and showed a recognition by TLR 2/6 [133]

Li et al. [202] have shown that *L. reuteri* RC-14, a human vaginal isolate, produced small signaling molecules that were able to interfere with the staphylococcal quorum-sensing system agr (a key regulator of virulence genes) and repress the expression of toxic shock syndrome toxin-1 (TSST-1) in *S. aureus* MN8, a prototype of menstrual TSS *S. aureus* strains.

L. rhamnosus GR-1 enhanced the activation of NF-κB and TNF release through increased levels of TLR4 on the bladder cells and altered subsequent release of cytokines from urothelial cells. By influencing immunological factors as TLR4, that is fundamental in the process of fighting pathogens, probiotic could facilitate pathogen recognition and infection clearance [203]

L. helveticus HY7801 inhibited the expression of pro-inflammatory cytokines including TNF-a, IL-1B and IL-6, and inflammatory enzymes COX-2 and INOS, as well as the activations of NF-kB in *Candida* infections. Moreover, *L. helveticus* HY7801 caused an increase in IL-10 cytokine expression in the vaginal tissues [173].

Wagner and Johnson [204] suggested that that *C. albicans* infection induced pro-inflammatory responses in vaginal epithelial cells, and *L. rhamnosus* GR-1 and estrogens suppressed expression of NF-κB-related inflammatory genes. Lactobacilli could modify epithelial cell cytokine production (e.g. inducing IL-1α and IL-1β expression) by activation of alternate signal transduction pathway (as MAPK/AP-1), which could be mechanisms for probiotic modulation of morbidity in vulvovaginal candidiasis.

Rizzo et al. [205] have shown that *Lactobacillus crispatus* ATCC 33820 influenced the innate immune response of HeLa epithelial cells to *C. albicans* infections through the involvement of TLR2/4, IL-8 and human B-defensin 2 and 3.

Evrard et al. [206] have demonstrated the dose-dependent immunomodulation of human dendritic cells by *L. rhamnosus* Lcr35. The strain, in high doses, increased the pro-Th1/Th17 cytokine levels (TNFa, IL-1B, IL-12p70, IL-12p40, and IL-23), but only caused a low increase in IL-10 concentration, indicating a semi-maturation of the cells, and a tendency to a pro-inflammatory effect. On the contrary, other authors [207,208] have suggested that *L. rhamnosus* affected macrophages-dendritic cells interaction

through a granulocyte colony-stimulating factor (G-CSF) modulation directed to the IL-12/23 p40 response of the dendritic cells.

Is important to point out also that vaginal microbiota affects the fetus [209-211]. Stencel-Gabriel et al. [209] have shown that lactobacilli in vagina affected the development of the neonatal immune system, because they have detected different degree of expression of CD45RO on neonatal CD4+Tcells and IL 12 production in CBMC (cord blood mononuclear cells) culture). On the other side, Bloise et al. [210] have evidenced, the *in vitro* effect of heat killed *Lactobacillus rhamnosus* GG (LGG) on primary trophoblast cells purified from normal human term placenta. LGG stimulated IL-4, IL-10 and urocortin release, while inhibited LPS-induced TNF-α release, suggesting an immunomodulatory effect in human placenta. These effects did not alter the basic trophoblast functions. The immunomodulatory effect of *L. rhmanosus* GR-1 on human placental trophoblast cells was evidenced by Yeganegi et al. [211], showing the activation of the Janus Kinases/Signal Transducers and Activators of Transcription (JAK/STAT) and mitogen-activated protein kinase (MAPK) pathways.

The described results indicate/suggest the signaling and the immunomodulatory effect of some specific *Lactobacillus* strains on the human female urogenital tract. Eventhough the upper tract is sterile, some of the cited studies have isolated DNA of some vaginal strains from placenta after the delivery [54,56]. These new evidences indicate how the indigenous microbiota or microbiome interacts with the different cells of the human urogenital tract, supported also by the hematogenous transfer of the microbiota from different tracts [54].

4.4. Compatibility of strains, safety and resistance to the environmental conditions

Once the potential probiotic strains are selected, based on the production of primary or secondary metabolites (antagonistic substances, biosurfactants) and surface properties (auto- and co-aggregation, biofilm formation and adhesion to eukaryotic cells or tissue components), some other studies need to be performed (Figure 1). As the beneficial properties are specifically related with a particular strain, the combination of strains in a multi-strain probiotic product is an interesting strategy looking for a synergistic effect [73,212]. In this sense, the first assay to be carried out is the compatibility between the beneficial strains [118].

Once selected the compatible strains, their safety characteristics must be assayed, according to recommendations of the regulatory agencies [61,64,68].

Eventhough the LAB are considered as GRAS, QPS and FGM [63], they must be assayed in some specific properties of safety, as follows:

Resistance to antibiotics. LAB are intrinsically resistant to vancomycin, but they shouldn´t carry plasmids or extrachromosal elements that could transfer the antibiotic resistance, either horizontally or transversally [213-216]. The strains must be assayed by applying different methodologies, because there are not clear limits of the sensitivity/resistance to antibiotics in the LAB group, as in the pathogens case. Clinical and Laboratory Standards Institute (CLSI) and the Standard Operating Procedure (SOP) gave some type of indications on this topic, and also some documents were published by the EFSA for microorganisms included in product for human and animal consumption [68]. The antibiotic resistance of the strains must be assayed both by phenotypic experiments and at the genetic level, to determine if the resistance genes are present, either by an incomplete cluster, or because they are not expressed in the assayed conditions [61,62,215,216].

Production of enzymes related to deleterious effects. Hemolysins, gelatinases or collagenases could produce damage into the host. Enzymes evaluation must be carried out by using methods recommended by regulatory agencies [62,64,68].

Animal studies for evaluation of safety. Eventhough the safety definition of LAB, it is very valuable to determine if the beneficial strains do not produce damage, adverse or collateral effects on the host once administered by the specific way. In this sense, mice are one of the most frequent experimental animals used with this purpose [76,176,178,217], and also rabbits and macaque models described before in item 4.3.6. The animal studies also help in the understanding of the mechanistic effect of some specific strains.

Referred to the resistance to the environmental conditions, if the strains are going to be administered locally at the urogenital tract, they must be assayed in their resistance to vaginal fluid and/or urine [218,219]. If they will be applied as oral capsule or foods, their survival must be studied in intestinal fluid or into their components, either in one phase assays, or in continuous models resembling the different areas of the intestinal tract [220-226].. The strains must survive under these conditions and express later their beneficial characteristics [219-226].

4.5. Technological properties of urogenital probiotics

Microbial cells are API of the probiotic products for pharmaceutical applications. As shown in Figure 1, one of the main challenges during the design of probiotic products is the inclusion of a high number of viable and active microorganisms resisting to the conditions of manufacturing process and shelf life of products and later to the environment or target tract or mucosa [64].

Therefore, the technological characterization of potential beneficial microorganisms is a basic evaluation required to complement the strains selection, in order to determine if they can be included in the design of probiotic products that will be elaborated at large-scale and later commercialized [64]. These requirements are supported on results of research studies and guidelines established by scientific organizations [61,62,65], which proclaim that the efficacy of probiotics depends on the number of viable cells [10^6-10^9 CFU per doses of formulation] reaching their target.

4.5.1. API optimal production for urogenital probiotics

In pharmaceutical biotechnology, the development of suitable strategies for optimal API production for vaginal probiotic products is a fundamental aspect. However, these studies have scarcely been reported to date, which can be possibly due to intellectual protection of results or patent processes. The effect of the culture conditions on the growth and inhibitory metabolite production of several potential probiotic vaginal lactobacilli, which were isolated from human vagina in Tucumán, Argentina, was reported [165,227,228]. In the specific case of bacteriocin-producing *L. salivarius* CRL 1328, different physical and chemical factors (e.g. initial pH of the culture media, incubation temperature and growth medium components) drastically affected the biomass and bacteriocin production [227]. Both bacterial cells and bacteriocin of *L. salivarius* CRL 1328 are candidates to be included in a pharmabiotic product to prevent or treat female urogenital infections. Therefore, to optimize simultaneously the production of both biomass and bacteriocin, culture media of lower cost than conventional laboratory media were formulated [227].

In order to potentially apply the results obtained at laboratory scale to the technological production of potential probiotic microorganisms and metabolites for pharmaceutical products, alternative low-cost ingredients of culture media (e.g. whey permeate, potato extracts, molasses, soy milk, corn syrup, etc.) [229] and different strategies of fermentation and biomass production must be evaluated. As example, whey protein concentrate supplemented with different substances, such as yeast extract and

Tween 80, supported the optimal growth of potential probiotic vaginal lactobacilli (unpublished data). Recently, Donnarumma et al. [230] reported that the optimal growth of potential probiotic vaginal *Lactobacillus crispatus* L1 was obtained in a semidefined medium containing soy peptone and yeast extract as nitrogen sources and glucose as carbon source. In order to overcome the inhibition of *L. crispatus* L1 growth caused by lactic acid produced during the culture, an *in situ* product removal fermentation process using a bioreactor with microfiltration modules was evaluated. This strategy allowed a 7-fold improvement of the *L. crispatus* L1 viable biomass yield compared to traditional batch processes.

4.5.2. Storage of API of interest and formulation of urogenital probiotics

Probiotic products with beneficial effects on the female urogenital tract can be mainly manufactured as solid, such as tablets, capsules and suppositories, among others, as mentioned in Tables 1-3. In these products, concentrated cultures of probiotic microorganisms must be included in matrixes with low water activity (aw). Potential probiotic strains should be compatible with the physical form and excipients of the final product and they should survive and maintain their beneficial properties during the storage of concentrated cultures and during the product shelf life [64,76].

The preservation of concentrated cultures can be carried out applying several methodologies, such as lyophilization, spray drying and microencapsulation [70,73]. However, the different steps of microbial preservation processes can cause structural and physiological cell damage through several mechanisms, affecting negatively the stability (viability and/or functionality) of microorganisms. Various factors such as water activity, pH, oxygen tension, temperature, osmotic pressure, intrinsic characteristics of strains, etc., affect the microbial survival during the preservation [231].

The challenge in the development of vaginal probiotics is to found production and storage strategies allowing to obtain physiologically more robust strains, in which the loss of viability and functionality is limited [62,64]. For example, microbial pre-adaptation at different temperatures or at different solute concentrations could increase the strain resistance to some stress conditions during drying and storage [232]. However, this type of studies was not published for vaginal probiotic microorganisms.

Table 4 shows several technological studies carried out in different probiotic or potentially probiotic microorganisms for vaginal pharmaceutical products [70,73,212,231,233-242]. To the best of our knowledge, the resistance of vaginal

probiotic strains to spray drying has not yet been reported. The freeze-drying of *Lactobacillu*s cultures, which could be incorporated into a suitable pharmaceutical form of vaginal application, has been proved successful to preserve the stability during the long-term storage of some potential probiotic strains [231,233]. The use of different protective compounds (carbohydrates and proteinaceous substances such as skim milk) improved the bacterial survival during the processes assayed [231,233].

On the other hand, the microencapsulation of probiotic strains can provide a means to optimize the delivery and bacterial survival in the site of action [70,234]. This technology confers to probiotic cells a physical barrier against external conditions, potentially enhancing their stability during the storage and under adverse conditions of the tract where will be applied. Microencapsulation of freeze-dried or non dried cultures was evaluated in order to obtain particulate-delivery systems with bioadhesive properties for urogenital probiotic products [70,234]. However, survival of encapsulated bacteria during storage was not yet reported.

Several studies describing the pharmaceutical formulation and/or API stability of potential vaginal probiotics were found in the literature (Table 4). The pharmaceutical forms assayed were: vaginal tablets (with and without retarding polymer), liquid systems such as vaginal gels and douches, vaginal capsules, ovules/suppositories and pod-intravaginal rings. In most of the pharmaceutical formulations, except in liquid systems and in a type of vaginal tablets, the viability and/or biological characteristics (e.g. antimicrobial activity and survival in fluid vaginal) of microorganisms was maintained during manufacture and storage for different time periods. Muller et al. [233] determined the stability of freeze-dried powders and vaginal pharmaceutical formulations according to the ICH Guideline Q1A [243], which requires a minimum of 12 months to test products under different storage conditions; however, the stability studies can be performed up to the expiration date (24 or 36 months). In order to reduce the length of the stability assays and the costs of laboratory technology, different mathematical models were proposed to predict the bacterial viability during storage.

Several excipients or active pharmaceutical ingredients of conventional vaginal products could complement the effects of probiotic microorganisms and enhance the benefits on the host, therefore being good candidates to be incorporated in the final formula. In this sense, Vera Pingitore et al. [73] designed urogenital pharmabiotics including beneficial strains combined with other ingredients of microbial origin (an antimicrobial metabolite) and substances with different functionalities (prebiotics,

vitamins, antioxidants). The compatibility of all the ingredients was carefully assayed, in order to preserve the API stability during the processes of production and long-time storage.

4.5.3. Probiotic products for the urogenital tract

According to the international requirements, a probiotic product must include the label describing the microbiological content, the name of the strains, the minimum numbers of viable bacteria at end of shelf-life, the storage conditions, and specific claims on their benefits on the host [61,62,65]. Tables 1-3 summarize the available information of probiotic or potential probiotic products (single- or multi-strain formulation, specific microbial strains, minimum numbers of viable cells, commercial name and manufacturer data) that were used in several clinical assays.

Most of the probiotic strains for the female urogenital tract are included in pharmaceutical forms for vaginal administration, e.g. as capsules (EcoVag®, Gynophilus®, LACTIN-V and Probaclac Vaginal), tablets (Gynoflor®, ActiCand 30, Normogin® and Florisia™), pessaries or powders (LACTIN-V powder, with a vaginal applicator). Moreover, probiotic capsules (LaciBios® femina, Lactogyn, prOVag® and Provinorm) and tablets for oral administration are commercialized. Most of the products detailed in Tables 1-3 contain between 10^7 and 10^9 viable cells of freeze-dried microorganisms and require to be stored usually refrigerated. However, the technological characteristics of the strains and the details of the production process are not available in most of the cases.

5. Concluding remarks

Different *Lactobacillus* species are the predominant microorganisms in the healthy human vaginal microbiome. Numerous *in vitro* and *in vivo* studies have evaluated the beneficial characteristics suggested or involved in the protective mechanisms of lactic acid bacteria in the urogenital tract, but still there are many gaps in the area. Technological studies have scarcely been reported to date, because most of the probiotic strains or products are protected by patent processes or under the "confidential clauses" of the manufacturers or pharmaceutical companies. The current state of the application of probiotics for the urogenital tract in medical clinical practice and evidence of their effects must be demonstrated, mainly referred to the stability of the vaginal microbiome and to the protection/prevention of specific syndromes or

infections, both at the vaginal and urinary tract. The host genetic polymorphism lately described is being also related to the susceptibility to infections, and must also be considered. Urogenital probiotics were clinically evaluated employing different bacterial strains, doses, schemes of treatment, routes and vehicles of administration; therefore unique recommendations for their use are precluded. The potential benefits of probiotics on the health of women around the world strongly deserve to carry out further studies to complement the current knowledge and to support the clinical applications of probiotics in the urogenital tract, either as preventive or therapeutic agents.

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References

[1] Human Microbiome Project Consortium, Structure, function and diversity of the healthy human microbiome, Nature. 486 (2012) 207-214.

[2] Integrative HMP (iHMP) Research Network Consortium, The Integrative Human Microbiome Project: dynamic analysis of microbiome-host omics profiles during periods of human health and disease, Cell Host Microbe, 16 (2014) 276-289.

[3] A.L. Goodman, N.P. McNulty, Y. Zhao, D. Leip, R.D. Mitra, C.A. Lozupone, R. Knight, J.I. Gordon, Identifying genetic determinants needed to establish a human gut symbiont in its habitat, Cell Host Microbe. 6 (2009) 279-289.

[4] R.D.Moloney, L. Desbonnet, G. Clarke, T.G. Dinan, J. Cryan, The microbiome: stress, health and disease, Mamm. Genome. 25 (2014) 49-74.

[5] K. Li, M. Bihan, B.A. Methé, Analyses of the stability and core taxonomic memberships of the human microbiome, PLoS One. 8 (2013) e63139.

[6] R.F. Lamont, J.D. Sobel, R.A. Akins, S.S. Hassan, T. Chaiworapongsa, J.P. Kusanovic, R. Romero, The vaginal microbiome: new information about genital tract flora using molecular based techniques, BJOG. 118 (2011) 533-549.

[7] J. Ravel, P. Gajer, Z. Abdo, G.M. Schneider, S.S. Koenig, S.L. McCulle, S. Karlebach, R. Gorle, J. Russell, C.O. Tacket, R.M. Brotman, C.C. Davis, K. Ault, L. Peralta, L.J. Forney, Vaginal microbiome of reproductive-age women, Proc. Natl. Acad. Sci. USA. 108 (2011) 4680-4687.

[8] R.M. Stumpf, B.A. Wilson, A. Rivera, S. Yildirim, C.J. Yeoman, J.D. Polk, B.A. White, S.R. Leigh., The primate vaginal microbiome: Comparative context and implications for human health and disease, Am. J. Phys. Anthropol. 152 (2013) 119-134.

[9] T.R. Klaenhammer, E. Altermann, E. Pfeiler, B.L. Buck, Y.J. Goh, S. O'Flaherty, R. Barrangou, T. Duong, Functional genomics of probiotic lactobacilli, J. Clin. Gastroenterol. 42 (2008) S160-S162.

[10] M. Kleerebezem, E.E. Vaughan, Probiotic and gut lactobacilli and bifidobacteria: molecular approaches to study diversity and activity, Annu. Ver. Microbiol. 63 (2009) 269-290.

[11] R. Kant, J. Blom, A. Palva, R.J. Siezen, W.M. de Vos, Comparative genomics of *Lactobacillus*, Microb. Biotechnol. 4 (2011) 323-332.

[12] G. Reid, J. Dols, W. Miller, Targeting the vaginal microbiota with probiotics as a means to counteract infections, Curr. Opin. Clin. Nutr. Metab. Care. 12 (2009) 583-587.

[13] S. Borges, J. Silva, P. Teixeira, The role of lactobacilli and probiotics in maintaining vaginal health, Arch. Gynecol. Obstet. 289 (2014) 479-489.

[14] R. Hickey, X. Zhou, J. Pierson, J. Ravel, L. Forney, Understanding vaginal microbiome complexity from an ecological perspective, Transl. Res. 160 (2012) 267-272.

[15] F.P. Douillard, W.M. de Vos, Functional genomics of lactic acid bacteria: from food to health, Microb. Cell Fact. 13 (2014) S8.

[16] R. Datcu, Characterization of the vaginal microflora in health and disease, Dan. Med. J. 61 (2014) B4830.

[17] X. Zhou, C.J. Brown, Z. Abdo, C.C. Davis, M.A. Hansmann, P. Joyce, J.A. Foster, L.J. Forney, Differences in the composition of vaginal microbial communities found in healthy Caucasian and black women, ISMEJ. 1 (2007) 121-133.

[18] X. Zhou, M.A. Hansmann, C.C. Davis, H. Suzuki, C.J. Brown, U. Schütte, J.D. Pierson, L.J. Forney, The vaginal bacterial communities of Japanese women resemble those of women in other racial groups, FEMS Immunol. Med. Microbiol. 58 (2010) 169-181.

[19] B. Ma, L. Forney, J. Ravel, The vaginal microbiome: rethinking health and disease, Ann. Rev. Microbiol. 66 (2012) 371-389.

[20] S. Pendharkar S, T. Magopane, P.G. Larsson, G. de Bruyn, G.E. Gray, L. Hammarström, H. Marcotte, Identification and characterisation of vaginal lactobacilli from South African women, BMC Infect. Dis. 13 (2013) 43.

[21] M.D. Martínez-Peña, G. Castro-Escarpulli, M.G. Aguilera-Arreola, *Lactobacillus* species isolated from vaginal secretions of healthy and bacterial vaginosis-intermediate Mexican women: a prospective study, BMC Infect. Dis. 13 (2013) 189.

[22] J.M. Fettweis, J.P. Brooks, M.G. Serrano, N.U. Sheth, P.H. Girerd, D.J. Edwards, J.F. Strauss 3rd, Differences in vaginal microbiome in African American women versus women of European ancestry. Vaginal Microbiome Consortium, Jefferson KK, Buck GA. Microbiology. 160 (2014) 2272-2282.

[23] J. Qin, R. Li, J. Raes, M. Arumugam, K.S. Burgdorf, C. Manichanh, et al. MetaHIT Consortium, A human gut microbial gene catalogue established by metagenomic sequencing, Nature. 464 (2010) 59-65.

[24] J.H. van de Wijgert, H. Borgdorff, R. Verhelst, T. Crucitti, S. Francis, H. Verstraelen, V. Jespers, The vaginal microbiota: what have we learned after a decade of molecular characterization?, PLoS One. 9 (2014) e105998.

[25] E. Shipitsyna, A. Roos, R. Datcu, A. Hallén , H. Fredlund, J.S. Jensen, L. Engstrand , M. Unemo, Composition of the vaginal microbiota in women of reproductive age-sensitive and specific molecular diagnosis of bacterial vaginosis is possible?, PLoS One. 8 (2013) e60670.

[26] J. Ravel, R.M. Brotman, P. Gajer, B. Ma, M. Nandy, D.W. Fadrosh, J. Sakamoto, S.S. Koenig, L. Fu, X. Zhou, R.J. Hickey, J.R. Schwebke, L.J. Forney, Daily temporal dynamics of vaginal microbiota before, during and after episodes of bacterial vaginosis, Microbiome. 1 (2013) 29.

[27] E. Motevaseli, M. Shirzad, R. Raoofian, S.M. Hasheminasab, M. Hatami M, M. Dianatpour, M.H. Modarressi, Differences in vaginal lactobacilli composition of Iranian healthy and bacterial vaginosis infected women: a comparative analysis of

their cytotoxic effects with commercial vaginal probiotics, Iran. Red. Crescent. Med. J. 15 (2013) 199-206.

[28] H. Mendes-Soares, H. Suzuki, R.J. Hickey, L.J. Forney, Comparative functional genomics of *Lactobacillus* spp. reveals possible mechanisms for specialization of vaginal lactobacilli to their environment, J. Bacteriol. 196 (2014) 1458-1470.

[29] S. Srinivasan, C. Liu, C.M. Mitchell, T.L. Fiedler, K.K. Thomas, K.J. Agnew, J.M. Marrazzo, D.N. Fredricks, Temporal variability of human vaginal bacteria and relationship with bacterial vaginosis, PLoS One. 5 (2010) e10197.

[30] R.M. Brotman, M.D. Shardell, P. Gajer, J.K. Tracy, J.M. Zenilman, J. Ravel, P.E. Gravitt, Interplay between the temporal dynamics of the vaginal microbiota and human papillomavirus detection, J. Infect. Dis. 210 (2014) 1723-1733.

[31] D.N. Fredricks, Molecular methods to describe the spectrum and dynamics of the vaginal microbiota, Anaerobe. 17 (2011) 191-195.

[32] R. Hummelen, J.M. Macklaim, J.E. Bisanz, J.A. Hammond, A. McMillan, R. Vongsa, D. Koenig, G.B. Gloor, G. Reid, Vaginal microbiome and epithelial gene array in post-menopausal women with moderate to severe dryness, PLoS One. 6 (2011) e26602.

[33] M.R. Walther-António, P. Jeraldo, M.E. Berg Miller, C.J. Yeoman, K.E. Nelson, B.A. Wilson, B.A.White, N. Chia, D.J. Creedon, Pregnancy's stronghold on the vaginal microbiome, PLoS One. 9 (2014) e98514.

[34] R.M. Brotman, M.D. Shardell, P. Gajer, D. Fadrosh, K. Chang, M.I. Silver, R.P. Viscidi, A.E. Burke, J. Ravel, P.E. Gravitt, Association between the vaginal microbiota, menopause status, and signs of vulvovaginal atrophy, Menopause. 21 (2014) 450-458.

[35] S.S. Witkin, S. Gerber, W.J. Ledger, Influence of interleukin-1 receptor antagonist gene polymorphism on disease, Clin. Infect. Dis. 34 (2002) 204-209.

[36] B. Ragnarsdóttir, N. Lutay, J. Grönberg-Hernandez, B. Köves, C. Svanborg, Genetics of innate immunity and UTI susceptibility, Nat. Rev. Urol. 12 (2011) 449-468.

[37] N.M. Jones, C. Holzman, K.H. Friderici, K. Jernigan, H. Chung, J. Wirth, R. Fischer, Interplay of cytokine polymorphisms and bacterial vaginosis in the etiology of preterm delivery, J. Reprod. Immunol. 87 (2010) 82-89.

[38] J. Liu, F. Hu, W. Liang, G. Wang, P.C. Singhal, G. Ding, Polymorphisms in the surfactant protein a gene are associated with the susceptibility to recurrent urinary tract infection in Chinese women, Tohoku J. Exp. Med. 221 (2010) 35-42.

[39] N.S. Nevadunsky, I. Korneeva, T. Caputo, S.S. Witkin, Mannose-binding lectin codos 54 genetic polymorphism and vaginal protein levels in women with gynecologic malignancies, Eur. J. Obstet. Gynecol. Reprod. Biol. 163 (2012) 216-218.

[40] S. Jaillon, F. Moalli, B. Ragnarsdottir, E. Bonavita, M. Puthia, F. Riva, E. Barbati, M- Nebuloni, L. Cvetko Krajinovic, A. Markotic, S. Valentino, A. Doni, S. Tartari, G. Graziani, A. Montanelli, Y. Delneste, C. Svanborg, C. Garlanda, A. Mantovani, The humoral pattern recognition molecule PTX3 is a key component of innate immunity against urinary tract infection, Immunity. 17 (2014) 621-632.

[41] B. Huang, J.M. Fettweis, J.P. Brooks, K.K. Jefferson, G.A. Buck, The changing landscape of the vaginal microbiome, Clin. Lab. Med. 34 (2014) 747-761.

[42] M.R. Genc, A. Onderdonk, Endogenous bacterial flora in pregnant women and the influence of maternal genetic variation, BJOG. 118 (2011) 154-163.

[43] L. Petricevic, K.J. Domig, F.J. Nierscher, M.J. Sandhofer, M. Fidesser, I. Krondorfer, P. Husslein, W. Kneifel, H. Kiss, Characterisation of the vaginal *Lactobacillus* microbiota associated with preterm delivery, Sci. Rep. 4 (2014) 5136.

[44] P. Mastromarino, B. Vitali, L. Mosca, Bacterial vaginosis: a review on clinical trials with probiotics, New Microbiol. 36 (2013) 229-238.

[45] M. de Vrese, Health benefits of probiotics and prebiotics in women, Menopause Int. 15 (2009) 35-40.

[46] B. Ragnarsdóttir, C. Svanborg, Susceptibility to acute pyelonephritis or asymptomatic bacteriuria: host-pathogen interaction in urinary tract infections, Pediatr. Nephrol. 27 (2012) 2017-2029.

[47] F.M. Wagenlehner, R. Bartoletti, M. Cek, M. Grabe, G. Kahlmeter, R. Pickard R, T.E. Bjerklund-Johansen, Antibiotic stewardship: a call for action by the urologic community, Eur. Urol. 64 (2013) 358-360.

[48] F.M. Wagenlehner, W. Vahlensieck, H.W. Bauer, W. Weidner, H.J. Piechota, K.G. Naber, Prevention of recurrent urinary tract infections, Minerva Urol. Nefrol. 65 (2013) 9-20.

[49] F.M. Wagenlehner, A. Pilatz, K. Naber, W. Weidner, Urinary tract infections, Aktuelle Urol. 45 (2014) 135-145.

[50] F.M. Wagenlehner, W. Weidner, A. Pilatz, K.G. Naber, Urinary tract infections and bacterial prostatitis in men, Curr. Opin. Infect. Dis. 27(2014) 97-101.

[51] V. Iacovelli, G. Gaziev, L. Topazio, P. Bove, G. Vespasiani, E. Finazzi Agrò, Nosocomial urinary tract infections: A review, Urologia, 23;81 (2014) 222-227.

[52] P.M. Grin, P.M. Kowalewska, W. Alhazzan, A.E. Fox-Robichaud, *Lactobacillus* for preventing recurrent urinary tract infections in women: meta-analysis, Can. J. Urol. 20 (2013) 6607-6614.

[53] J. Renard, S. Ballarini, T. Mascarenhas, M. Zahran, E. Quimper, J. Choucair, C.E. Iselin, Recurrent lower urinary tract infections have a detrimental effect on patient quality of life: a prospective, observational study, Infect. Dis .Ther. (2014) In press.

[54] M.S. Payne, S. Bayatibojakhi, Exploring preterm birth as a polymicrobial disease: an overview of the uterine microbiome, Front. Immunol. 5 (2014) 595.

[55] S. Witkin, The vaginal microbiome, vaginal anti-microbial defence mechanisms and the clinical challenge of reducing infection-related preterm birth, BJOG, 122 (2015) 213-218.

[56] I. Solt, The human microbiome and the great obstetrical syndromes: A new frontier in maternal-fetal medicine, Best Pract. Res. Clin. Obstet. Gynaecol. (2014) In press.

[57] A. Sultan, M. Rizvi, F. Khan, H. Sami, I. Shukla, H.M. Khan, Increasing antimicrobial resistance among uropathogens: Is fosfomycin the answer?, Urol. Ann. 7 (1015) 26-30.

[58] R. Cantón, Antibiotic resistance genes from the environment: a perspective through newly identified antibiotic resistance mechanisms in the clinical setting, Clin. Microbiol. Infect. 1 (2009) 20-25.

[59] P. Lichtenberger, T.M. Hooton, Antimicrobial prophylaxis in women with recurrent urinary tract infections, Int. J. Antimicrob. Agents, 38 (2011) 36-41.

[60] S.E. Geerlings, M.A. Beerepoot, J.M. Prins, Prevention of recurrent urinary tract infections in women: antimicrobial and nonantimicrobial strategies, Infect. Dis. Clin. North Am. 28 (2014) 135-147.

[61] FAO/WHO, Evaluation of health and nutritional properties of powder milk and live lactic acid bacteria, Food and Agriculture Organization of the United Nations and World Health Organization Expert Consultation Report. (2001) http://www.fao.org/es/ESN/Probio/probio.htm

[62] C. Hill, F. Guarner, G. Reid, G.R. Gibson, D.J. Merenstein, B. Pot, L. Morelli, R.B. Canani, H.J. Flint, S. Salminen, P.C. Calder, M.E. Sanders, Expert consensus

document: The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic, Nat. Rev. Gastroenterol. Hepatol. 11 (2014) 506-514.

[63] European Food Safety Authority. Introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA-Opinion of the Scientific Committee. (2007) http://www.efsa.europa.eu/en/efsajournal/doc/587.pdf.

[64] G. Reid, M.E. Sanders, H.R. Gaskins, G.R. Gibson, A. Mercenier, R. Rastall, M. Roberfroid, I. Rowland, C. Cherbut, T.R. Klaenhammer, New Scientific paradigms for probiotics and prebiotics, J. Clin. Gastroenterol. 37 (2003) 105-118.

[65] ISAPP (International Scientific Association for Probiotics and Prebiotics), Prebiotics: A Consumer Guide for Making Smart Choices. (2009) www.isapp.net

[66] P.G. Larsson, E. Brandsborg, U. Forsum, S. Pendharkar, K.K. Andersen, S. Nasic, L. Hammarström, H. Marcotte, Extended antimicrobial treatment of bacterial vaginosis combined with human lactobacilli to find the best treatment and minimize the risk of relapses, BMC Infect. Dis. 11 (2011) 223.

[67] V. Rousseau, J.P. Lepargneur, C. Roques, M. Remaud-Simeon, F. Paul, Prebiotic effects of oligosaccharides on selected vaginal lactobacilli and pathogenic microorganisms, Anaerobe. 11 (2005) 145-153.

[68] EFSA (European Food Safety Authority), Technical guidance prepared by the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) on the update of the criteria used in the assessment of bacterial resistance to antibiotics of human or veterinary importance, The EFSA Journal, 732 (2008) 1-15.

[69] F. Shanahan, C. Stanton, P. Ross, C. Hill, Pharmabiotics: Bioactives from mining host-microbe-dietary interactions, Funct. Food Rev. 1 (2009) 20-25.

[70] D. Pliszczak, S. Bourgeois, C. Bordes, J.P. Valour, M.A. Mazoyer, A.M. Orecchioni, E. Nakache, P. Lantéri, Improvement of an encapsulation process for the preparation of pro- and prebiotics-loaded bioadhesive microparticles by using experimental design, Eur. J. Pharm. Sci. 44 (2011) 83-92.

[71] E. Vera Pingitore, E. Bru, M.E. Elena Nader-Macías, Effect of lyophilization and storage temperature on the activity of salivaricin CRL 1328, a potential bioactive ingredient of a urogenital probiotic product, J. Gen. Appl. Microbiol. 58 (2012) 71-81.

[72] A. Palmeira-de-Oliveira, B.M. Silva, R. Palmeira-de-Oliveira, J. Martinez-de-Oliveira, L. Salgueiro, Are plant extracts a potential therapeutic approach for genital infections?, Curr. Med. Chem. 20 (2013) 2914-2928.

[73] E. Vera Pingitore, M.S. Juárez Tomás, B. Wiese, M.E. Nader-Macías, Design of novel urogenital pharmabiotic formulations containing lactobacilli, salivaricin CRL 1328 and non-microbial compounds with different functionalities, Drug Dev. Ind. Pharm. (2014) In press.

[74] M. Ventura, S. O'Flaherty, M.J. Claesson, F. Turroni, T.R. Klaenhammer, D. van Sinderen, P.W. O'Toole, Genome-scale analyses of health-promoting bacteria: probiogenomics, Nat. Rev. Microbiol. 7 (2009) 61-71.

[75] M. Ventura, F. Turroni, C. Canchaya, E.E. Vaughan, P.W. O'Toole, D. van Sinderen, Microbial diversity in the human intestine and novel insights from metagenomics, Front. Biosci. (Landmark Ed). 14 (2012) 3214-3221.

[76] M.E.F. Nader-Macías, C.S. Ruiz De, V.S. Ocaña, M.S. Juárez Tomás, Advances in the knowledge and clinical applications of lactic acid bacteria as probiotics in the urogenital tract, Curr. Women's Health Rev. 4 (2008) 240-257.

[77] R.C.R. Martinez, S.A. Franceschini, M.C. Patta, S.M. Quintana, R.C. Candido, J.C. Ferreira, E.C. De Martinis, G. Reid, Improved treatment of vulvovaginal candidiasis with fluconazole plus probiotic *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14, Lett. Appl. Microbiol. 48 (2009) 269-274.

[78] R.C.R. Martinez, S.A. Franceschini, M.C. Patta, S.M. Quintana, B.C. Gomes, E.C. De Martinis, G. Reid, Improved cure of bacterial vaginosis with single dose of tinidazole (2 g), *Lactobacillus rhamnosus* GR-1, and *Lactobacillus reuteri* RC-14: a randomized, double-blind, placebo-controlled trial, Can. J. Microbiol. 55 (2009) 133-138.

[79] C.S. Bradshaw, M. Pirotta, D. De Guingand, J.S. Hocking, A.N. Morton, S.M. Garland, G. Fehler, A. Morrow, S. Walker, L.A. Vodstrcil, C.K. Fairley, Efficacy of oral metronidazole with vaginal clindamycin or vaginal probiotic for bacterial vaginosis: randomised placebo-controlled double-blind trial, PLoS One. 7 (2012) e34540.

[80] O. Bodean, O. Munteanu, C. Cirstoiu, D. Secara, M. Cirstoiu, Probiotics - a helpful additional therapy for bacterial vaginosis, J. Med. Life. 6 (2013) 434-436.

[81] S. Ehrström, K. Daroczy, E. Rylander, C. Samuelsson, U. Johannesson, B. Anzén, C. Pahlson, Lactic acid bacteria colonization and clinical outcome after probiotic

supplementation in conventionally treated bacterial vaginosis and vulvovaginal candidiasis, Microbes Infect. 12 (2010) 691-699.

[82] V. Marcone, G. Rocca, M. Lichtner, E. Calzolari, Long-term vaginal administration of *Lactobacillus rhamnosus* as a complementary approach to management of bacterial vaginosis, Int. J. Gynaecol. Obstet. 110 (2010) 223-226.

[83] A. Hemmerling, W. Harrison, A. Schroeder, J. Park, A. Korn, S. Shiboski, A. Foster-Rosales, C.R. Cohen, Phase 2a study assessing colonization efficiency, safety, and acceptability of *Lactobacillus crispatus* CTV-05 in women with bacterial vaginosis, Sex. Transm. Dis. 37 (2010) 745-750.

[84] B.M. Ngugi, A. Hemmerling, E.A. Bukusi, G. Kikuvi, J. Gikunju, S. Shiboski, D.N. Fredricks, C.R. Cohen, Effects of bacterial vaginosis-associated bacteria and sexual intercourse on vaginal colonization with the probiotic *Lactobacillus crispatus* CTV-05, Sex. Transm. Dis. 38 (2011) 1020-1027.

[85] S. Kovachev, R. Dobrevski-Vacheva, Effect of *Lactobacillus casei* var *rhamnosus* (Gynophilus) in restoring the vaginal flora by female patients with bacterial vaginosis-randomized, open clinical trial, Akush. Ginekol. (Sofiia). 52 (2013) 48-53.

[86] A.E. Stapleton, M. Au-Yeung, T.M. Hooton, D.N. Fredricks, P.L. Roberts, C.A. Czaja, Y. Yarova-Yarovaya, T. Fiedler, M. Cox, W.E. Stamm, Randomized, placebo-controlled phase 2 trial of a *Lactobacillus crispatus* probiotic given intravaginally for prevention of recurrent urinary tract infection, Clin. Infect. Dis. 52 (2011) 1212-1217.

[87] M. Strus, A. Chmielarczyk, P. Kochan, P. Adamski, Z. Chełmicki, A. Chełmicki, A. Pałucha, P.B. Heczko, Studies on the effects of probiotic *Lactobacillus* mixture given orally on vaginal and rectal colonization and on parameters of vaginal health in women with intermediate vaginal flora, Eur. J. Obstet. Gynecol. Reprod. Biol. 163 (2012) 210-215.

[88] W. Ya, C. Reifer, L.E. Miller, Efficacy of vaginal probiotic capsules for recurrent bacterial vaginosis: a double-blind, randomized, placebo-controlled study, Am. J. Obstet. Gynecol. 203 (2010) 120.e1-e6.

[89] R. Hemalatha, P. Mastromarino, B.A. Ramalaxmi, N.V. Balakrishna, B. Sesikeran, Effectiveness of vaginal tablets containing lactobacilli versus pH tablets on vaginal health and inflammatory cytokines: a randomized, double-blind study, Eur. J. Clin. Microbiol. Infect. Dis. 31 (2012) 3097-3105.

[90] G.G. Donders, B. Van Bulck, P. Van de Walle, R.R. Kaiser, G. Pohlig, S. Gonser, F. Graf, Effect of lyophilized lactobacilli and 0.03 mg estriol (Gynoflor®) on vaginitis and vaginosis with disrupted vaginal microflora: a multicenter, randomized, single-blind, active-controlled pilot study, Gynecol. Obstet. Invest. 70 (2010) 264-272.

[91] M.R. Sudha, A.K. Maurya, Effect of oral supplementation of the probiotic capsule UB-01BV in the treatment of patients with bacterial vaginosis, Benef. Microbes. 3 (2012) 151-155.

[92] Z. Ling, X. Liu, W. Chen, Y. Luo, L. Yuan, Y. Xia, K.E. Nelson, S. Huang, S. Zhang, Y. Wang, J. Yuan, L. Li, C. Xiang, The restoration of the vaginal microbiota after treatment for bacterial vaginosis with metronidazole or probiotics, Microb. Ecol. 65 (2013) 773-780.

[93] G. Vujic, A. Jajac Knez, V. Despot Stefanovic, V. Kuzmic Vrbanovic, Efficacy of orally applied probiotic capsules for bacterial vaginosis and other vaginal infections: a double-blind, randomized, placebo-controlled study, Eur. J. Obstet. Gynecol. Reprod. Biol. 168 (2013) 75-79.

[94] F. Vicariotto, M. Del Piano, L. Mogna, G. Mogna, Effectiveness of the association of 2 probiotic strains formulated in a slow release vaginal product, in women affected by vulvovaginal candidiasis: a pilot study, J. Clin. Gastroenterol. 46 (2012) S73-S80.

[95] N. Stojanović, D. Plećaš, S. Plešinac, Normal vaginal flora, disorders and application of probiotics in pregnancy, Arch. Gynecol. Obstet. 286 (2012) 325-332.

[96] B. Vitali, F. Cruciani, M.E. Baldassarre, T. Capursi, E. Spisni, M.C. Valerii, M. Candela, S. Turroni, P. Brigidi, Dietary supplementation with probiotics during late pregnancy: outcome on vaginal microbiota and cytokine secretion. BMC Microbiol. 12 (2012) 236.

[97] F. Facchinetti, G. Dante, L. Pedretti, P. Resasco, E. Annessi, D. Dodero, The role of oral probiotic for bacterial vaginosis in pregnant women. A pilot study, Minerva Ginecol. 65 (2013) 215-221.

[98] L. Hanson, L. Vandevusse, M. Duster, S. Warrack, N. Safdar, Feasibility of oral prenatal probiotics against maternal group B *Streptococcus* vaginal and rectal colonization, J. Obstet. Gynecol. Neonatal Nurs. 43 (2014) 294-304.

[99] L. Krauss-Silva, M.E.L. Moreira, M.B. Alves, A. Braga, K.G. Camacho, M.R. Batista, A. Almada-Horta, M.R. Rebello, F. Guerra, A randomised controlled trial of

probiotics for the prevention of spontaneous preterm delivery associated with bacterial vaginosis: preliminary results, Trials. 12 (2011) 239.

[100] S.B. Kavak, E. Kavak, R. Ilhan, R. Atilgan, O. Arat, U. Deveci, E. Sapmaz, The efficacy of ampicillin and *Lactobacillus casei rhamnosus* in the active management of preterm premature rupture of membranes remote from term, Drug Des. Devel. Ther. 8 (2014) 1169-1173.

[101] M.A.J. Beerepoot, G. ter Riet, S. Nys, W.M. van der Wal, C.A. de Borgie, T.M. de Reijke, J.M. Prins, J. Koeijers, A. Verbon, E. Stobberingh, S.E. Geerlings, Lactobacilli vs antibiotics to prevent urinary tract infections: a randomized, double-blind, noninferiority trial in postmenopausal women, Arch. Intern. Med. 172 (2012) 704-712.

[102] J.E. Bisanz, S. Seney, A. McMillan, R. Vongsa, D. Koenig, L. Wong, B. Dvoracek, G.B. Gloor, M. Sumarah, B. Ford, D. Herman, J.P. Burton, G. Reid, A systems biology approach investigating the effect of probiotics on the vaginal microbiome and host responses in a double blind, placebo-controlled clinical trial of post-menopausal women, PLoS One 9 (2014) e104511.

[103] M. Parma, M. Dindelli, L. Caputo, A. Redaelli, L. Quaranta, M. Candiani, The role of vaginal *Lactobacillus rhamnosus* (Normogin ®) in preventing bacterial vaginosis in women with history of recurrences , undergoing surgical menopause: a prospective pilot study, Eur. Rev. Med. Pharmacol. Sci. 17 (2013) 1399-1403.

[104] U. Jaisamrarn, S. Triratanachat, S. Chaikittisilpa, P. Grob, V. Prasauskas, N. Taechakraichana, Ultra-low-dose estriol and lactobacilli in the local treatment of postmenopausal vaginal atrophy, Climacteric. 16 (2013) 347-355.

[105] G. Donders, P. Neven, M. Moegele, A. Lintermans, G. Bellen, V. Prasauskas, P. Grob, O. Ortmann, S. Buchholz, Ultra-low-dose estriol and *Lactobacillus acidophilus* vaginal tablets (Gynoflor(®)) for vaginal atrophy in postmenopausal breast cancer patients on aromatase inhibitors: pharmacokinetic, safety, and efficacy phase I clinical study, Breast Cancer Res. Treat. 145 (2014) 371-379.

[106] S.F. Kotarski, D.C. Savage, Models for study of the specificity by which indigenous lactobacilli adhere to murine gastric epithelia, Infect. Immun. 26 (1979) 966-975.

[107] S. Yildirim, C.J. Yeoman, M. Sipos, M. Torralba, B.A. Wilson, T.L. Goldberg, R.M. Stumpf, S.R. Leigh, B.A. White, K.E. Nelson, Characterization of the fecal

microbiome from non-human wild primates reveals species specific microbial communities, PLoS One. 5 (2010) e13963.

[108] S.Y. Doerflinger, A.L. Throop, M.M. Herbst-Kralovetz, Bacteria in the vaginal microbiome alter the innate immune response and barrier properties of the human vaginal epithelia in a species-specific manner, J. Infect. Dis. 209 (2014) 1989-1999.

[109] M.E. Nader-Macias, M.E. Lopez-Bocanera, C. Silva-Ruiz, A. Pesce-Ruiz Holgado, Isolation of lactobacilli from the urogenital tract of mice. Elaboration of beads for their inoculation, Microbiologie-Aliments-Nutrition. 10 (1992) 43-47.

[110] C. Silva de Ruiz, M.R. Rey, A.A. Ruiz Holgado, M.E. Nader-Macias, Experimental administration of estradiol on the colonization of *Lactobacillus fermentum* and *Escherichia coli* in the urogenital tract of mice, Biol. Pharm. Bull. 24 (2001) 127-134.

[111] V.S. Ocaña, E. Bru, A.A. Holgado, M.E. Nader-Macias, Surface characteristics of lactobacilli isolated from human vagina, J Gen Appl Microbiol. 212 (1999) 203-212.

[112] R. Martín, N. Soberón, M. Vaneechoutte, A.V. Flórez, F. Vázquez, J.E. Suárez, Characterization of indigenous vaginal lactobacilli from healthy women as probiotic candidates, Int. Microbiol. 11 (2008) 261-266.

[113] Y. Gao, Y. Lu, K.L. Teng, M.L. Chen, H.J. Zheng, Y.Q. Zhu, J. Zhong, Complete genome sequence of *Lactococcus lactis* subsp. *lactis* CV56, a probiotic strain isolated from the vaginas of healthy women, J. Bacteriol. 193 (2011) 2886-2887.

[114] J.M. Macklaim, G.B. Gloor, K.C. Anukam, S. Cribby, G. Reid, At the crossroads of vaginal health and disease, the genome sequence of *Lactobacillus iners* AB-1, Proc. Natl. Acad. Sci. U S A, 108 (2011) 4688-4695.

[115] S. Malik, M.I. Petrova, I.J.J. Claes, T.L. Verhoeven, P. Busschaert, M. Vaneechoutte, B. Lievens, I. Lambrichts, R.J. Siezen, J. Balzarini, J. Vanderleyden, S. Lebeer, The highly autoaggregative and adhesive phenotype of the vaginal *Lactobacillus plantarum* strain CMPG5300 is sortase dependent, Appl. Environ. Microbiol. 79 (2013) 4576-4585.

[116] C. Sabia, I. Anacarso, A. Bergonzini, R. Gargiulo, M. Sarti, C. Condò, P. Messi, S. de Niederhausern, R. Iseppi, M. Bondi, Detection and partial characterization of a bacteriocin-like substance produced by *Lactobacillus fermentum* CS57 isolated from human vaginal secretions, Anaerobe. 26 (2014) 41-45.

[117] M.S. Juárez Tomás, D. Zonenschain, L. Morelli, ME. Nader-Macías, Characterisation of potentially probiotic vaginal lactobacilli isolated from Argentinean women, Br. J. Biomed. Sci. 62 (2005) 170-174.

[118] M.S. Juárez Tomás, C.I. Saralegui Duhart, P.R. De Gregorio, E. Vera Pingitore, M.E. Nader-Macías, Urogenital pathogen inhibition and compatibility between vaginal *Lactobacillus* strains to be considered as probiotic candidates, Eur. J. Obstet. Gynecol. Reprod. Biol. 159 (2011) 399-406.

[119] M.C. Leccese Terraf, L.M. Mendoza, M.S. Juárez Tomás, C. Silva, M.E. Nader-Macías, Phenotypic surface properties (aggregation, adhesion and biofilm formation) and presence of related genes in beneficial vaginal lactobacilli, J. Appl. Microbiol. 117 (2014) 1761-1772.

[120] K.C. Anukam, J.M. Macklaim, G.B. Gloor, G. Reid, J. Boekhorst, B. Renckens, S.A. van Hijum, R.J. Siezen, Genome sequence of *Lactobacillus pentosus* KCA1: vaginal isolate from a healthy premenopausal woman, PLoS One. 8 (2013) e59239.

[121] S. Malik, R.J. Siezen, B. Renckens, M. Vaneechoutte, J. Vanderleyden, S. Lebeer, Draft genome sequence of *Lactobacillus plantarum* CMPG5300, a human vaginal isolate, Genome Announc. 2 (2014) e01149-14.

[122] A. McMillan, J.M. Macklaim, J.P. Burton, G. Reid, Adhesion of *Lactobacillus iners* AB-1 to Human fibronectin: a key mediator for persistence in the vagina?, Reprod. Sci. 20 (2012) 791-796.

[123] K. Selle, T.R. Klaenhammer, Genomic and phenotypic evidence for probiotic influences of *Lactobacillus gasseri* on human health, FEMS Microbiol. Rev. 37 (2013) 915-935.

[124] B.R. Johnson, T.R. Klaenhammer, Impact of genomics on the field of probiotic research: historical perspectives to modern paradigms, Antonie Van Leeuwenhoek, 106 (2014) 141-156.

[125] R. Martín, N. Soberón, F. Vázquez, J.E. Suárez, Vaginal microbiota: composition, protective role, associated pathologies, and therapeutic perspectivas, Enferm. Infecc. Microbiol. Clin. 26 (2008) 160-167.

[126] C.R. Wira, K.S. Grant-Tschudy, M.A. Crane-Godreau, Epithelial cells in the female reproductive tract: a central role as sentinels of immune protection, Am. J. Reprod. Immunol. 53 (2005) 65-76.

[127] G. Kunz, M. Noe, M. Herbertz, G. Leyendecker, Uterine peristalsis during the follicular phase of the menstrual cycle: effects of oestrogen, antioestrogen and oxytocin, Hum. Reprod. Update 4 (1998) 647-654.

[128] G. Kunz, M. Herbertz, M. Noe, G. Leyendecker, Sonographic evidence for the involvement of the utero-ovarian counter-current system in the ovarian control of directed uterine sperm transport, Hum. Reprod. Update. 4 (1998) 667-672.

[129] L.K. Hansen, N. Becher, S. Bastholm, J. Glavind, M. Ramsing, C.J. Kim, R. Romero, J.S. Jensen, N. Uldbjerg, The cervical mucus plug inhibits, but does not block, the passage of ascending bacteria from the vagina during pregnancy, Acta Obstet. Gynecol. Scand. 93 (2014) 102-108.

[130] U. Hynönen, R. Kant, T. Lähteinen, T.E. Pietilä, J. Beganović, H. Smidt, K. Uroić, S. Avall-Jääskeläinen, A. Palva, Functional characterization of probiotic surface layer protein-carrying *Lactobacillus amylovorus* strains, BMC Microbiol. 14 (2014) 199.

[131] R. Martín, C. Martín, S. Escobedo, J.E. Suárez, L.M. Quirós, Surface glycosaminoglycans mediate adherence between HeLa cells and *Lactobacillus salivarius* Lv72, BMC Microbiol. 13 (2013) 210.

[132] R. Martín, B. Sánchez, J.E. Suárez, M.C. Urdaci, Characterization of the adherence properties of human lactobacilli strains to be used as vaginal probiotics, FEMS Microbiol. Lett. 328 (2012) 166-173.

[133] V. Abramov, V. Khlebnikov, I. Kosarev, G. Bairamova, R. Vasilenko, N. Suzina, A. Machulin, V. Sakulin, N. Kulikova, N. Vasilenko, A. Karlyshev, V. Uversky, M.L. Chikindas, V. Melnikov, Probiotic properties of *Lactobacillus crispatus* 2,029: homeostatic interaction with cervicovaginal epithelial cells and antagonistic activity to genitourinary pathogens, Probiotics and Antimicrobial Proteins. (2014) In press.

[134] S. Coudeyras, G. Jugie, M. Vermerie, C. Forestier, Adhesion of human probiotic *Lactobacillus rhamnosus* to cervical and vaginal cells and interaction with vaginosis-associated pathogens, Infect. Dis. Obstet. Gynecol. 2008 (2008) 549640.

[135] V. Ocaña, M.E. Nader-Macías, Adhesion of *Lactobacillus* vaginal strains with probiotic properties to vaginal epithelial cells, Biocell. 25 (2001) 265-273.

[136] J.A. Younes, H.C. van der Mei, E. van den Heuvel, H.J. Busscher, G. Reid, Adhesion forces and coaggregation between vaginal staphylococci and lactobacilli, PLoS One 7 (2012) e36917.

[137] S. Katharios-Lanwermeyer, C. Xi, N.S. Jakubovics, A.H. Rickard, Mini-review: Microbial coaggregation: ubiquity and implications for biofilm development, Biofouling. 30 (2014) 1235-51.

[138] H. Ekmekci, B. Aslim, S. Ozturk, Characterization of vaginal lactobacilli coaggregation ability with *Escherichia coli*, Microbiol. Immunol. 53 (2009) 59-65.

[139] V.S. Ocaña, M.E. Nader-Macías, Vaginal lactobacilli: self and co-aggregating ability, Br. J. Biomed. Sci. 59 (2002) 183-190.

[140] P.R. De Gregorio, M.S. Juárez Tomás, M.C. Leccese Terraf, M.E. Nader-Macias, *In vitro* and *in vivo* effects of beneficial vaginal lactobacilli on pathogens responsible for urogenital tract infections, J. Med. Microbiol. 63 (2014) 685-696.

[141] A. McMillan, M. Dell, M.P. Zellar, S. Cribby, S. Martz, E. Hong, J. Fu, A. Abbas, T. Dang, W. Miller, G. Reid, Disruption of urogenital biofilms by lactobacilli, Colloids Surf. B. Biointerfaces. 86 (2011) 58-64.

[142] M.C. Leccese Terraf, M.S. Juárez Tomás, M.E. Nader-Macías, C. Silva de Ruiz, Screening of biofilm formation by beneficial vaginal lactobacilli and influence of culture media components, J. Appl. Microbiol. 113 (2012) 1517-1529.

[143] G.T. Spear, A.L. French, D. Gilbert, M.R. Zariffard, P. Mirmonsef, T.H. Sullivan, W.W. Spear, A. Landay, S. Micci, B.H. Lee, B.R. Hamaker, Human α-amylase present in lower-genital-tract mucosal fluid processes glycogen to support vaginal colonization by *Lactobacillus*, J. Infect. Dis. 210 (2014) 1019-1028.

[144] W.G. Lewis, L.S. Robinson, N.M. Gilbert, J.C. Perry, A.L. Lewis, Degradation, foraging, and depletion of mucus sialoglycans by the vagina-adapted *Actinobacterium Gardnerella vaginalis*, J. Biol. Chem. 288 (2013) 12067-12079.

[145] S.S. Witkin, H. Mendes-Soares, I.M. Linhares, A. Jayaram, W.J. Ledger, L.J. Forney, Influence of vaginal bacteria and D- and L-lactic acid isomers on vaginal extracellular matrix metalloproteinase inducer: implications for protection against upper genital tract infections, MBio. 4 (2013) e00460-13.

[146] E. Zakaria Gomaa, Antimicrobial and anti-adhesive properties of biosurfactant produced by lactobacilli isolates, biofilm formation and aggregation ability, J. Gen. Appl. Microbiol. 59 (2013) 425-436.

[147] M.M. Velraeds, B. van de Belt-Gritter, H. C. van der Mei, G. Reid, and H. J. Busscher, Interference in initial adhesion of uropathogenic bacteria and yeasts to silicone rubber by a *Lactobacillus acidophilus* biosurfactant, J. Med. Microbiol. 47 (1998) 1081-1085.

[148] E.J. Gudiña, V. Rocha, J.A. Teixeira, L.R. Rodrigues, Antimicrobial and antiadhesive properties of a biosurfactant isolated from *Lactobacillus paracasei* ssp. *paracasei* A20, Lett. Appl. Microbiol. 50 (2010) 419-424.

[149] D.E. O'Hanlon, T.R. Moench, R.A. Cone, In vaginal fluid, bacteria associated with bacterial vaginosis can be suppressed with lactic acid but not hydrogen peroxide, BMC Infect. Dis. 11 (2011) 200.

[150] M. Aldunate, D. Tyssen, A. Johnson, T. Zakir, S. Sonza, T. Moench, R. Cone, G. Tachedjian, Vaginal concentrations of lactic acid potently inactivate HIV, J. Antimicrob. Chemother. 68 (2013) 2015-2025.

[151] M.A. Graver, J.J. Wade, The role of acidification in the inhibition of *Neisseria gonorrhoeae* by vaginal lactobacilli during anaerobic growth, Ann. Clin. Microbiol. Antimicrob. 10 (2011) 8.

[152] P. Mirmonsef, A.L. Hotton, D. Gilbert, D. Burgad, A. Landay, K.M. Weber, M. Cohen, J. Ravel, G.T. Spear, Free glycogen in vaginal fluids is associated with *Lactobacillus* colonization and low vaginal pH, PLoS One. 9 (2014) e102467.

[153] A.C. Vallor, M.A. Antonio, S.E. Hawes, S.L. Hillier, Factors associated with acquisition of, or persistent colonization by, vaginal lactobacilli: role of hydrogen peroxide production, J. Infect. Dis. 184 (2001) 1431-1436.

[154] D.E. O'Hanlon, B.R. Lanier, T.R. Moench, R.A. Cone, Cervicovaginal fluid and semen block the microbicidal activity of hydrogen peroxide produced by vaginal lactobacilli. BMC Infect. Dis. 10 (2010) 120.

[155] P.A. Cadieux, J. Burton, E. Devillard, G. Reid, *Lactobacillus* by-products inhibit the growth and virulence of uropathogenic *Escherichia coli*, J. Physiol. Pharmacol. 60 (2009) 13-18.

[156] F. Atassi, A.L. Servin, Individual and co-operative roles of lactic acid and hydrogen peroxide in the killing activity of enteric strain *Lactobacillus johnsonii* NCC933 and vaginal strains *Lactobacillus gasseri* KS120.1 against enteric, uropathogenic and vaginosis-associated pathogens, FEMS Microbiol. Lett. 304 (2010) 29-38.

[157] S.E. Dover, A.A. Aroutcheva, S. Faro, M.L. Chikindas, Natural antimicrobials and their role in vaginal health: a short review, Int. J. Probiotics Prebiotics. 3 (2008) 219-230.

[158] Y. Turovskiy, R.D. Ludescher, A.A. Aroutcheva, S. Faro, M.L. Chikindas, Lactocin 160, a bacteriocin produced by vaginal *Lactobacillus rhamnosus*, targets

cytoplasmic membranes of the vaginal pathogen, *Gardnerella vaginalis*, Probiotics Antimicrob. Proteins. 1 (2009) 67-74.

[159] K.S. Noll, M.N. Prichard, A. Khaykin, P.J. Sinko, M.L. Chikindas, The natural antimicrobial peptide subtilosin acts synergistically with glycerol monolaurate, lauric arginate, and ε-poly-L-lysine against bacterial vaginosis-associated pathogens but not human lactobacilli, Antimicrob. Agents Chemother. 56 (2012) 1756-1761.

[160] Y. Turovskiy, M.L. Chikindas, Zinc lactate and sapindin act synergistically with lactocin 160 against *Gardnerella vaginalis*, Probiotics Antimicrob. Proteins. 3 (2011) 144-149.

[161] Y. Turovskiy, T. Cheryian, A. Algburi, R.E. Wirawan, P. Takhistov, P.J. Sinko, M.L. Chikindas, Susceptibility of *Gardnerella vaginalis* biofilms to natural antimicrobials subtilosin, ε-poly-L-lysine, and lauramide arginine ethyl ester. Infect. Dis. Obstet. Gynecol. 2012 (2012) 284762.

[162] S. Borges, J. Barbosa, J. Silva, P. Teixeira, Evaluation of characteristics of *Pediococcus* spp. to be used as a vaginal probiotic, J. Appl. Microbiol. 115 (2013) 527-538.

[163] G. Stoyancheva, M. Marzotto, F. Dellaglio, S. Torriani, Bacteriocin production and gene sequencing analysis from vaginal *Lactobacillus* strains, Arch. Microbiol. 196 (2014) 645-653.

[164] V. Ocaña, A. Ruiz Holgado, M.E. Nader-Macías, Selection of vaginal-H_20_2 generating *Lactobacillus* species for probiotic use, Curr Microbiol. 38 (1999) 279-284.

[165] M.S. Juárez Tomás, V.S. Ocaña, B. Wiese, M.E. Nader-Macías, Growth and lactic acid production by vaginal *Lactobacillus acidophilus* CRL 1259, and inhibition of uropathogenic *Escherichia coli*, J. Med. Microbiol. 52 (2003) 1117-1124.

[166] V.S. Ocaña, A. Pesce de Ruiz Holgado, M.E. Nader-Macías, Characterization of a bacteriocin-like substance produced by a vaginal *Lactobacillus salivarius* strain, Appl. Environ. Microbiol. 65 (1999) 5631-5635.

[167] E. Vera Pingitore, E.M. Hébert, M.E. Nader-Macías, F Sesma, Characterization of salivaricin CRL 1328, a two-peptide bacteriocin produced by *Lactobacillus salivarius* CRL 1328 isolated from the human vagina, Res. Microbiol. 160 (2009) 401-408.

[168] K.V. Reddy, C Aranha, S.M. Gupta, R.D. Yedery, Evaluation of antimicrobial peptide nisin as a safe vaginal contraceptive agent in rabbits: *in vitro* and *in vivo* studies, Reproduction. 128 (2004) 117-126.

[169] S.E. Dover, A.A. Aroutcheva, S. Faro, M.L.Chikindas, Safety study of an antimicrobial peptide lactocin 160, produced by the vaginal *Lactobacillus rhamnosus*, Infect. Dis. Obstet. Gynecol. 2007 (2007) 78248.

[170] M. Mota-Meira, H. Morency, M.C. Lavoie, *In vivo* activity of mutacin B-Ny266, J. Antimicrob. Chemother. 56 (2005) 869-871.

[171] J. P.Lepargneur, V. Rousseau, Rôle protecteur de la flore de Döderlein. J. Gynecol. Obstet. Biol. Reprod. 31 (2002) 485-494.

[172] H.M. Joo, Y.J. Hyun, K.S. Myoung, Y.T. Ahn, J.H. Lee, C.S. Huh, M.J. Han, D.H. Kim, *Lactobacillus johnsonii* HY7042 ameliorates *Gardnerella vaginalis*-induced vaginosis by killing *Gardnerella vaginalis* and inhibiting NF-κB activation, Int. Immunopharmacol. 11 (2011) 1758-1765.

[173] H.M. Joo, K.A. Kim, K.S. Myoung, Y.T. Ahn, J.H. Lee, C.S. Huh, M.J. Han, D.H. Kim, *Lactobacillus helveticus* HY7801 ameliorates vulvovaginal candidiasis in mice by inhibiting fungal growth and NF-κB activation. Int. Immunopharmacol. 14 (2012) 39-46.

[174] B. Brichacek, L.A. Lagenaur, P.P Lee, D. Venzon, D.H. Hamer, *In vivo* evaluation of safety and toxicity of a *Lactobacillus jensenii* producing modified cyanovirin-N in a rhesus macaque vaginal challenge model, PLoS One. 8 (2013) e78817.

[175] M.E. Nader-Macías, C. Silva-Ruiz, M.E. Lopez-Bocanera, A. Pesce-Ruiz Holgado, Behaviour of lactobacilli on prevention and therapeutic effects on urinary tract infections (UTI) in mice, Anaerobe. 2 (1996) 85-93.

[176] C. Silva de Ruiz, R. Rey, M.E. Nader-Macías, Structural and ultrastructural studies of the urinary tract of mice inoculated with *Lactobacillus fermentum*, Br. J. Urol. 91 (2003) 878-882.

[177] E. Vintiñi, V.S. Ocaña, M.E. Nader-Macías, Effect of lactobacilli administration in the vaginal tract of mice. Evaluation of side-effects and local immune response by the local administration of selected strains, in: J.F.T. Spencer, A. Ragout de Spencer (Eds.), Public Health Microbiology. Methods and Protocols. Methods in Molecular Biology, Humana Press, New York, 2004, 268, pp. 401-410.

[178] P.R. De Gregorio, M.S. Juárez Tomás, V. Santos, M.E. Nader-Macías, Beneficial lactobacilli: effects on the vaginal tract in a murine experimental model, Antonie van Leeuwenhoek, 102 (2012) 569-580.

[179] G. Zárate, V. Santos, M.E. Nader-Macias, Protective effect of vaginal *Lactobacillus paracasei* CRL 1289 against urogenital infection produced by *Staphylococcus aureus* in a mouse animal model, Infect. Dis. Obstet. Gynecol. 2009 (2009) 48358.

[180] C.R. Wira, R.M. Rossoll, R.C., Young Polarized uterine epithelial cells preferentially present antigen at the basolateral surface: role of stromal cells in regulating class II-mediated epithelial cell antigen presentation, J. Immunol. 175 (2005) 1795-1804.

[181] D.K. Hickey, M.V. Patel, J.V. Fahey, C.R. Wira, Innate and adaptive immunity at mucosal surfaces of the female reproductive tract: stratification and integration of immune protection against the transmission of sexually transmitted infections, J. Reprod. Immunol. 88 (2011) 185-194.

[182] C. Wira, J. Fahey, P. Wallace, G. Yeaman, Effect of the menstrual cycle on immunological parameters in the human female reproductive tract, J. Acquir. Immune Defic. Syndr. 38 (2005) S34-S36.

[183] S.S. Witkin, I.M. Linhares, A.M. Bongiovanni, C. Herway, D. Skupski, Unique alterations in infection-induced immune activation during pregnancy, BJOG. 118 (2011) 145-153.

[184] P. Mirmonsef, D. Gilbert, M.R. Zariffard, B.R. Hamaker, A. Kaur, A.L. Landay, G.T. Spear, The effects of commensal bacteria on innate immune responses in the female genital tract, Am. J. Reprod. Immunol. 65 (2011) 190-195.

[185] M.R. Jones, A.S. Neish, Recognition of bacterial pathogens and mucosal immunity. Microreview, Cell. Microbiol. 13 (2011) 670-676.

[186] S. O'Flaherty, D.M. Saulnier, B. Pot, J. Versalovic, How can probiotics and prebiotics impact mucosal immunity?, Gut Microbes. 1 (2010) 293-300.

[187] K. Nasu, H. Narahara, Pattern recognition via the toll-like receptor system in the human female genital tract, Mediators Inflamm. 2010 (2010) 976024.

[188] B.D. Taylor, T. Darville, R.E. Ferrell, R.B. Ness, S.F. Kelsey, C.L. Haggerty, Cross-sectional analysis of Toll-like receptor variants and bacterial vaginosis in African-American women with pelvic inflammatory disease, Sex. Transm. Infect. 90 (2014) 563-566.

[189] D.O. Ochiel, R.M. Rossoll, T.M. Schaefer, C.R. Wira, Effect of oestradiol and pathogen-associated molecular patterns on class II-mediated antigen presentation and immunomodulatory molecule expression in the mouse female reproductive tract, Immunol. 135 (2012) 51-62.

[190] N. Iijima, J.M. Thompson, A. Iwasak, Dendritic cells and macrophages in the genitourinary tract, Mucosal Immunol. 1 (2008) 451-459.

[191] N. Iijima, M.M. Linehan, S. Saeland, A. Iwasaki, Vaginal epithelial dendritic cells renew from bone marrow precursors, Proc. Natl. Acad. Sci. USA 104 (2007) 19061-19066.

[192] S.R. Hedges, F. Barrientes, R.A. Desmond, J.R. Schwebke, Local and systemic cytokine levels in relation to changes in vaginal flora, J. Infect. Dis. 193 (2006) 556-562.

[193] M.B. Parr, E.L. Parr, Langerhans cells and T lymphocyte subsets in the murine vagina and cervix, Biol. Reprod. 44 (1991) 491-498.

[194] D. Duluc, J. Gannevat, E. Anguiano, S. Zurawski, M. Carley, et al. Functional diversity of human vaginal APC subsets in directing T-cell responses. Mucosal Immunol. 6 (2013) 626-638.

[195] E.L. Parr, M.B. Parr, Immunoglobulin G is the main protective antibody in mouse vaginal secretions after vaginal immunization with attenuated herpes simplex virus type 2, J. Virol. 71 (1997) 8109-8115.

[196] S. Gupta, S. Agrawal, S. Gollapudi, Increased activation and cytokine secretion in B cells stimulated with leptin in aged humans, Immun. Ageing. 10 (2013) 1-3.

[197] P.R. De Stasio, M.W. Taylor, Specific effect of interferon on the herpes simplex virus type 1 transactivation event, J. Virol. 64 (1990) 2588-2593.

[198] D.M. Koelle, L. Corey, Herpes simplex: insights on pathogenesis and possible vaccines. Annu. Rev. Med. 59 (2008) 381-395.

[199] S.L. Swain, K.K. McKinstry, T.M. Strutt, Expanding roles for $CD4^+$ T cells in immunity to viruses. Nat. Rev. Immunol. 12 (2012) 136-148.

[200] S.L. Bixler, J.J. Mattapallil, Loss and dysregulation of Th17 cells during HIV infection. Clin. Dev. Immunol. 2013 (2013) 852418.

[201] A. Singh, M. Vajpayee, S.A. Ali, N.K. Chauhan, Cellular interplay among Th17, Th1, and Treg cells in HIV-1 subtype "C" infection, J. Med. Virol. 86 (2014) 372-384.

[202] J. Li, W. Wang, S.X. Xu, N.A. Magarvey, J.K.. McCormick, *Lactobacillus reuteri*-produced cyclic dipeptides quench agr-mediated expression of toxic shock syndrome toxin-1 in staphylococci, Proc. Natl. Acad. Sci. U.S.A. 108 (2011) 3360-3365.

[203] M. Karlsson, N. Scherbak, G. Reid, J. Jass, *Lactobacillus rhamnosus* GR-1 enhances NF-kappaB activation in *Escherichia coli*-stimulated urinary bladder cells through TLR4, BMC Microbiol. 12 (2012) 15.

[204] R. Wagner, S.J. Johnson, Probiotic *Lactobacillus* and estrogen effects on vaginal epithelial gene expression responses to *Candida albicans*, J. Biomed. Sci. 19 (2012) 58.

[205] A. Rizzo, A. Losacco, C.R. Carratelli, *Lactobacillus crispatus* modulates epithelial cell defense against *Candida albicans* through Toll-like receptors 2 and 4, interleukin 8 and human β-defensins 2 and 3, Immunol. Lett. 156 (2013) 102-109.

[206] B. Evrard, S. Coudeyras, A. Dosgilbert, N. Charbonnel, J. Alamé, A. Tridon, C. Forestier, Dose-dependent immunomodulation of human dendritic cells by the probiotic *Lactobacillus rhamnosus* Lcr35, PLoS One. 6 (2011) e18735.

[207] A.J. Martins, S. Spanton, H.I. Sheikh, S.O. Kim, The anti-inflammatory role of granulocyte colony-stimulating factor in macrophage-dendritic cell crosstalk after *Lactobacillus rhamnosus* GR-1 exposure, J. Leukoc. Biol. 89 (2011) 907-915.

[208] L. Vong, R.J. Lorentz, A. Assa, M. Glogauer, PM. Sherman, Probiotic *Lactobacillus rhamnosus* inhibits the formation of neutrophil extracellular traps, J. Immunol. 192 (2014) 1870-1877.

[209] K. Stencel-Gabriel, I. Gabriel, A. Wiczkowski, M. Paul, A. Olejek, Prenatal priming of cord blood T lymphocytes by microbiota in the maternal vagina, Am. J. Reprod. Immunol. 61 (2009) 246-252.

[210] E. Bloise, M. Torricelli, R. Novembri, L.E. Borges, P. Carrarelli, F.M. Reis, F. Petraglia, Heat-killed *Lactobacillus rhamnosus* GG modulates urocortin and cytokine release in primary trophoblast cells, Placenta. 31 (2010) 867-872.

[211] M. Yeganegi, C.G. Leung, A. Martins, S.O. Kim, G. Reid, J.R. Challis, A.D. Bocking, *Lactobacillus rhamnosus* GR-1-induced IL-10 production in human placental trophoblast cells involves activation of JAK/STAT and MAPK pathways, Reprod. Sci. 17 (2010) 1043-1051

[212] M.C. Verdenelli, M.M. Coman, C. Cecchini, S. Silvi, C. Orpianesi, A. Cresci, Evaluation of antipathogenic activity and adherence properties of human

Lactobacillus strains for vaginal formulations. J. Appl. Microbiol. 116 (2014) 1297-1307.

[213] R.R. Nelson, Intrinsically vancomycin-resistant Gram positive-organisms: clinical relevance and implications for infection control, J. Hosp. Infect. 42 (1999) 275-282.

[214] S. Harbarth, U. Theuretzbacher, J. Hackett, on behalf of the DRIVE-AB consortium, Antibiotic research and development: business as usual?, J. Antimicrob. Chemother. 2015, In press.

[215] L.M. Bebell, A.N. Muiru, Antibiotic Use and Emerging Resistance: How Can Resource-Limited Countries Turn the Tide? Glob. Heart. 9(2014) 347-358. Review.

[216] H. Aarts, A. Margolles, Antibiotic resistance genes in food and gut (non-pathogenic) bacteria. Bad genes in good bugs, Front. Microbiol. 5 (2015) 754.

[217] P.R. De Gregorio, M.S. Juárez Tomás, M.C. Leccese Terraf, M.E.F. Nader-Macías, Preventive effect of *Lactobacillus reuteri* CRL1324 on Group B *Streptococcus* vaginal colonization in an experimental mouse model, J. Appl. Microbiol. (2014) In press.

[218] J. das Neves, C.M. Rocha, M.P. Gonçalves, R.L. Carrier, M. Amiji, M.F. Bahia, B. Sarmento, Interactions of microbicide nanoparticles with a simulated vaginal fluid, Mol. Pharm. 9 (2012) 3347-3356.

[219] M.S. Juárez Tomás, M.E. Nader-Macías, Effect of a medium simulating vaginal fluid on the growth and expression of beneficial characteristics of potentially probiotic lactobacilli, in A. Méndez-Vilas (Ed.), Communicating Current Research and Educational Topics and Trends in Applied Microbiology, Microbiology Book Series, Formatex, Badajoz, 2007, pp. 732–739.

[220] S. Delgado, A.M. Leite, P. Ruas-Madiedo, B. Mayo, Probiotic and technological properties of *Lactobacillus* spp. strains from the human stomach in the search for potential candidates against gastric microbial dysbiosis, Front. Microbiol. 5 (2014) 766.

[221] G. Weiss, L. Jespersen, Transcriptional analysis of genes associated with stress and adhesion in *Lactobacillus acidophilus* NCFM during the passage through an *in vitro* gastrointestinal tract model, J. Mol. Microbiol. Biotechnol. 18 (2010) 206-214.

[222] D.M. Saulnier, F. Santos, S. Roos, T.A. Mistretta, J.K. Spinler, D. Molenaar, B. Teusink, J. Versalovic, Exploring metabolic pathway reconstruction and genome-wide expression profiling in *Lactobacillus reuteri* to define functional probiotic features, PLoS One. 6 (2011) e18783.

[223] E. Hamon, P. Horvatovich, E. Izquierdo, F. Bringel, E. Marchioni, D. Aoudé-Werner, S. Ennahar, Comparative proteomic analysis of *Lactobacillus plantarum* for the identification of key proteins in bile tolerance, BMC Microbiol. 11 (2011) 63.

[224] K. Koskenniemi, K. Laakso, J. Koponen, M. Kankainen, D. Greco, P. Auvinen, K. Savijoki, T.A. Nyman, A. Surakka, T. Salusjärvi, W.M. de Vos, S. Tynkkynen, N. Kalkkinen, P. Varmanen, Proteomics and transcriptomics characterization of bile stress response in probiotic *Lactobacillus rhamnosus* GG, Mol. Cell. Proteomics. 10 (2011) M110.002741.

[225] K. Lee, H.G. Lee, Y.J. Choi, Proteomic analysis of the effect of bile salts on the intestinal and probiotic bacterium *Lactobacillus reuteri*, J. Biotechnol. 137 (2008) 14-19.

[226] E. Denou, B. Berger, C. Barretto, J.M. Panoff, F. Arigoni, H. Brüssow, Gene expression of commensal *Lactobacillus johnsonii* strain NCC533 during *in vitro* growth and in the murine gut, J. Bacteriol. 189 (2007) 8109-8119.

[227] M.S. Juárez Tomás, E. Bru, B. Wiese, A.P. de Ruiz Holgado, M.E. Nader-Macías, Influence of pH, temperature and culture media on the growth and bacteriocin production of vaginal *Lactobacillus salivarius* CRL 1328, J. Appl. Microbiol. 93 (2002) 714-724.

[228] M.S. Juárez Tomás, E. Bru, B. Wiese, M.E.F. Nader-Macías, Optimization of low-cost culture media for the production of biomass and bacteriocin by a urogenital *Lactobacillus salivarius* strain, Probiotics Antimicrob. Proteins. 2 (2010) 2-11.

[229] L. Lavari, R. Páez, A. Cuatrin, J. Reinheimer, G. Vinderola, Use of cheese whey for biomass production and spray drying of probiotic lactobacilli, J. Dairy Res. 81 (2014) 267-274.

[230] G. Donnarumma, A. Molinaro, D. Cimini, C. De Castro, V. Valli, V. De Gregorio, M. De Rosa, C. Schiraldi, *Lactobacillus crispatus* L1: high cell density cultivation and exopolysaccharide structure characterization to highlight potentially beneficial effects against vaginal pathogens, BMC Microbiol. 14 (2014) 137.

[231] M.S. Juárez Tomás, E. Bru, G. Martos, M.E. Nader-Macías, Stability of freeze-dried vaginal *Lactobacillus* strains in the presence of different lyoprotectors, Can. J. Microbiol. 55 (2009) 544-552.

[232] S. Mills, C. Stanton, G.F. Fitzgerald, R.P. Ross, Enhancing the stress responses of probiotics for a lifestyle from gut to product and back again, Microb. Cell Fact. 10 (2011) S19.

[233] C. Muller, V. Busignies, V. Mazel, C. Forestier, A. Nivoliez, P. Tchoreloff, Mechanistic approach to stability studies as a tool for the optimization and development of new products based on *L. rhamnosus* Lcr35® in compliance with current regulations, PLoS One. 8 (2013) e79041.

[234] M.J. Martín Villena, M.E. Morales Hernández, P. Gálvez Martín, B. Clares Naveros, M.A. Ruiz Martínez, Desarrollo de una técnica para la microencapsulación de probióticos. Development of a technique for microencapsulation of probiotics, ARS Pharmaceutica. 51 Suppl 3 (2010) 479-484.

[235] S. Uehara, K. Monden, K. Nomoto, Y. Seno, R. Kariyama, H. Kumon, A pilot study evaluating the safety and effectiveness of *Lactobacillus* vaginal suppositories in patients with recurrent urinary tract infection, Int. J. Antimicrob. Agents. 28 (2006) 30-34.

[236] S. Kaewnopparat, N. Kaewnopparat, Formulation and evaluation of vaginal suppositories containing *Lactobacillus*, WASET. 55 (2009) 25-28.

[237] F. Rodrigues, M.J. Maia, J. das Neves, B. Sarmento, M.H. Amaral, M.B.P.P. Oliveira, Vaginal suppositories containing *Lactobacillus acidophilus*: development and characterization, Drug Dev. Ind. Pharm. (2014) In press.

[238] S. Borges, P. Costa, J. Silva, P. Teixeira, Effects of processing and storage on *Pediococcus pentosaceus* SB83 in vaginal formulations: lyophilized powder and tablets, Biomed. Res. Int. 2013 (2013) 680767.

[239] S. Borges, P. Teixeira, *Pediococcus pentosaceus* SB83 as a potential probiotic incorporated in a liquid system for vaginal delivery, Benef. Microbes. 5 (2014) 421-426.

[240] A. Nivoliez, O. Camares, M. Paquet-Gachinat, S. Bornes, C. Forestier, P. Veisseire, Influence of manufacturing processes on in vitro properties of the probiotic strain *Lactobacillus rhamnosus* Lcr35®, J. Biotechnol. 160 (2012) 236-241.

[241] C. Muller, V. Mazel, C. Dausset, V. Busignies, S. Bornes, A. Nivoliez, P. Tchoreloff, Study of the *Lactobacillus rhamnosus* Lcr35® properties after compression and proposition of a model to predict tablet stability, Eur. J. Pharm. Biopharm. (2014) In press.

[242] M. Gunawardana, M.M. Baum, T.J. Smith, J.A. Moss, An intravaginal ring for the sustained delivery of antibodies, J. Pharm. Sci. (2014) In press.

[243] CPMP/ICH/2736/99, ICH Q1A (R2) Note for guidance on stability testing: Stability testing of new drug substances and products. European Medicines Agency. (2003).

Figure captions

Figure 1. Steps in the selection of probiotic microorganisms for the human female urogenital tract (adapted from reference [64]).

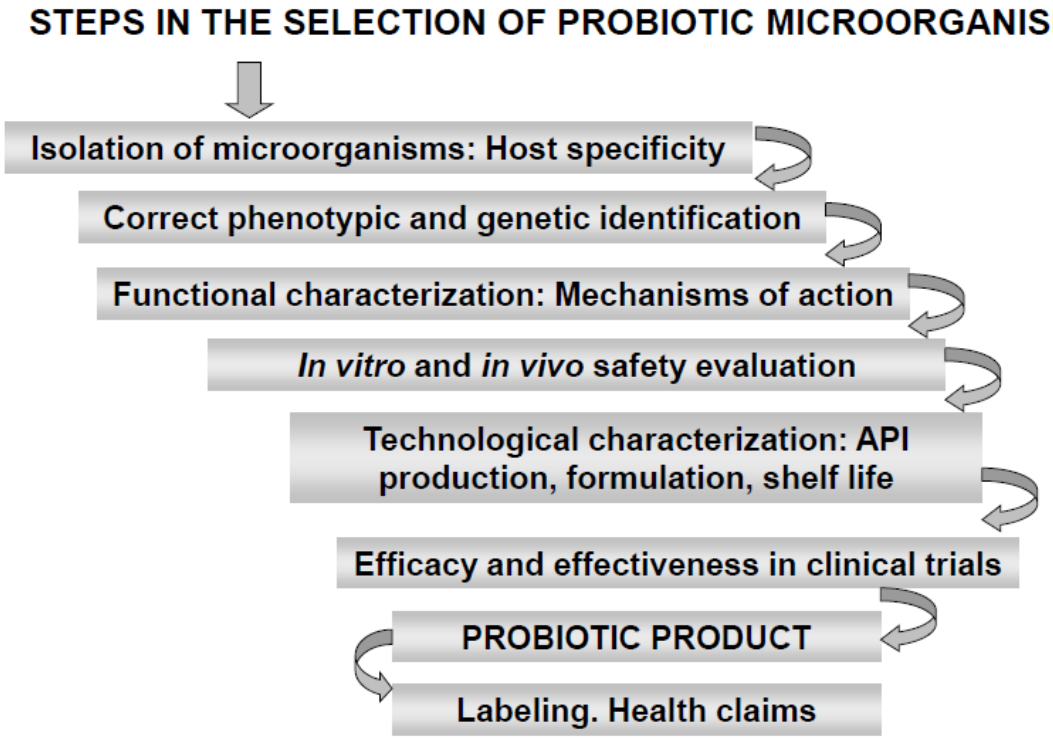


FIGURE 1

Table 1. Effects of probiotic or potential probiotic microorganisms on the human urogenital tract: Clinical assays performed in fertile, non-pregnant women

Potential clinical application	Probiotic API/Pharmaceutical form or product	Type of study, type and number of patients and treatment scheme	Results	Reference
Treatment adjunct to antifungal treatment (with fluconazole) of vulvovaginal candidiasis (VVC)	*Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14 (Chr. Hansen, Denmark; minimum 10^9 CFU of each strain per capsule), included in oral gelatin capsules.	Randomized, double-blind and placebo-controlled trial. Fifty-five women diagnosed with VVC were treated with a single dose of fluconazole (150 mg) supplemented with either 2 placebo capsules or 2 oral probiotic capsules every morning for 4 weeks.	Significantly lower presence of cultivable yeast and lower vaginal discharge associated with itching and burning vaginal feeling, dyspareunia and dysuria in the probiotic group than the placebo group.	[77]
Treatment adjunct to antimicrobial treatment (with tinidazole) of bacterial vaginosis (BV)	*L. rhamnosus* GR-1 and *L. reuteri* RC-14 (Chr. Hansen, Denmark; minimum 10^9 colony forming units (CFU) of each strain per capsule), included in oral gelatin capsules	Randomized, double-blind, placebo-controlled trial. Sixty-four women diagnosed with BV were treated with a single dose of tinidazole (2 g) supplemented with either 2 placebo capsules or 2 oral probiotic capsules every morning for 4 weeks.	Significantly higher cure rate of BV and higher numbers of women with normal vaginal microbiota in the probiotic group than the placebo group.	[78]
Treatment adjunct to extended antimicrobial treatment (with clindamycin and metronidazole) of BV	**Commercial products:** 1) *Lactobacillus gasseri* DSM 14869 and *L. rhamnosus* DSM 14870 (10^8 CFU/capsule) in EcoVag® vaginal capsules (Bifodan A/S, Denmark); 2) *L. rhamnosus* GR-1 and *L. reuteri* RC-14 (at least 10^9 CFU/capsule) in LaciBios® femina oral capsules (ASA Sp. z o.o., Głubczyce, Poland). **New *Lactobacillus* mixtures**: Mix 1, *L. crispatus* (*Lc*) 4R5, *L. gasseri* (*Lg*) 20M39, and *L. jenseneii* (*Lj*) 22B42; mix 2, *Lc* 23B33, *Lg* 6M9, *Lj* 12B1; mix 3, *Lc* 21M49, *Lg* 6M9 and *Lc* 8R6; mix 4, *Lg* DSM 14869, *L. rhamnosus* DSM 14870 and *Lg* DSM 15527; mix 5: *Lg* DSM	Consecutive treatment, open label, follow-up study. 63 women with BV received the following treatment: clindamycin (seven days course of daily 2% vaginal clindamycin cream together with oral clindamycin 300 mg BID for 7 days); then, probiotic capsules for 5 days; after the first menstruation, metronidazole vaginal gel for 5 days followed by 5 more days with probiotic administration; after the second menstruation, new course of vaginal metronidazole gel. **Groups assayed:** group 0, EcoVag® vaginal capsules; group 1, 2, 3 and 4, vaginal capsules containing the mix 1, 2, 3 and 4, respectively; group 5, oral capsules containing the mix 5; group 6, LaciBios® femina oral capsules.	High cure rate after 6, 12 and 24 months. There was no significant difference in cure rate depending on which *Lactobacillus* strains were given to the women or if the women were colonized by lactobacilli.	[66]

	14869, *L. rhamnosus* DSM 14870, *Lg* DSM 15527. These mixtures were included in gelatine capsules (10^9 freeze-dried bacteria per capsule)			
Treatment adjunct to antimicrobial treatment (with metronidazole) to reduce the BV recurrence rates	*Lactobacillus acidophilus* KS400 (at least 10^7 CFU/dose) combined with ultra-low-dose estriol (0.03 mg), in a single vaginal pessary (Medinova AG, Switzerland)	Randomized placebo-controlled double-blind trial. 408 women with BV were included in analyses and were treated with oral metronidazole (400 mg) for 7 days together with either 2% vaginal clindamycin-cream for 7 days or vaginal probiotic or placebo for 12 days.	No significant differences in one- and 6-month BV recurrence between the different treatment groups. Therefore, the combination of the oral metronidazole therapy and vaginal clindamycin, or oral metronidazole with a vaginal probiotic, did not reduce BV recurrence.	[79]
Treatment adjunct to antimicrobial treatment (with metronidazole) of BV	*L. acidophilus* (7.5 x 10^8 CFU) and *Lactobacillus bifidus* (2.5 x 10^8 CFU) in oral tablets; and *L. rhamnosus* B, *L. acidophilus*, *Streptococcus thermophilus* and *Lactobacillus bulgaricus* (10 milliard CFU in total) in vaginal capsules	173 women with BV were treated with oral metronidazole 500mg twice daily for 7 days associated with topical metronidazole cream for 5 days. A group of patients did not receive any probiotic treatment. Other group of patients received the oral probiotic treatment for 10 days, taking 2 capsules daily at 2 hours after the ingestion of the antibiotic. The remaining patients used 1 vaginal capsule daily, for 6 days. The therapy was repeated for 3 consecutive menstrual periods.	Lower recurrence rates in patients who received probiotic oral capsules while taking an antibiotic. Three or more BV relapse episodes per year in more than a half of women who did not use any probiotic product.	[80]
Restoration of the vaginal microbiota and reduction of recurrences in women with BV and/or VVC after treatment with clindamycin and/or clotrimazol	*L gasseri* LN40, *Lactobacillus fermentum* LN99, *Lactobacillus casei* subsp. *rhamnosus* LN113 and *Pediococcus acidilactici* LN23 (10^8-10^9 CFU of freeze-dried bacteria) in vaginal capsules (Ellen AB, Sweden)	Randomized double-blind placebo controlled study. After conventional treatment of BV (local clindamycin 100 mg ovules) and/or VVC (clotrimazol 200 mg vaginal tablets) for 3 days, 95 women assigned to receive probiotic vaginal capsule (n = 60) or placebo (n = 35) twice daily, for 5 consecutive days	High number of patients colonized by LN strains 2-3 days after administration, but the number decreased after one menstruation and after six months after administration. High cure rate in women receiving LN strains. Lower occurrence of malodorous discharge and somewhat fewer recurrences in probiotic group compared with placebo.	[81]
Restoration of vaginal ecosystem balance and reduction of the recurrence of BV after traditional oral treatment	*L. rhamnosus* (>40000 CFU/tablet) in Normogin® vaginal tablets (Baldacci, Pisa, Italy)	49 women diagnosed with BV were treated with a twice daily dose of 500 mg oral metronidazole for 7 days. Then, only a group of patients (n = 24) was treated with a once-weekly probiotic vaginal application for 6	Balanced vaginal ecosystem in almost all patients of probiotic group during 12 months. Significant increase in the number of women with abnormal flora over time, in the group that did not receive probiotic	[82]

with metronidazole		months.	administration after metronidazole therapy.	
Restoration of the vaginal lactobacilli population in women with BV after conventional antibiotic treatment with metronidazole gel	*L. crispatus* CTV-05 (2×10^9 CFU/dose) LACTIN-V powder that is administered using a prefilled tampon-like vaginal applicator (Osel, Inc., Santa Clara, CA, USA)	Phase 2a trial to determine the colonization efficiency, safety, tolerability, and acceptability of LACTIN-V administered by a vaginal applicator. Twenty-four women with BV (previously treated with 0.75% topical metronidazole gel for 5 consecutive days) were assigned to placebo group or probiotic group for administration of placebo or LACTIN-V, respectively, for 5 initial consecutive days, followed by a weekly application over 2 weeks.	A high number of women of probiotic group were colonized with *L. crispatus* CTV-05 at Day 10 or Day 28. Some minimal adverse effects in probiotic and placebo groups, but LACTIN-V was well tolerated and accepted. In women not colonized with CTV-05, higher vaginal concentration of certain BV-associated bacteria (*Gardnerella vaginalis* and *Atopobium* spp.)	[83,84]
Restoration of vaginal ecosystem balance after traditional treatment of BV with metronidazole	*L. rhamnosus* Lcr35® (at least 10^8 CFU/tablet) in Gynophilus® vaginal capsules (Probionov, Aurillac, France)	Randomized, open clinical trial. 60 women with bacterial vaginosis were treated with two daily oral doses of metronidazole 500 mg for 5 days, and with local application of metronidazole ovules 1000 mg at the 1st and the 3rd day. After antimicrobial treatment, a group (n = 30) was treated with probiotic vaginal capsules two daily doses for 7 days.	Higher clinical and microbiological efficacy when probiotic administration was added to the standard antimicrobial therapy. Restoration of the microbial balance in the vaginal ecosystem in most of patients of probiotic group, which could prevent BV relapses.	[85]
Prevention of recurrent urinary tract infection (UTI)	*L. crispatus* CTV-05 (10^8 CFU/capsule) in LACTIN-V vaginal gelatin capsules (Osel, Inc., Santa Clara, CA, USA)	Double-blind placebo-controlled trial. One hundred women with a history of recurrent UTI received antimicrobials for acute UTI and then were randomized to receive either LACTIN-V or placebo daily for 5 days, then once weekly for 10 weeks.	Significant reduction in recurrent UTI (associated with vaginal colonization of *L. crispatus*) in probiotic group compared with placebo group.	[86]
Improvement of vaginal ecosystem status	*L. fermentum* 57A, *L. plantarum* 57B and *L. gasseri* 57C (1×10^8 CFU of freeze-dried lactobacilli) in prOVag® oral capsules (IBSS BIOMED S.A., Poland)	37 initial patients (25 women finalized the study) with intermediate vaginal microbiota received probiotic oral capsules once a day for 60 days.	Between the 20th and 70th days of the study, vaginal and rectal colonization of lactobacilli administered. Normalization of vaginal health parameters (decrease of vaginal pH and increase of total numbers of lactobacilli). Absence of adverse events.	[87]
Prevention of recurrent BV	*L. rhamnosus*, *L acidophilus*, and *S. thermophilus* (8 billion CFU/capsule) in Probaclac Vaginal capsules (Nicar Laboratories, Inc, Blainville,	Double-blind, randomized, placebo-controlled study. One hundred twenty healthy women with a history of recurrent BV were assigned to daily vaginal prophylaxis with 1 probiotic capsule (n = 58 women) or 1 placebo capsule	Lower recurrence rates for BV and *Gardnerella vaginalis* incidence through 11 months in probiotic group compared to placebo group. Absence of adverse effects in the two groups assayed.	[88]

	Quebec, Canada)	(n = 62 women) for 7 days on, 7 days off, and 7 days on.		
Treatment and prevention of BV	*Lactobacillus brevis* CD2, *Lactobacillus salivarius* subsp. *salicinius* FV2, *Lactobacillus plantarum* FV9 (10^9 CFU/tablet) in Florisia™ vaginal tablets (CD Pharma India, Pvt. Ltd)	Randomized, double-blind study in sixty-seven patients with BV, 50 with intermediate microbiota and 42 with normal vaginal microbiota. Administration of one lactobacilli tablet or pH tablet inserted into the vagina daily at bedtime for 8 days.	High tolerability and absence of adverse effects in probiotic group. Higher BV cure rate and reduction in IL-1β and IL-6 vaginal pro-inflammatory cytokines in probiotic group. In healthy subjects, higher BV preventive effect of probiotic tablets than that of pH tablets.	[89]
Short-term treatment of BV and aerobic vaginitis	*L. acidophilus* KS400 (at least 10^7 CFU/tablet) combined with ultra-low-dose estriol (0.03 mg), in Gynoflor® vaginal tablets (Medinova AG, Switzerland)	Multicenter, randomized, single-blind, active-controlled pilot study. Forty-six women with bacterial vaginosis or aerobic vaginitis were treated with either one Gynoflor® vaginal tablet or suppository containing 500 mg metronidazole daily for 12 or 6 days, respectively.	After 3-7 days post-treatment, similar improvement of vaginal microbiota status in probiotic and metronidazole groups. After 1 month, lower positive effect with probiotic treatment compared to that with metronidazole.	[90]
Treatment of BV	*L. acidophilus* UBLA-34, *L. rhamnosus* UBLR-58, *L. reuteri* UBLRu-87, *L. plantarum* UBLP-40, *L. casei* UBLC-42, *L. fermentum* UBLF-31, *Bifidobacterium bifidum* UBBB-55 (10^9 CFU of each strain/capsule) in potential probiotic oral capsules UB-01BV (Provinorm, manufactured at Unique Biotech Limited, Hyderabad, India)	30 women diagnosed with BV (presenting symptoms such as white discharge, pH > 4.7, increased discharge, odour, colour of discharge and pruritus) were assigned to receive two potential probiotic oral capsules UB-01BV a day for 7 days	Significant positive response (reduction in BV symptoms) in all subjects at the end of the treatment. Absence of adverse effects.	[91]
Treatment of BV	*Lactobacillus delbrueckii* subsp. *lactis* DM8909 (10^9 CFU) in vaginal capsules	One hundred fifteen women, consisting of 30 healthy subjects, 30 BV-positive control subjects, 30 subjects with BV treated with a 7-day intravaginal metronidazole regimen (500 mg once daily), and 25 subjects with BV treated with a 10-day probiotics regimen (once daily), were analyzed.	At day 30, significant higher BV cure rate in the probiotics-treated subjects compared to the metronidazole-treated subjects. Reduction in the taxa diversity and eradication of most of the BV-associated phylotype after metronidazole treatment. In probiotic group, gradual and steady re-establishment of vaginal	[92]

			homeostasis.	
Treatment of BV, VVC and trichomoniasis	*L. rhamnosus* GR-1 and *L. reuteri* RC-14 (> 10^9 CFU of each strain per capsule), included in oral capsules (Lactogyn, JGL, Rijeka, Croatia).	Double-blind, randomized, multicentric, placebo-controlled study. 544 women diagnosed with vaginal infection (bacterial vaginosis, candidiasis, trichomoniasis or combination of these conditions) received either two probiotic (395 subjects) or placebo capsules (149 subjects) per day over a period of 6 weeks.	Restitution to balanced vaginal microbiota in a significantly higher number of subjects in the probiotic group, compared to the placebo group.	[93]
Treatment of VVC	*L. fermentum* LF10 (DSM 19187) and *L. acidophilus* LA02 (DSM 21717) (at least 0.4 billion live cells of each strain) in slow release effervescent vaginal tablets (ActiCand 30 product; Probiotical, Novara, Italy)	30 women with VVC (including 8 menopausal women) received an acute treatment for 4 weeks: administration of a vaginal tablet once a day for 7 days, followed by 1 tablet every 3 nights for a further 3-week application. In the following month, patients used 1 tablet per week.	After 28 days and 2 months, significant resolution of VVC in most subjects.	[94]

Table 2. Effects of probiotic microorganisms on the human urogenital tract: Clinical assays performed in pregnant women

Potential clinical application	Probiotic API/Pharmaceutical form or product	Type of study, type and number of patients and treatment scheme	Results	Reference
Preventing of the occurrence of abnormal vaginal microbiota and of the alteration of parameters relevant to preterm birth	*L. rhamnosus* BMX 54 (>40000 CFU) in Normogin® vaginal tablets (Baldacci, Pisa, Italy)	Sixty pregnant women were assigned randomly to the untreated group (n = 30) or probiotic group (n = 30) for vaginal application of one probiotic tablet, once a week for 12 weeks.	Effects evidenced in the probiotic group, unlike untreated group: Prevention of the increase of pathogenic microorganisms in vaginal and/or cervical swabs, non significant changes in pH values or vaginal discharge, and non significant modifications of cervical parameters that could represent risk factors of vulnerability to preterm delivery.	[95]
Modulation of the vaginal microbiota and cytokine secretion, with potential implications in preventing preterm birth	*Lactobacillus paracasei*, *Lactobacillus plantarum*, *Lactobacillus acidophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Bifidobacterium longum*, *Bifidobacterium breve*, *Bifidobacterium infantis* and *Streptococcus thermophilus* (at least 900 billion viable of lyophilized bacteria) in VSL#3 sachet (VSL Pharmaceuticals, Inc.,Towson, MD, USA) for oral administration.	Pilot, not randomized, controlled and perspective study. 27 healthy women during last trimester of pregnancy were assigned to the control group (n = 12; without any dietary supplementation) or probiotic group (n = 15) for oral administration of 1 sachet once/day of VSL#3 for 4 weeks from the 33rd to the 37th week of gestation.	Effects observed in probiotic group, unlike control group: Positive changes in the composition of the vaginal microbiota (prevention of the decrease of *Bifidobacterium* and of the increase of *Atopobium*) and significant decreases in some pro-inflammatory vaginal cytokines.	[96]
Treatment of BV	*Lactobacillus plantarum*, *Lactobacillus acidophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Bifidobacterium longum*, *Bifidobacterium breve*, *Bifidobacterium infantis* and *Streptococcus thermophilus*	Fourty pregnant women (between 10th and the 34th week of gestation) with a diagnosis of BV were assigned to: A) probiotic group for oral administration of VSL#3®, 2 tablets a day for 5 days, followed by 1 tablet daily for 10 days, or B) clindamycin group for vaginal administration of clindamycin 100 mg daily for 15 days.	After treatment, vaginal discharge was absent in probiotic group, and reduced in clindamycin group. Improvement of constipation only in the probiotic group. Vaginal swabs resulted negative in both groups in particular for *Gardnerella vaginalis.*	[97]

	(at least 225 billion viable of lyophilized bacteria) in VSL#3 capsules (VSL Pharmaceuticals, Inc.,Towson, MD, USA) for oral administration.			
Reduction of Group B *Streptococcus* (GBS) vaginal colonization	*L. acidophillus* (> 7.5 x 10^9 CFU), *Bifidobacterium lactis* (> 6.0 x 10^9 CFU) and *B. longum* (> 1.5 x 10^9 CFU) included as freeze-dried cultures in Florajen3 oral capsules (American Lifeline Inc., USA).	Open-label, two-group quasi-experiment pilot study. Administration of oral probiotic (Florajen3) taken once daily to ten pregnant participants; 10 participants served as controls.	Absence of adverse effects or minor side effects in probiotic group; one half reported improved gastrointestinal symptoms. Positive qualitative prenatal GBS cultures at 36 weeks of gestation in two women in each group. However, lower quantitative GBS colony counts in the probiotic group.	[98]
Prevention of spontaneous preterm delivery associated with bacterial vaginosis (BV)	*Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14 (more than 10^6 colony forming units (CFU) of each strain), included in oral capsules	Randomized controlled trial. Pregnant women at less than 20 weeks of gestation, with no indication of elective preterm delivery, with asymptomatic bacterial vaginosis/intermediate-degree infections. 317 and 323 patients, respectively assigned to the placebo and probiotic groups, finished the trial. Oral administration of two capsules per day up to the 24th to 26th week of gestation (treatment duration: 6-12 weeks).	No significant differences in the relative risk of spontaneous premature birth between probiotic-treated and placebo groups.	[99]
Ampicillin prophylaxis accompanied by *Lactobacillus casei rhamnosus* to prolong the latency period (period through which the fetus is exposed to a potentially unfavorable intrauterine environment) in patients with preterm premature rupture of membranes (PPROM) remote	*L. casei rhamnosus* (>40000 CFU) in Vagi-Flora® vaginal capsules (Laboratoires Lyocentre, Aurillac, France)	Retrospective study. Pregnant patients who were admitted for PPROM at 23–31 weeks of pregnancy (without infection findings at admission) were classified into two groups: group 1 (n = 20), patients who received four doses of 1 g/day ampicillin, and group 2 (n = 20), patients who received four doses of 1 g/day ampicillin plus a transvaginal application of 1 capsule/day, until labor of *L. casei rhamnosus.*	Gestational weeks at delivery (28.1 ± 0.3 weeks versus 31.5 ± 0.4 weeks), latency periods (12.3 ± 1.5 days versus 41.4 ± 4.4 days), 5-minute APGAR scores (indicating how well the newborn is doing outside the mother's uterus), and birth weights were significantly higher in group 2. White blood cells (WBC) and neutrophil counts at delivery, ΔWBC levels, ΔC-reactive protein levels and Δneutrophil levels were significantly lower in group 2 at delivery.	[100]

Table 3. Effects of probiotic microorganisms on the human urogenital tract: Clinical assays performed in post-menopausal women

Potential clinical application	Probiotic API/Pharmaceutical form or product	Type of study, type and number of patients and treatment scheme	Results	Reference
Non-antibiotic prophylaxis for recurrent urinary tract infections (UTIs)	*Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14 (Chr. Hansen, Denmark; minimum 10^9 colony forming units (CFU) of each strain), included in oral capsules	Double-blind non-inferiority trial, in 252 postmenopausal women with recurrent UTIs. For 12 months of prophylaxis, administration of: 1) trimethoprim-sulfamethoxazole (480 mg) 1 tablet at night and 1 placebo capsule twice daily or 2) probiotic oral capsules twice daily and 1 placebo tablet at night.	Probiotics did not meet the non-inferiority criteria in the prevention of UTIs when compared with trimethoprim-sulfamethoxazole. At least 1 symptomatic UTI occurred in 69.3% and 79.1% of the antimicrobial- and lactobacilli-treated patients, respectively. However, unlike trimethoprim-sulfamethoxazole, lactobacilli do not increase antibiotic resistance in *Escherichia coli* from feces and urine.	[101]
Positive modification of the vaginal microbiome and host response	*L. rhamnosus* GR-1 and *L. reuteri* RC-14 (Chr. Hansen, Denmark; minimum 2.56 x 10^9 colony forming units (CFU) of each strain), included in vaginal capsules	Double blinded, placebo-controlled crossover study, in fourteen post-menopausal women (natural menopause) with an intermediate Nugent Score (4-6). Administration of probiotic vaginal capsules or placebo twice a day for 3 days.	When compared to placebo, the probiotic treatment did not result in an improved Nugent score. However, subtle molecular changes were evidenced: an increase in the relative abundance of *Lactobacillus* (associated with an increase in lactate levels), a decrease in *Atopobium* and a modulator effect on immune response.	[102]
Long-lasting treatment to prevent bacterial vaginosis (BV) in women with history of recurrences, undergoing surgical menopause	*L. rhamnosus* (at least 10^6 CFU of live and lyophilized bacteria) in Normogin® vaginal tablets (Baldacci, Pisa, Italy)	22 patients affected by recurrent BV and treated for surgical menopause for benign pathology. Administration of one tablet/day for 6 days, then two tablets per week for 2 months and then one tablet once a week till 6 months.	One episode of recurrence in 7 out of 21 women that completed the treatment; two episodes of BV during the year successive to menopause in 2 patients. Absence of side effects.	[103]
Local treatment and relapse prevention of post-menopausal vaginal atrophy	*Lactobacillus acidophilus* KS400 (10^8 CFU) combined with ultra-low-dose estriol (0.03 mg), in Gynoflor® vaginal tablets (Medinova AG, Switzerland)	Double-blind, randomized, placebo-controlled study (Controlled phase-initial therapy) followed by an open-label follow-up (Open phase-test medication initial and maintenance therapy). 87 postmenopausal women (natural or surgical menopause) with vaginal atrophy symptoms and Vaginal Maturation Index (VMI) of ≤ 40% completed the study. Administration of one vaginal	Controlled phase: Increase in VMI in probiotic-treated women compared to placebo. Open phase: Further increase in VMI after initial therapy and during maintenance therapy. Maturation of epithelium, followed by improvement of clinical symptoms and normalization of the vaginal ecosystem.	[104]

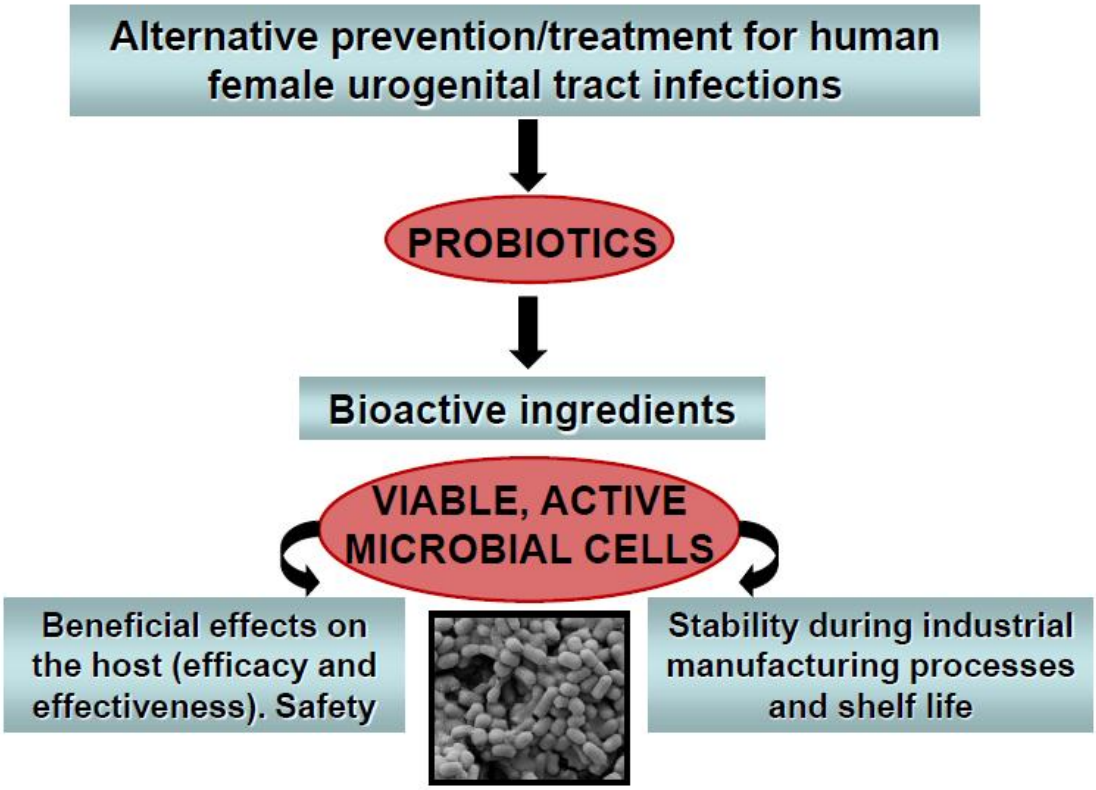
		tablet daily for 12 days (initial therapy with test medication or placebo in first phase), followed by one tablet on two consecutive days weekly for 12 weeks (maintenance therapy).		
Treatment of atrophic vaginitis in breast cancer (BC) patients taking non-steroidal aromatase inhibitor (NSAIs)	*Lactobacillus acidophilus* KS400 (10^8 CFU) combined with ultra-low-dose estriol (0.03 mg), in Gynoflor® vaginal tablets (Medinova AG, Switzerland)	Phase I pharmacokinetic (PK) study assessed circulating estrogens in 16 BC patients during a NSAI treatment with vaginal atrophy. Application of one daily probiotic vaginal tablet for 28 days followed by a maintenance therapy of 3 tablets weekly for 8 weeks.	Small and transient increases in serum estriol, but not estrone or estradiol. Resolution or improvement of vaginal atrophy in all women. The product was well tolerated and can be considered as safe and efficacious.	[105]

Table 4. Technological studies of probiotic or potential probiotic microorganisms for their inclusion in vaginal pharmaceutical products

Process	Microorganism (API)	Type of study	Results	Reference
Freeze-drying (for potential inclusion in a pharmaceutical formulation)	*Lactobacillus paracasei* CRL 1289, *Lactobacillus acidophilus* CRL 1266, *L. acidophilus* CRL 1251, *Lactobacillus gasseri* CRL 1259, *Lactobacillus johnsonii* CRL 1294 and *Lactobacillus salivarius* CRL 1328	Survival and expression of beneficial characteristics during freeze-drying and storage at 4°C for 24 months, in the presence of lyoprotectors (lactose and sucrose suspended in water or reconstituted skim milk).	High degree of survival and expression of potentially probiotic characteristics (production of antimicrobial substances or auto-aggregation capabilities) of most strains after freeze-drying and storage, in the presence of lactose and sucrose in water or in skim milk.	[231]
	Lactobacillus rhamnosus Lcr35®	Viability in laboratory freeze-dried powders (obtained in De Man-Rogosa-Sharpe and reconstituted milk) and stored at different temperatures (20, 25 and 30°C) for 12 months, according the ICH Guideline Q1A. Application of the Arrhenius model to predict strain stability during storage.	Higher lost of bacterial viability at higher temperature and at higher length of storage. The Arrhenius model applied was suitable to predict the loss of bacterial viability under different storage conditions.	[233]
Microencapsulation (for potential inclusion in a pharmaceutical formulation)	*Lactobacillus fermentum* CECT5716	Development of an emulsification/gelation process to encapsulate a freeze-dried probiotic strain, using sodium alginate..	High amount of viable *L. fermentum* CECT5716 in the microparticles obtained.	[234]
	Lactobacillus rhamnosus, *L. salivarius*, *Lactobacillus brevis* and *Lactobacillus plantarum*	Optimization of the emulsification/gelation process to encapsulate probiotic suspensions and prebiotics, using bioadhesive polymers (pectin and hyaluronic acid sodium salt).	High number of encapsulated viable bacteria in the final microparticles. Complete release of probiotic strains in a simulated vaginal fluid at 37°C. The bioadhesive delivery system obtained could be incorporated in vaginal gels or tablets.	[70]
Pharmaceutical formulation and shelf life	*Lactobacillus crispatus* GAI 98332	Elaboration of vaginal suppositories containing freeze-dried bacterium mixed and solidified with Witepsol H15. Bacterial viability in suppositories stored at 4°C, during 8 weeks.	The number of viable *L. crispatus* GAI 98332 did not decrease over time during storage at 4°C.	[235]
	Lactobacillus paracasei HL32	Development of different vaginal suppositories containing freeze-dried lactobacilli: 1) a conventional suppository with Witepsol H-15 as a base, 2) a conventional suppository with mixed	The hollow-type suppository with mixed PEGs as the base gave the highest release of *L. paracasei* HL32 and was microbiological stable after storage at 2-8°C over the period of 3 months.	[236]

	polyethylene glycols (PEGs) as a base, 3) a hollow-type suppository with Witepsol H-15 as a base and 4) a hollow-type suppository with mixed PEGs as a base. Bacterial viability during storage at ambient temperature (30 ± 2°C) and 2-8°C for 3 months.		
Lactobacillus acidophilus (unspecified strain)	Development of different vaginal suppositories containing freeze-dried lactobacilli: solid body and hollow-type suppositories with PEG 400 and 4000 or Witepsol H12. Characterization of suppositories: organoleptic properties, mass uniformity, disintegration, breaking strength and *L. acidophilus in vitro* release.	PEG 400, PEG 4000 and Witepsol H12 showed the absence of toxicity when tested using different vaginal cell lines. Obtained vaginal suppositories presented uniform and mild texture and a content of about 1 x 10^8 colony-forming units, were completely disintegrated in simulated vaginal environment in less than 60 min and provided sustained *in vitro* release of *L. acidophilus*. There was no significant loss of freeze-dried viable bacteria during release assays.	[237]
Pediococcus pentosaceus SB83	Survival and antilisterial activity after freeze-drying and manufacture of vaginal formulations (lyophilized powder with excipients and tablets with and without hydroxypropyl methylcelluloses as retarding polymer) and during storage at 4°C and at room temperature for 12 months.	Higher survival of *P. pentosaceus* SB83 during storage at 4°C, in the different vaginal formulations. Lost of antilisterial activity in the tablets; antimicrobial activity was maintained in lyophilized bacteria after storage at 4°C for 12 months.	[238]
Pediococcus pentosaceus SB83	Survival and functional properties of bacterial suspension in a liquid system (glycerol for eventually formulating a vaginal gel) during storage at 4°C and at room temperature	The vaginal probiotic incorporated into glycerol was able to survive in simulated vaginal fluid. The viability and antilisterial activity declined during storage at different temperatures; viability loss was total after 13 weeks at 4°C	[239]
Lactobacillus rhamnosus Lcr35®	Stability of strain biological properties during manufacture of vaginal capsules (GYNOPHILUS Lcr Regenerans®).	Compared with the native strain, bacteria from the vaginal formulation GYNOPHILUS evidenced: increased ability to metabolize	[240]

		glycogen and to produce lactic acid, and higher *in vitro* growth inhibition of the pathogen *Candida albicans*.	
Lactobacillus rhamnosus Lcr35®	Bacterial viability, genetic stability and maintenance of biological strain properties during tableting at different compression pressures. Viability in accelerated storage conditions. Application of a mathematical model to predict strain viability during manufacture process.	Greater bacterial viability in tablets than in freeze-dried powders. After compaction, genetic stability of Lcr35® strain and maintenance of its ability to inhibit the *C. albicans* growth. Development of a new mathematical model combining compression and temperature parameters to predict the bacterial viability at any pressure and time.	[241]
Lactobacillus gasseri ATCC 33323	Development of a novel pod-intravaginal ring (IVR) technology where polymer-coated tablets ("pods") of the probiotic strain are embedded in silicone IVRs. *In vitro* API release through a delivery channel and viability.	Release of a high number of bacterial cells per day (depending on the diameter of the delivery channel) for up to 21 days, in a sustained and controlled way. Bacteria in the IVR pods remained viable throughout the *in vitro* studies and formed biofilms on the surfaces of the devices.	[242]
L. salivarius CRL 1328, *L. gasseri* CRL 1266 and their combination	API compatibility during freeze-drying and storage in gelatin capsules at 4°C for 12 months, in different formulations including potential probiotic strains, a bacteriocin (salivaricin CRL 1328), lactose, inulin and ascorbic acid.	Compatibility of both microorganisms in multi-strain formulations together with lactose, inulin and ascorbic acid. Only *L. salivarius* CRL 1328 could be included in a single-strain formulation together with salivaricin and non-microbial substances.	[73]
Combination (SYNBIO®) of *L. rhamnosus* IMC 501® and *L. paracasei* IMC 502®	Preparation of ovules and douches using lyophilized microorganisms included in different matrixes.	Witepsol® ovules have proved the best formulation in terms of probiotic viability	[212]



Graphical abstract