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The role of lactic acid production by probiotic *Lactobacillus* species in vaginal health

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Abstract

Vaginal eubiosis is characterised by beneficial lactobacillus-dominated microbiota. In contrast, vaginal dysbiosis (e.g. bacterial vaginosis, BV), characterised by an overgrowth of multiple anaerobes, is associated with an increased risk of adverse urogenital and reproductive health outcomes. A major distinguishing feature between the vaginal environment in states of eubiosis and dysbiosis is a high concentration of lactic acid, produced by lactobacilli, that acidifies the vagina in eubiosis versus a sharp drop in lactic acid and an increase in pH in dysbiosis. Here we review the antimicrobial, antiviral and immunomodulatory properties of lactic acid and the use of lactic acid and lactobacilli probiotics in preventing or treating BV.

Keywords: Microbiota; Lactobacilli; Probiotics; Lactic acid; Metabolites; Vagina

1. Introduction

Vaginal eubiosis is characterised by the presence of beneficial lactic-acid-producing microbiota predominantly from the genus *Lactobacillus*. *Lactobacillus* spp., naturally or administered as probiotics, may establish vaginal eubiosis by killing dysbiotic microbes, and many types of pathogens, with acidic lactic acid. They may also release other antimicrobial factors such as bacteriocins [1,2]. While many lactobacillus-based probiotics have been selected on the basis of hydrogen peroxide (H_2O_2) production, recent studies demonstrate that lactic acid is a major antimicrobial factor produced by lactobacilli [3,4]. This review focuses on the antimicrobial, antiviral and immunomodulatory properties of lactic acid, the major organic acid metabolite produced by lactobacilli. The differential effects reported for lactic acid isomers and their protonated forms will be discussed, as well as how lactobacilli generate lactic acid, by using amylase breakdown products of glycogen, We also review clinical studies that have evaluated the use of lactic acid or lactobacilli probiotics in preventing or treating bacterial vaginosis, and studies that are providing a clearer understanding of the properties of lactic acid production by *Lactobacillus* spp. which could lead to the development of improved vaginal probiotics.

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2. Vaginal eubiosis and dysbiosis

The vagina and ectocervix, comprising the lower female reproductive tract (FRT), are composed of a rarely keratinised stratified squamous epithelium, resting on a lamina propria, that is bathed in mucous and colonised with microbiota that can have commensal (benefit of one of the organisms, without effect for the other), mutualistic (both organisms benefit) or parasitic (microbes profit at the expense of the host) roles. The vaginal microbiota, during eubiosis in reproductive-age women, is typically dominated by the distinct *Lactobacillus* spp., *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus iners* and *Lactobacillus jensenii*, respectively, most of which produce large amounts of lactic acid [5–7]. In contrast, vaginal dysbiosis is characterised by the presence of polymicrobial populations with either a modest lactobacillus load (intermediate microbiota) or no lactobacilli (bacterial vaginosis, BV) [5,8,9]. BV is a very common yet poorly understood condition in reproductive-age women that is associated with adverse sexual and reproductive health outcomes [10]. BV can be classified as either asymptomatic or symptomatic, where diagnosis of the latter includes the presence of a malodorous abnormal vaginal discharge [11]. The observation that the vaginal microbiome in asymptomatic women is largely composed of bacteria capable of producing lactic acid leads to the assertion that lactic acid has a potential key ecological function in vaginal microbiomes associated with eubiosis.

The vaginal microbiomes harboured by women can differ based on cultural, behavioural and genetic factors. Caucasian women are predominantly colonised with *L. crispatus* microbiomes, while African and Hispanic women tend to be colonised with *L. iners* or polymicrobial microbiomes [5,12]. The vaginal microbial communities differ in their stability in the face of intrinsic (e.g. sex hormones, menses) and extrinsic (e.g. sexual intercourse, vaginal hygiene practices) disturbances. *L. crispatus* dominated microbiomes are relatively stable [13], while most, but not all, strains of *L. iners* appear to be less effective at preventing episodes of bacterial dysbiosis [13,14].

3. Vaginal microbiomes associated with eubiosis and dysbiosis have distinct organic acid metabolite profiles

Major distinguishing features of women with vaginal microbiomes associated with eubiosis, i.e. lactobacillus-dominated vaginal microbiomes versus dysbiosis typified by BV, are a vaginal pH $\leq$ 4.5 and a distinct bacterial organic acid metabolite profile, specifically the alpha hydroxy acid, lactic acid, versus short chain fatty acids (SCFAs) such as acetic acid [15]. Organic acid metabolite changes reported for lactobacillus-dominated vaginal microbiomes compared to BV include a dramatic drop in lactic acid from ~110 mM to <20 mM and corresponding increases in the SCFAs acetic acid (0–4 to 40–120 mM), propionic acid (<1 to 2–4 mM), butyric acid (<1 to 2–4 mM), and succinic acid (<1–20 mM), as well as amines that may contribute to the increase of vaginal pH [13,16,17]. Vaginal SCFAs and succinic acid are weaker acids than lactic acid (acid dissociation constant, pKa 3.89), i.e. acetic acid (pKa 4.76), propionic acid (pKa 4.87), butyric acid (pKa 4.82) and succinic acid (pKa 4.16 and 5.61). The association of high lactic acid levels with lactobacillus-dominated microbiota suggests that this organic acid metabolite contributes to the beneficial properties ascribed to lactobacilli, such as decreased susceptibility of the human host to urogenital pathogens, which would be a desirable characteristic for a vaginal probiotic.

4. Lactic acid is the major acidifier of the lower FRT when lactobacilli dominate

Lactobacilli are aerotolerant anaerobes that produce lactic acid through the fermentation of glucose [18]. Vaginal microbiota dominated by distinct *Lactobacillus* spp. acidify the vagina to different pH levels, with the acidity achieved by *L. crispatus*, on average, being the highest compared to *L. iners*, *L. jensenii* and *L. gasseri* [5]. In women with lactobacillus-dominated microbiota, the lactic acid concentration is inversely correlated with pH, indicating that lactic acid is primarily responsible for acidification of the vagina [19]. O'Hanlon et al. [19] demonstrated that the average concentration of vaginal lactic acid in women with lactobacillus-dominated microbiota, defined by a Nugent score of 0–3, is 1.0% ± 0.2% (w/v) and pH is 3.5 ± 0.3 (mean ± SD). This lactic acid concentration is 11 times higher and pH considerably lower than previously reported [20], as these recent measurements were restricted to women with Nugent scores of 0–3, and took into consideration the loss of CO_2 and minimised exposure of cervicovaginal secretions to aerobic conditions [19].

Vaginal lactic acid exists as L- and D-isomers predominantly produced by lactobacilli with <15% of L-lactic produced by vaginal epithelial cells [21]. The ratio of D-to L-lactic acid and the asymptotic pH are characteristics of specific *Lactobacillus* species and strains [21–23], i.e., lactobacilli acidifying the vagina slow down their production of lactic acid until their production rate matches the rate at which lactic acid is lost from the vagina [22]. At this steady-state, virtually all the lactobacilli are still viable and growing slowly [22]. In axenic cultures, *L. crispatus* and *L. gasseri* produce both D- and L-lactic acid, while *L. iners* produces only the L-isomer and *L. jensenii* only the D-isomer [23].

The well-established higher protection of *L. crispatus*, compared to *L. iners* against uropathogens [24,25] and adverse pregnancy outcomes [26] has been attributed to a greater protective role of D-lactic acid than of the L-isomer [23]. The study by Witkin et al. [23] showed that *L. iners* produces less lactic acid than *L. crispatus*, although it should be kept in mind that these were in vitro studies, which may not fully recapitulate the in vivo situation. Indeed, one would expect lower vaginal pH when dominated by *L. crispatus*. However, while Ravel et al. [5] reported that the average pH for women with *L. iners*-dominated vaginal microbiota was higher than those dominated by *L. crispatus*, similar pH values were observed

for some of the individual ethnic groups. This might be explained as study bias caused by the use of pH gloves, which cannot measure pH below 4.0 [5].

The protonated form of lactic acid predominates at pH values below pKa 3.9 and the lactate anion (charged, unprotonated) at pH values above the pKa. In vitro studies show that lactic acid and the lactate anion have distinct properties. We have demonstrated that the protonated form of lactic acid and not the lactate anion has antimicrobial and immunomodulatory activities, with similar properties reported for the L- and D-lactic acid isomers [3,27]. Unlike the lactate anion, protonated lactic acid is membrane-permeant and likely enters cells without using monocarboxylate transporters or the GPR81 receptor that binds lactate [28,29]. Upon entry, lactic acid acidifies the cytosol of most bacteria interfering with intracellular function and leading to cell death [30], and also acts by permeabilising bacterial membranes [31]. To maximise the antibacterial properties of lactic acid, lactobacillus-based probiotics would need to acidify the vagina to pH $\leq$ 3.9 to achieve a condition where the protonated form of lactic acid dominates.

5. Lactic acid is a major antimicrobial factor produced by lactobacilli

Until recently the prevailing view was that H_2O_2 is the major antimicrobial factor produced by lactobacilli [32,33]. Previous studies with H_2O_2 were performed under aerobic conditions despite the conditions in the vagina being hypoxic [32,33]. A dominant antimicrobial role for H_2O_2 lacks plausibility due to several recent findings. Under the hypoxic conditions found in the vagina, lactobacilli produce little or no H_2O_2 [34]. Hydrogen peroxide is inactivated by the powerful antioxidant effect of cervicovaginal fluid and semen [35]. Physiological concentrations of H_2O_2 are not bactericidal against BV-associated bacteria and, as exogenous H_2O_2 is added, it adversely affects the viability of vaginal lactobacilli more than BV-associated bacteria [35]. In contrast to H_2O_2, lactic acid at physiological concentrations (e.g. 110 mM) even at pH 4.5 mediates a potent 10^6-fold decrease in the viability of 17 different BV-associated bacteria while not affecting the viability of four vaginal *Lactobacillus* spp. in vitro [3]. Notably the antimicrobial activity of lactic acid is orders of magnitude greater than media acidified to pH 4.5 with HCl alone, or with acetic acid, and the bactericidal activity is mediated by the protonated form of lactic acid, not the lactate anion [3]. In addition, in close to ex vivo conditions, lactic acid alone, without bacteriocins, is effective against BV-microbes in BV secretions (Kevin DeLong, unpublished data).

Lactic acid and not H_2O_2 is active against bacterial STIs. Lactic acid production by *L. crispatus* and *L. gasseri*, and not H_2O_2, inactivates *Chlamydia trachomatis* [4,36] and *Neisseria gonorrhoeae* [37] as well as *Escherichia coli* in vitro [38]. Lactic acid, produced by *L. crispatus*, targets the growth of bacteria in living tissue, as demonstrated by inhibition of *N. gonorrhoeae* and *Gardnerella vaginalis*, in a porcine vaginal mucosa model [39]. These in vitro and ex vivo studies suggest that lactic acid either delivered directly or by a probiotic strain has the potential to maintain vaginal eubiosis or to reverse dysbiosis and protect against bacterial STIs. The early clinical studies, showing that women with H_2O_2 producing lactobacilli are at reduced risk of dysbiotic microbiota, indirectly suggest that this association might arise from vaginal microbiota dominated by *Lactobacillus* spp., such as *L. crispatus*, that can produce H_2O_2 under aerobic conditions [4].

Other urogenital opportunistic pathogens include Group B *Streptococcus* (GBS) and *Candida albicans* that cause neonatal morbidity and mortality [40] and vulvovaginal candidiasis (VVC) [41], respectively. While GBS are acid-tolerant and produce lactic acid as a virulence factor [42], the concentration of lactic acid produced is lower compared to lactobacilli grown under the same culture conditions [43]. Lactobacilli inhibit GBS in vitro [43–45], and this inhibition is associated with the production of lactic acid and a decrease in pH, implicating the protonated form of lactic acid [43,45]. However, whether lactic acid produced by commensal or lactobacillus-based probiotics has a role in reducing urogenital colonisation by GBS in vivo is unclear [43,46].

The role of lactobacilli as a barrier to preventing VVC due to *C. albicans* or *Candida glabrata* is controversial. Based largely on in vitro studies, it has been proposed that lactobacilli have a role in preventing VVC by either producing antimicrobial factors or by competing with *Candida* spp. for adhesion sites on the mucosa [47]. In this regard, there is evidence that the growth of *C. albicans* is inhibited by lactic acid produced by lactobacilli at low pH [48] or is not inhibited [45]. Vaginal *Candida* spp. demonstrate different levels of acid tolerance and adapt to low acidity through several mechanisms, including high plasma membrane proton pump activity [49]. Thus, the ability of lactic acid to inhibit *Candida* growth may depend on the level of acid tolerance of an individual species. Despite these in vitro data, epidemiological studies do not support a protective role for vaginal lactobacilli against VVC. Colonisation of the vagina with *Candida* spp. is more common in women with lactobacillus-dominated vaginal microbiota compared to women with BV [25]. In addition, a prospective study showed that vaginal lactobacilli does not protect against VVC, while BV affords protection [50]. Despite these observations, lactobacillus-based probiotics are being pursued as an adjunct to conventional antimicrobial treatment for recurrent VVC [51,52].

6. HIV virucidal activity of lactic acid

Women colonised vaginally with lactobacillus-dominated microbiota are less likely to acquire HIV from their male partners [53]. In addition, reduced viral shedding into the lower FRT is observed in HIV-infected women with lactobacillus-dominated microbiota [24,54,55] that could protect against the sexual transmission of HIV to their male partners and to vaginally delivered neonates. We have shown that physiological concentrations of lactic acid have broad-spectrum HIV virucidal activity that is dramatically more rapid and potent than media acidified to the same pH with HCl

or with acetic acid [27]. Both the D- and L-lactic acid isomers demonstrate similar HIV-1 virucidal activity at 1% (w/w) although L-lactic acid is 17-fold more potent than D-lactic acid at the threshold concentration of 0.3% (w/w) against HIV_{Ba-L} [27]. Similar to its bactericidal activity, the HIV virucidal activity of lactic acid is mediated by the protonated form [27].

The virucidal activity of lactic acid is observed in the presence of genital secretions. Potent HIV-1 virucidal activity is maintained in the presence of 50% cervicovaginal secretions, and 0.75% seminal plasma (SP) at a final concentration of 1% (w/w) L-lactic acid [27]. However, virucidal activity in SP is attenuated at lower lactic acid concentrations due to the strong buffering effect of semen that increases pH [27]. A mixture of vaginal microbiota organic acids (i.e. acetic acid and other SCFAs as well as succinic acid) normally present in the context of BV, when tested at a pH typical of BV (i.e. pH 5.0), fails to inactivate HIV-1 [56]. In contrast, HIV-1 is potently inactivated when a mixture of vaginal microbiota organic acids (including lactic acid), present when lactobacilli dominate, is tested at a pH typical of eubiosis (i.e. pH 3.8) [56]. The lack of HIV-1 virucidal activity of the acids under BV conditions is a combination of the lower inherent virucidal potency of SCFAs and succinic acid compared to lactic acid and diminished levels of the active protonated forms of SCFAs and succinic acid at pH values above their pKa [56].

HIV-1 inactivation by lactic acid is irreversible [27], but does not lead to disintegration of HIV-1 or loss of the gp120 surface protein from the virion [57]. Our ongoing studies suggest that the mechanism of HIV-1 inactivation is likely multifactorial, potentially either directly affecting the functioning of viral surface and fusion proteins and/or, indirectly, by altering the integrity of the lipid envelope as well as negating the functioning of viral proteins encased within the viral core (Aldunate, unpublished data). Generation of HIV with reduced susceptibility to lactic acid will be important to dissect the most prominent inhibitory mechanisms of this vaginal organic acid.

HIV transmission can occur through cell-free and cell-associated virus that is either shed into the vagina of HIV-infected women or deposited by semen that neutralises the acidity in the vaginal tract, thereby transiently negating the pH-dependent virucidal activity of lactic acid [27,58]. Lymphocytes, monocytes and macrophages, permissive to HIV infection, are rapidly immobilised by pH less than 5.8 and killed when the pH drops below 5.5 [59]. Thus, in HIV-infected women, acidification of the vagina by lactic acid produced by lactobacilli would be expected to reduce the load of both cell-free and cell-associated HIV, thereby decreasing the risk of female-to-male HIV transmission. Furthermore, it has been proposed that cell-associated HIV may be more likely to establish infection compared to cell-free virus in the context of male-to-female HIV transmission [58].

Acidified cervicovaginal mucus (CVM) from lactobacillus-dominated vaginal microbiota immobilises HIV-1, in contrast to neutralised CVM [57]. The increased D/L lactic acid ratio is implicated as a surrogate marker for a factor produced by *L. crispatus* that hinders and traps HIV-1 particles [57,60,61]. Immobilisation by this factor could impair the ability of the virus to reach HIV target cells in the vaginal mucosa, and indicates that *L. crispatus*, which is also the most potent vaginal acidifier [5], may represent a superior probiotic species compared to other vaginal lactobacilli.

7. Inhibitory effects of lactic acid on HSV

BV is an independent predictor of herpes simplex virus 2 (HSV-2) genital shedding [62], while women with vaginal microbiota dominated by lactobacilli are less likely to be infected with HSV-2 [24]. These observations suggest that vaginal lactobacilli and their products may have a role in inactivating this viral STI. Lactobacilli inhibit HSV-2 through virucidal-dependent and independent mechanisms, the latter involving inhibition of viral entry and replication that is linked to either the adhesion capacity of the lactobacillus strain or through the direct effects of lactic acid [63,64]. The HSV-2 inhibitory activity of lactic acid correlates with acidic pH, again indicating that it is the protonated form of lactic acid that is mediating the effect [64,65]. Lactic acid also has HSV-1 virucidal activity at pH < 4.5 [65]. Distinct from previous studies with HSV-2 and HIV-1 [27,65], we observed similar HSV-2 virucidal potency when medium was acidified to the same pH with lactic acid and HCl. Reasons for this disparity might be explained by the experimental systems employed, which performed HSV-2 inactivation in HCl-acidified phosphate-buffered saline [65] compared to clarified HCl-acidified culture medium with an osmolality approaching that of cervicovaginal fluid for our studies on HSV-2 and HIV [27]. Since lactic acid can inhibit HSV-1 and HSV-2 in vitro, this suggests that either a lactic-acid-producing probiotic or direct vaginal delivery of lactic acid might reduce HSV-1 and HSV-2 levels in the lower FRT.

8. Immunomodulatory properties of lactic acid

Lactobacilli are generally associated with a non-inflammatory vaginal environment [66–68], while the presence of polymicrobial vaginal microbiota (e.g. BV) is linked to a pro-inflammatory milieu that is associated with increased acquisition of HIV and other STIs [69]. Moreover, young African women with *L. crispatus*-dominated microbiota did not acquire HIV, in contrast to women harbouring *L. iners* and diverse bacterial communities dominated by anaerobes [70]. Proteomics analyses of cervicovaginal fluid have linked increased inflammatory cytokines and vaginal bacterial diversity with changes in mucin proteins, proteases, protease inhibitors and mucosal barrier proteins [71–73]. Changes in the mucosal barrier proteins include a decrease in the cornified envelope factors involucrin (INV) and small proline-rich protein 1A (SPR1A) involved in wound healing repair [73]. This observation is consistent with the ability of soluble factors produced by *G. vaginalis*, but not *L. iners*, to inhibit wound healing, as determined in cervical cell monolayers [73]. The higher levels of proinflammatory mediators promoting recruitment and activation of HIV target cells, as

reported by Gosmann et al. [70], and disruption of vaginal epithelium integrity that would promote pathogen invasion, provide plausible mechanisms for increased susceptibility to HIV and other STIs in women with vaginal dysbiosis compared to those with eubiosis.

The cervicovaginal epithelium provides an immunological as well as a physical barrier to pathogens in the lower FRT. In vitro studies show that certain strains of lactobacilli dampen pro-inflammatory responses elicited by Toll-like receptor (TLR) agonists from cervicovaginal epithelial cells [74–78]. Our studies indicate that these immunomodulatory effects may in part be ascribed to lactic acid [79,80]. We have shown a dramatic increase in the anti-inflammatory cytokine, interleukin-1 receptor antagonist (IL-1RA), without a significant increase in the pro-inflammatory IL-1β, from human cervicovaginal epithelial cells cultured in transwells, when treated apically with lactic acid under physiological conditions that are tolerated by these cells (0.3% lactic acid, pH 3.9) [79,80]. Furthermore, protonated lactic acid treatment of cervicovaginal epithelial cells dampens the production of pro-inflammatory cytokines and chemokines elicited by the TLR 1/2 agonist Pam3CSK4 that mimics the viral and bacterial pathogen-associated molecular patterns (PAMPs) of HIV (i.e. gp120) and BV-associated bacteria [79–82]. The anti-inflammatory effect of lactic acid is distinct from low pH alone, since acidifying the media to the same pH with HCl does not cause the same effects [79,80]. Of note, similar immune modulatory responses were observed for both the L- and D-lactic acid isomers [79,80].

Our data are distinct from other studies reporting pro-inflammatory effects of lactic acid on immune and vaginal epithelial cells [83,84], which appears to be at odds with the observed non-inflammatory effects of vaginal lactobacilli [74–78]. This apparent discrepancy may be explained by the use of lactic acid concentrations that are cytotoxic under the conditions employed in vitro or that lactic acid is not tested at physiological concentrations and low pH observed in women with lactobacillus-dominated microbiota, where the protonated lactic acid and not the lactate anion predominates [19].

While our investigations have not revealed major differences between the immunomodulatory properties of the two lactic acid isomers on cervicovaginal epithelial cells, two studies have found differences. The first reported that treatment of vaginal epithelial cells with D-lactic acid, but not L-lactic acid, blocks chlamydia infection through an effect on the epithelial cells that is pH-dependent and that is distinct from bactericidal activity [85]. The second study showed that D-lactic acid has a role in modulating the ability of L-lactic acid to elicit the production of the extracellular matrix metalloproteinase inducer (EMMPRIN) from vaginal epithelial cells [23]. EMMPRIN has two functions. It is an essential cofactor for monocarboxylate transporter-1 (MCT-1) that transports lactate anions out of the cell, thereby preventing cell death that might result from increased cytosolic acidity [23]. EMMPRIN also induces the production of matrix metalloproteinase-8 (MMP-8) that is implicated in degrading the cervical plug, and therefore EMMPRIN upregulation might eventually lead to free passage of bacteria from the lower to the upper FRT, leading to infection and preterm birth in pregnant women [86].

L. crispatus, which is more protective than *L. iners* against vaginal dysbiosis [14], produces more D-vs. L-lactic acid, while *L. iners* produces no D-lactic acid [23]. L-lactic acid levels and the ratio of L-to D-lactic acid were reported as being elevated in women with cytolytic vaginosis, although this did not correlate with a significant increase in EMMPRIN and MMP-8 [87]. These studies have led to the notion that D-lactic acid might have superior protective properties compared to L-lactic acid, although the lactic acid isomers have similar HIV-1 virucidal and bactericidal activity against BV-associated bacteria [3,27]. Transcriptional analyses of cervicovaginal cells treated with D-versus L-lactic acid and different ratios of these isomers, as well as delineating the effects of the lactate anion and the uncharged lactic acid forms, will be needed to establish whether differences exist in their immunomodulatory and signalling properties on cervicovaginal epithelial cells. These studies could inform the selection of lactobacillus strains as probiotics to address particular aspects of vaginal health, depending on the ratio of lactic acid isomers produced and the level of acidification achieved.

9. Glycogen is associated with increased levels of lactobacilli and lactic acid in the vagina

Oestrogen rises during puberty and coincides with an increased glycogen deposition in vaginal epithelial cells as well as colonisation of the lower FRT with lactobacilli [88] although next-generation sequencing studies report that microbiomes dominated by *Lactobacillus* spp. are present before menarche during the early to middle stages of puberty [89]. Free glycogen could be released from vaginal epithelial cells through detachment mediated by MMP-8 and hyaluronidase-1, as well as by cell lysis through the action of high concentrations of lactic acid and membrane-degrading cytolysins produced by lactobacilli [87,90]. Women have higher levels of vaginal glycogen than female macaques and other primates and mammals [73,91], that likely explains the lack of lactobacillus dominance, and less acidic vaginal pH in non-human species. Lactobacillus levels strongly correlate with low vaginal pH and glycogen levels [92,93], with higher levels of glycogen corresponding to a higher load of *L. crispatus* and *L. jensenii* but not *L. iners* [92].

Vaginal lactobacilli do not directly metabolise glycogen [94]; rather, they metabolise the human α-amylase breakdown products, glucose and maltose [95,96] through fermentation that produces lactic acid. Lactic acid is also an energy source for some BV-associated bacteria further depleting lactic acid levels [15,18]. Glycogen increases during pregnancy and decreases in menopause coincident with the rise and fall of oestrogen [97]. Median levels of free glycogen in genital fluid of premenopausal and postmenopausal women are 0.065 and 0.002 μg per microliter, respectively and positively correlate with lactobacillus levels [98]. In addition, in pre-menopausal women, there is a strong negative correlation between free glycogen levels in genital fluid and vaginal pH [93]. Glycogen

is also an energy source for BV-associated bacteria [99] and BV is linked to depleted levels of free glycogen levels in the vagina [92,96]. It has been proposed that higher loads of BV-associated bacteria consume glycogen more rapidly compared to the lower-loaded lactobacillus-dominated microbiota, and that this glycogen depletion likely starves lactobacilli of an energy source. Given the role of glycogen as an energy source for lactobacilli, treating women with vaginal glycogen might enhance colonisation with lactobacilli and promote vaginal health [92] although whether glycogen would also give BV-associated bacteria a growth advantage is unclear. However, Gynoflor, a combination of live lactobacilli and low-dose oestriol, that can promote glycogen production, has been reported to normalise vaginal microbiota in women with vaginal infections, including BV following standard anti-infective therapy [100].

10. Use of probiotics for treating and preventing bacterial vaginosis

Selection of lactobacillus-based probiotics has largely been centred on their in vitro characteristics, including production of antimicrobial factors (H_2O_2, bacteriocins), ability to adhere to vaginal epithelial cells and antimicrobial activity against pathogens. Alternative desirable criteria for probiotics include their ability to strongly acidify the vagina with lactic acid so as to have positive effects on the host, such as immunomodulatory activities, the ability to survive and outcompete other bacteria, co-aggregation and susceptibility to antibiotics, apart from those used for treating BV [101–104]. Several lactobacillus strains and species (e.g. *Lactobacillus rhamnosus* GR-1, *Lactobacillus reuteri* RC-14, *Lactobacillus acidophilus*, *L. brevis*, *Lactobacillus plantarum*, *L. gasseri*, *L. crispatus*, *Lactobacillus fermentum*) have been evaluated as vaginal probiotics for treating or preventing BV [101,103,105]. These lactobacillus-based probiotics have been delivered directly to the vaginal tract by capsules, applicators and tampons loaded with freeze-dried lactobacilli or orally with the premise that the oral probiotic can be transferred from the gastrointestinal tract through the rectum to the lower FRT.

Lactobacillus-based probiotics, formulated with single or multiple strains, have been evaluated in several randomised clinical trials for BV treatment, alone or as adjuncts to antibiotic therapy (either metronidazole or clindamycin), and for the prevention of BV recurrence following antibiotic treatment [106–128]. Probiotics are well-tolerated and there are increasing reports of positive effects of probiotics for BV cure or reduced recurrence. However, there are also some well-designed studies that have failed to demonstrate significant effects [105,117]. Many candidate probiotic lactobacillus strains are not adapted to grow and survive in the lower FRT. Conversely, probiotics based on *Lactobacillus* spp. similar to those found in the lower FRT (i.e. LACTIN-V, comprising the *L. crispatus* CTV-05 strain), may not stably colonise the vaginal mucosa due to competition with endogenous *L. crispatus*, and colonisation can be negatively impacted by the microbial exchange that occurs during sexual activity [115,129,130]. The lack of consistent clinical benefit observed in some, but not all, probiotic trials is likely to be attributable to a number of factors, including the use of differing probiotic lactobacillus strains including non-vaginal species, their mode of delivery, varied trial methodologies, distinct clinical populations and differing and often non-standardized BV endpoints [101,105,109,131–133].

Adequately powered clinical trials are needed with well-characterised probiotics, in defined study populations with clear inclusion criteria and trial methodology, standardised endpoints and an adequate duration of follow-up in which to evaluate a clinically meaningful treatment effect. In this regard, LACTIN-V delivered via a vaginal applicator is currently being evaluated in an NIAID-sponsored multicentre phase II-b randomised double-blind placebo-controlled trial in the USA to assess its safety and efficacy in preventing BV recurrence compared to placebo following a 5-day course with metronidazole gel (https://clinicaltrials.gov/ct2/show/study/NCT02766023). Study subjects are non-pregnant women of reproductive age who will receive LACTIN-V (2×10^9 cfu/dose) applied vaginally for 5 days, then twice weekly for 10 weeks. Primary endpoint is the reduction of the 12-week incidence of BV recurrence compared to placebo. Secondary objectives include colonisation of LACTIN-V, its fluctuation over 12 weeks in response to menses and coitus as well as long-term LACTIN-V at 24 weeks.

11. Intravaginal delivery of lactic acid for treating and preventing BV

The use of lactic acid, which can be considered as a "postbiotic" metabolite with favourable antimicrobial and immunomodulatory properties, presents an alternative approach that circumvents the regulatory and colonisation stability challenges presented by probiotics. Given that the production of 'lactic acid' is greater in most vaginal microbiota associated with eubiosis [5], lactic acid may represent a strategy to restore vaginal microbiota function independently of concerns surrounding introduction of probiotics that may be foreign and present other adverse effects to women.

Topical lactic-acid-containing gels have been evaluated in several clinical trials for BV treatment. This includes Lactacyd vaginal gel (LVG), containing 225 mg lactic acid and 5 g of the prebiotic glycogen to promote lactobacillus growth [134–137]. LVG was assessed for efficacy and tolerability in 90 Filipino women when given as an adjunct to metronidazole for BV treatment in a multicentre, open-labelled, controlled, randomised three-arm comparative study [136]. The three study arms were oral metronidazole (500 mg twice a day) for 7 days, LVG at bedtime for 7 days, and the combination of metronidazole and LVG. The study cohort comprised women >18 years of age with BV defined by the Amsel criteria. LVG was well-tolerated compared to metronidazole antibiotic therapy. While the combination of metronidazole and LVG showed some evidence of being superior for treating BV compared to LVG or metronidazole alone, significant changes were only reported for two of the four Amsel criteria. The

combination of metronidazole and LVG was superior to metronidazole alone in reducing the proportion of women with a whiff positive test at day 14 compared to metronidazole ($p = 0.0410$) as well as LVG ($p = 0.0134$) alone [136]. A significant decrease in women with clue cell positivity was observed in all treatment arms at day 3 ($p < 0.001$). While vaginal pH decreased for all three groups ($p < 0.001$), there was no significant difference in the percentage of women with pH < 4.7 among the treatment groups at day 14. An earlier open label study in 54 women of reproductive age with BV and 42 control women found that daily treatment with 5 ml of lactic acid gel (pH 3.5) for 7 days was as effective as oral metronidazole for the treatment of BV based on the absence of three criteria (i.e. positive amine test, clue cells in vaginal fluid, and pH $\geq$ 5.0) at 1 week after start of treatment [134].

A third study evaluated lactic acid gel (Lactal) for treatment and the prevention of BV recurrence [135]. Women with a history of recurrent BV were treated daily with lactic acid gel for 7 days, after which they entered a double blind clinical trial where they were randomised to intermittent treatment by intravaginal insertion of 5 ml of lactic acid gel or placebo gel once daily every evening for 3 consecutive days immediately following menstruation. BV, as defined by the Amsel criteria, resolved in 88% (15/17) of women in the treatment group compared to 10% (1/10) in the placebo group after 6 months ($p < 0.001$) [135]. Lactobacilli were also shown to recolonise the lower FRT in 83% of the treated women compared to 10% in the placebo group [135]. Intermittent application of vaginal Lactal was found to be free of side effects [135]. Recolonisation by lactobacilli after only a few days was reported in a smaller study of 10 pregnant women who were intermittently treated with 5 ml of the same lactic acid gel (pH 3.8) [138].

Collectively, these trials indicate that intravaginally delivered lactic acid may have a positive effect on resolving symptomatic BV. Gels containing lactic acid are available over the counter in some countries to treat symptoms associated with BV [139]. However, these gels are not approved for BV treatment by regulatory authorities such as the Center for Disease Control in the U.S. Given the promising results from the abovementioned trials, further investigations in a larger randomised, double-blind placebo controlled trials are warranted to provide definitive data for the use of lactic acid gels to be adopted in mainstream clinical practice for BV.

Alternative strategies have been developed for sustained release delivery of lactic acid rather than daily dosing. These include an oligomeric lactic acid (OMLA) pessary that can be administered once or twice a week [140], poly(ethylene glycol) (PEG) nanocarrier-based degradable hydrogels with lactic acid covalently linked or passively trapped [141] and a lactic-acid-releasing intravaginal ring [142]. OMLA forms a mucoadhesive gel when it comes in contact with vaginal mucous, and lactic acid is subsequently hydrolysed and released from the OMLA [140]. In a randomised open label study, the OMLA pessary was reported to be safe and efficacious in clearing BV, as defined by less than three Amsel's criteria. In the three arm efficacy evaluation ($n = 105$), the one-week BV clearance ratios in women administering the pessary once a week was 70.6% ($p < 0.001$ vs. untreated control), compared to 80% ($p < 0.001$ vs. untreated control) for women using the pessary twice a week [140]. A reservoir ring, releasing racemic DL-lactic acid, was evaluated in a phase I clinical trial in six healthy premenopausal women, demonstrating that it was safe as determined by colposcopic monitoring for visible cervicovaginal mucosal changes [142]. Further studies with lactic acid gels and sustained delivery vehicles are needed to determine whether lactic acid can normalise the vaginal microbiota and reverse the proinflammatory milieu in women with vaginal dysbiosis. While intermittent delivery of lactic acid alone may prove to be effective in the treatment of BV and to prevent BV recurrence [135,140], it is likely to be most effective when used in combination with antimicrobials for the treatment of BV [136].

12. Conclusions

Probiotic lactobacilli that produce lactic acid with the desired D-to L-lactic acid ratios may be considered "lactic acid" factories that can deliver sustained amounts of this metabolite to the cervicovaginal mucosa. However, challenges remain with regard to advancement of probiotics through regulatory pathways as "biotherapeutics" that are adopted in mainstream clinical practice. Other challenges include achieving stable colonisation of the desired probiotic in the face of intrinsic and extrinsic vaginal disturbances, as well as identifying the ideal strains for women, many of whom differ with respect to their "normal microbiota", perhaps requiring a personalised medicine approach. The multifaceted antiviral, antibacterial and immunomodulatory properties of lactic acid in the context of the lower FRT warrant further investigation. Studies are needed to delineate the activities of the protonated acid versus the lactate anion, as well as the specific properties of the D and L-lactic acid isomers, on epithelial and immune cells relevant to the lower FRT for selection of the most optimum probiotic strain. Several clinical trials show a positive signal for the ability of lactic acid to re-establish vaginal eubiosis and relieve symptoms in women with BV. Future work will be needed to evaluate lactic acid containing gels, pessaries and rings in robust and sufficiently powered trials that use standardised endpoints for BV. It will also be essential to determine their impact on the vaginal microbiome, e.g. using next-generation sequencing technologies, and to evaluate their influence on immune mediators in the lower FRT, if we are to advance their use for the treatment and prevention of BV as either bona fide alternatives or adjuncts to antimicrobial therapy.

Conflict of interest

G.T. is a co-inventor on patent applications claiming the anti-inflammatory effects of lactic acid (Patent Application AU201501042 and US Patent Application 20150306053).

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