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Lactobacillus iners, the unusual suspect

Mario Vaneechoutte

Laboratory of Bacteriology Research, Faculty of Medicine and Health Sciences, Ghent University, De Pintelaan 185, Medical Research Building 2, Ghent University Hospital, B-9000 Gent, Belgium

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Abstract

Lactobacillus iners is an unusual *Lactobacillus* species which does not grow on de Man Rogosa Sharpe agar, does not produce D-lactic acid, and only limited amounts of hydrogen peroxide. Its production of inerolysin, a cytotoxin, is also unusual for a lactobacillus. Epidemiological studies point to an ambiguous role for this species, which is quite often recovered in high numbers from vaginal dysbiosis and offers limited protection against vaginal dysbiosis and, subsequently, against sexually transmitted infections and adverse pregnancy outcome. Several data indicate that *L. iners* might even contribute to the onset and maintenance of vaginal dysbiosis and be a risk factor for adverse pregnancy outcome.

Keywords: *Lactobacillus iners*; Vaginal dysbiosis; Inerolysin; Adverse pregnancy outcome

1. Introduction

In cases of vaginal dysbiosis, urogenital infection and sexually transmitted infection (STI), the usual suspects are represented by several viruses (e.g., HIV, HPV and HSV), bacteria (e.g., *Atopobium vaginae*, *Chlamydia trachomatis*, *Escherichia coli, Gardnerella vaginalis*, *Mollicutes*, *Neisseria gonorrhoeae*, *Streptococcus agalactiae* and *Treponema pallidum*), unicellular eukaryotes (e.g., *Trichomonas vaginalis*) and fungi (e.g., *Candida albicans*). However, with regard to the etiology of vaginal dysbiosis, *Lactobacillus iners*, besides being an unusual lactobacillus, is an unusual suspect [1].

L. iners was first described in 1999 [2] in vaginal and urinary tract samples. At the same time, Antonio et al. [3] cultured a *Lactobacillus* species, designated 'L. 1086V', of which only 9% of the isolates produced H_2O_2, compared to 95 and 94% of the *L. crispatus* and *L. jensenii* isolates, respectively. This species was the third most prevalent species, present in 44 (15%) of the 302 subjects, with 36% of the 44 'L. 1086V'-colonized women presenting with bacterial vaginosis. This species was later shown to correspond to *L. iners* [Sharon Hillier, pers. comm.]. Another early study pointing to the high prevalence of *L. iners* was that of Tärnberg et al. [4].

2. Epidemiological data on the role of *L. iners* in the vaginal econiche

2.1. L. iners *predominance may vary between different populations*

The vaginal microbial community (VMC) is typically characterized by the predominance of one or few species of lactobacilli [e.g., Refs. [3–8]]. Since the advent of metagenome sequencing technology, *L. iners* is considered to be the most prevalent lactobacillus in the vaginal econiche [e.g., Refs. [9,10]]. It should be noticed that several of the studies that support the predominance of *L. iners* included numerous black women [e.g., Refs. [9,11–15]] or analyzed the VMC of postmenopausal women [16,17].

On the other hand, other studies reported the predominant presence of *L. iners* in three out of 24 Caucasian subjects with established low Nugent scores [5]. In a Brazilian study,

E-mail address: mario.vaneechoutte@ugent.be.

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L. iners was predominant in women with vaginal eubiosis, as well in women with vaginal dysbiosis and with vulvovaginal candidiasis [18]. The VMC of two out of five Chinese women, for whom Nugent scoring was carried out but not reported, was shown to be dominated by *L. iners*, and three other were dominated by *L. crispatus* [8]. More than 60% of 80 Korean women were found to have *L. iners* as the predominant vaginal species, but Nugent scores were not reported [19]. Zhou et al. [20] found equal numbers of Caucasian and black women colonized predominantly by *L. iners*, but again Nugent score data were lacking. In a Dutch population, *L. iners* was present in significantly higher loads than *L. crispatus* in women with bacterial vaginosis (BV), but a higher load of *L. iners* compared to *L. crispatus* was established, also in nearly half of the women without BV [21].

Our own data (from Belgium) [6,7,22–24], and those from a British study [25] in pregnant women, indicate that, in case of a low Nugent score, there is a predominance of *L. crispatus* or, less frequently, of *L. jensenii*.

Recently, Beamer et al. [26] correctly pointed out that several of the studies that reported a higher prevalence of *L. iners* in asymptomatic black women than in asymptomatic white women incorrectly considered all women without symptoms as 'healthy', whereas it is well-established that up to half of women with BV are asymptomatic as well. Indeed, since the prevalence of asymptomatic BV is higher among black women, and the prevalence and frequently also the sample load of *L. iners* is high in (asymptomatic) BV, the prevalence of *L. iners* among 'healthy' women will be high when asymptomatic women with BV are included in this category. After excluding asymptomatic BV and the presence of STI, Beamer et al. [26] found limited differences between the vaginal microbiomes of white and black women. They attributed the higher prevalence of *L. iners* among black women to the higher prevalence of BV among them, which in turn was linked to social and behavioral factors, or possibly to higher incidence of STIs among their male partners. The higher prevalence of BV (and thus possibly of *L. iners*) among black women may also be explained by a different genetic background, since Ness et al. [27] could not find behavioral factors as the explanation for the higher prevalence of BV in black women. On the other hand, the latter authors did not include, e.g., concurrent partnership as one of the parameters in their study, which might leave open the possibility that the differing risk for BV – and the prevalence of *L. iners* – is still explainable as grounded in ethnic and not purely racial differences.

In summary, it remains difficult to decide whether a higher number of *L. iners* among black women is due to more BV, itself explained by other reasons, or whether more BV in black women is due to stronger colonization of less protective *L. iners*, which raises the question as to why a less protective species such as *L. iners* may be more prevalent among black women. Most importantly, the association between *L. iners* and (asymptomatic) BV remains, after correcting for possible racial differences, although the species is also prevalent in women with vaginal eubiosis.

Future studies should define the type of VMC on the basis of microscopy instead of on the basis of the absence/presence of signs/symptoms and they must document the presence of symptoms or signs for each participant, and clearly indicate the race/ethnicity of each individual.

2.2. Epidemiological data indicate an association of L. iners with vaginal dysbiosis

Several authors have concluded that *L. iners* is simply the most prevalent commensal vaginal lactobacillus, and the species has even been considered as a vaginal probiotic [28].

However, already in the study of Antonio et al. [3], an association between *L. iners* and vaginal dysbiosis was established, since 36% of 44 *L. iners*-positive women had BV compared to only 9% (9/96) and 7% (5/69) of women with, respectively, *L. crispatus* and *L. jensenii*. The presence of this lactobacillus in large numbers during vaginal dysbiosis was also shown by cloning studies [7,29,30].

Shipitsyna et al. [31] reported high loads of *L. crispatus* and *L. iners* in 79 women with low Nugent scores, versus depletion of *L. crispatus* but a predominance of *L. iners* and *G. vaginalis* in 11 women with intermediate Nugent scores, versus depletion of lactobacilli in 73 women with high Nugent scores. We reported comparable findings [22], except that we also found high numbers of *L. iners* in several of the women with BV. Wertz et al. [12] found *L. iners* to be the only lactobacilli in women with BV; moreover, *L. iners* was the dominant species in the two women with BV.

Tamrakar et al. [32] suggested that the presence of *L. iners* may be correlated with vaginal colonization of Japanese pregnant women by BV-related bacteria, such as BVAB2, *Megasphaera* and *Leptotrichia*. All recent metagenome sequencing studies also indicated the presence of *L. iners* in conditions of vaginal dysbiosis, as recently reviewed [33,34]. For example, Srinivasan et al. [35] found that *L. iners* was present in 86.3% of women with BV, accounting for 17.9% of the reads.

Intriguingly, the seminal study of Swidsinski et al. [36] indicated the presence of variable numbers of lactobacilli in *G. vaginalis*-dominated vaginal biofilms using a *Lactobacillus* genus-specific probe. Similar studies with *L. iners*-specific probes should confirm the strong suspicion that it is predominantly *L. iners* that is involved.

2.3. L. iners is incompatible with L. crispatus

In our studies, *L. crispatus* and *L. iners* were almost mutually exclusive [7,22,24], and in one study, we also concluded that *L. gasseri* and *L. iners* were mutually exclusive [22]. Both cloning-based studies of Fredricks et al. [29] and Verhelst et al. [7] found no *L. crispatus* at all in women with BV, but frequently *L. iners*. It should be mentioned that Fredericks et al. [29] found that several women with normal VMC carried both *L. crispatus* and *L. iners*. The study by Zhou et al. [20] reported ten women with combined *L. crispatus* and *L. iners* clones, but similar studies by the same group [37,38] established almost complete mutual

exclusivity between *L. iners* and *L. crispatus* (the latter eventually accompanied by *L. gasseri*, *L. jensenii* and/or *L. vaginalis*). Although Zhou et al. [37] reported 'healthy women' with almost exclusively *L. iners* clones, the vaginal health status of the women was based only on self-reported absence of complaints, whereas the co-occurrence of e.g. *Megasphaera* in these women supports the suspicion that they had asymptomatic BV.

Srinivasan et al. [35] found that *L. crispatus* had a strong positive correlation with *L. jensenii* and *L. gasseri*, but was negatively correlated with *L. iners*. Also, Dols et al. [39] concluded that there was a virtually complete negative correlation between *L. crispatus* and *L. iners* among BV-negative women. Also, Wertz et al. [12] found mutual exclusion of *L. crispatus* and *L. iners*.

Yamamoto et al. [38] hypothesized that frequent occurrence of near monocultures of *L. crispatus* and *L. iners* indicates their ability to outcompete other bacteria, although this stance is not supported by the observation that *L. iners* easily co-exists with numerous BV-associated species in large numbers. France et al. [40] hypothesize that the trend toward mutual incompatibility that is observed is due to strong overlap in the econiche between *L. iners* and *L. crispatus*. However, both species may well have adapted to very different econiches, since the dynamics of the vaginal habitat make possible the existence, within the same individual, of very different econiches at different time points. For example, *L. crispatus* may dominate during the menstrual cycle outside the menses, whereas *L. iners* is present predominantly under conditions of vaginal dysbiosis such as during menses, after coitus, and in cases of bacterial vaginosis, as was observed by Lopes et al. [24] and Jespers et al. [41].

Indeed, *L. iners* numbers have been shown to vary during the menstruation cycle [24,43,44], replacing *L. crispatus* during menses, frequently in conjunction with *G. vaginalis* and/or *A. vaginae* [22,41], and resulting in high Nugent scores, as was also apparent from the study of Brotman et al. [45]. Interestingly, high levels of glycogen have been reported to correspond to higher levels of *L. crispatus* and *L. jensenii*, but not of *L. iners* [42].

These observations also indicate that the moment of sampling relative to the menstrual cycle is important. Therefore, future studies should preferably be longitudinal, cover several cycles and document sexual activity.

In summary, many studies indicate that *L. iners* rarely co-occurs in large numbers with large numbers of other lactobacilli, possibly because it is outcompeted by the other *Lactobacillus* species, although it may be the sole predominant species, and although it thrives frequently and in large numbers during conditions of vaginal dysbiosis, i.e. at elevated pH and in the company of numerous cells of very different non-*Lactobacillus* species.

2.4. L. iners *offers less protection against vaginal dysbiosis and STI*

The detailed studies by Gajer et al. [44] and Macklaim et al. [46] confirmed previous, preliminary results obtained in pregnant women [23] that an *L. crispatus*-dominated VMC most often undergoes transition into an *L. iners*-dominated or mixed lactobacilli VMC, whereas an *L. iners*-dominated VMC is more likely to undergo transition into a BV-associated VMC. Borgdorff et al. [47] concluded that *L. crispatus*-dominated, and to a lesser extent *L. iners*-dominated, cervicovaginal microbiomes are associated with a lower prevalence of HIV/STIs and a lower likelihood of genital HIV-1 RNA shedding. Nunn et al. [48] present evidence that it is not pH, but D-lactic acid production that is the basic factor in protection against HIV-infection, by increasing the impermeability of the cervicovaginal mucus, which leads to the assumption that *L. iners*, which does not produce D-lactic acid [49], will offer limited protection.

In summary, it is well-established that *L. iners*-dominated VMC offers limited protection against vaginal dysbiosis and STIs.

2.5. L. iners *offers less protection against and may be a risk factor for adverse pregnancy outcome*

Aagaard et al. [50] found that pregnancy consistently strongly promotes the prevalence of lactobacilli, confirming earlier studies [51] that indicated that women are less likely to develop BV during pregnancy, a condition which is also characterized by stronger vaginal acidification, which may be a consequence of higher glycogen levels, which follow in turn from high estradiol levels [34].

However, as outlined above, most studies indicate that, when *L. iners* is predominant, the vaginal microbiome is more likely to shift towards dysbiosis, unlike when *L. crispatus* is dominant, and this is also and, more importantly, the case during pregnancy. Verstraelen et al. [23] established a tenfold-increased risk of conversion to vaginal dysbiosis during the third trimester when *L. gasseri* or *L. iners* dominated the VMC of pregnant women during the first trimester (Relative Risk (RR) 10.41, 95% CI 1.39–78.12, $p = 0.008$), whereas *L. crispatus*-dominated VMC during the first trimester represented a fivefold decreased risk (RR 0.20, 95% CI 0.05–0.89, $p = 0.04$) of conversion to vaginal dysbiosis.

Van de Wijgert and Jespers [52] mention other studies, although underpowered and not taking into account *Candida* colonization, that indicate that women who delivered preterm were more likely to have *L. iners* dominance [53] or (a trend towards) increased vaginal bacterial diversity [54,55]. For example, Petricevic et al. [53] found that *L. iners* was predominantly present in 11 out of 13 (85%) women who delivered preterm, whereas predominant *L. iners* was detected in only 16 out of 98 (16%) women who delivered at term ($p < 0.001$); these authors suggested that the presence of dominating *L. iners* in vaginal smears of healthy women in early pregnancy might be associated with preterm delivery.

Most recently, Kindinger et al. [56] reported that *L. iners* dominance of the VMC at 16 weeks of gestation is a risk factor for preterm birth, as it was significantly associated with both a short cervix (<25 mm, $n = 15$, $P < 0.05$) and preterm birth (<34 weeks, $n = 18$, $P < 0.01$, 69% PPV). In contrast,

L. crispatus dominance was strongly predictive of term birth (n = 127, 98% PPV). Vaginal progesterone administration did not alter vaginal bacterial community structure nor reduce *L. iners*-associated preterm birth (<34 weeks).

Interestingly, Kindinger et al. [56] also reported that cervical shortening and preterm birth were not associated with vaginal dysbiosis (at 16 weeks of gestation).

By bringing together observations that seem at first not directly linked, we speculate that a pathogenic role of *L. iners* in preterm delivery may indeed be likely. First, there is the remark by Donders et al. [57] with regard to the so-called 'intermediate microflora' (Nugent score of 4–6), stating that it has become clear that this is not a transition state from normal to BV, as is implicitly suggested by the designation, but rather, may represent 'partial BV', i.e., a mixture of vaginal micro-areas with BV and zones with predominance of lactobacilli, together with other VMC abnormalities. The *Lactobacillus* species involved might be predominantly *L. iners*, given its status as the predominant lactobacillus in 'BV' and the fact that it is prevalent during transitions of the VMC [e.g., Ref. [58]]. Furthermore, some large, randomized, controlled studies of treatment of BV showed that metronidazole during pregnancy failed to prevent preterm birth in most women with BV (cited by Ref. [57]), and *L. iners*, which was shown to be metronidazole-resistant – like the few other vaginal lactobacilli tested [59], becomes the predominant species after metronidazole treatment [35,60]. And finally, there is the increased inflammation that may be associated with *L. iners* colonization (see below). Although still speculative, different lines of research findings seem to add weight to the notion that it may be *L. iners* colonization during pregnancy, rather than BV, that is associated with increased risk of preterm delivery, as established recently in a more direct manner [56].

2.6. *Other associations with the involvement of* L. iners

Neggers et al. [61] concluded that, among 1521 women (of which 86% were African Americans), increased dietary fat intake was associated with increased risk of BV and severe BV, whereas increased intake of folate, vitamin A and calcium may decrease the risk of severe BV. Possibly of related interest, it is worth mentioning that a Korean study indicated a higher prevalence of *L. iners* in women with high BMI: Women under 49, but not elderly women (age > 49 years) with an *L. iners*-dominated vaginal microbiome had a significant association with obesity (odds ratio [OR], 7.55 [95% confidence interval [CI], 1.18 to 48.2]), compared to women with an *L. crispatus*-dominated vaginal microbiome [62]. The same group characterized the VMC of 70 women with cervical intra-epithelial neoplasia (CIN) and 50 control women, using metagenome analysis, and found that the predominance of *A. vaginae*, *G. vaginalis* and *L. iners* with concomitant paucity of *L. crispatus* in the cervix was associated with CIN risk [63]. Again, it is difficult to assess whether these associations can be ascribed to the presence of *L. iners* or to conditions of vaginal dysbiosis in general.

2.7. L. iners *and recovery from vaginal dysbiosis*

Several authors have suggested that *L. iners* may initiate recovery from vaginal dysbiosis as a result of its resilience. It has been proposed that *L. iners* may become or is a dominant species when the microbiome is in a transitional stage [58], while others found that *L. iners* was predominant in all women who had been treated with metronidazole for BV [35,60], that the concentrations of *L. iners* tended to increase with metronidazole treatment for BV [43] and that *L. iners* was predominant in women after remission from BV [64].

Macklaim et al. [46], comparing the vaginal metatranscriptome between women with and without dysbiosis, found differential expression of 10% of *L. iners* genes. This led the authors to hypothesize that *L. iners* is able to adapt to dysbiosis by modifying its gene expression of metabolism for glycogen and glycerol usage and for cytolysis [46]; that same group suggested elsewhere that *L. iners*, by being the primary lactobacillus present and because of its ability to adapt its gene expression to the conditions in the vagina, may provide a nidus for recovery from BV and restoration of homeostasis [65]. Although such a recovery role for *L. iners* cannot be excluded, the evidence remains circumstantial, and this scenario is somehow counterintuitive with the recurrent observation that colonization with *L. iners* provides less protection against dysbiosis (see 2.5) and with the possible association of *L. iners* with *G. vaginalis*-dominated biofilm (see 2.2).

3. Unusual characteristics of *L. iners*

3.1. L. iners *has unusual culture characteristics*

It is well-known that *L. iners* has been largely overlooked in culture-based studies, especially when media without blood were used. *L. iners* does not grow on de Man Rogosa Sharpe (MRS) agar, that was developed exactly as a medium for selective recovery of lactobacilli. On blood containing agar, *L. iners* grows as small colonies.

Even on blood-containing media, there may be a negative culture bias due to confusion of *L. iners* with *G. vaginalis*. Indeed, when retesting *G. vaginalis* isolates from a previous collection at our lab, and from the collection that was used by Piot et al. [66] to develop their *G. vaginalis* biotyping scheme, we found that both collections contained several isolates of *L. iners* that had been misidentified as *G. vaginalis* (unpublished data), a finding which also urges reconsideration of the *G. vaginalis* biotyping scheme.

3.2. L. iners *has ambiguous cell morphology*

Apparently, *L. iners* is also regularly overlooked by microscopy as well. The frequent predominance of *L. iners* in cases of BV is intriguing, since this condition is, almost by definition, characterized by depletion of lactobacilli upon microscopy of Gram-stained vaginal smears. This begs the question: if *L. iners* is present in so many women with vaginal dysbiosis (high prevalence at the population level), and

frequently in large numbers (predominant in individual women), how can we explain that microscopy indicates the absence of the lactobacillar cell type in cases of BV?

We previously published a series of pictures of Gram stains of cultured *L. iners*, showing that all of the cells were staining Gram-negative [22]. To exclude bias (e.g. due to possible overdecoloration during that specific batch of staining), we recently repeated this experiment and stained *L. iners*, *L. crispatus* and *Streptococcus agalactiae* in the same staining batch and prepared artificial mixtures of the three species. The microscopic images of these artificial mixtures (Fig. 1) clearly confirm that *L. iners*, cultured anaerobically on 5% sheep blood agar, stains Gram-negative and that cell morphology can be rather coccal. Together with the well-known Gram-variable staining of *G. vaginalis*, explained by its thin cell wall [67], these findings might explain why BV is usually referred to as characterized by 'overgrowth of anaerobic Gram-negatives', whereas two of the most predominant species, i.e. *G. vaginalis* and *L. iners*, are phylogenetically both Gram-positives.

However, in case Gram-negative staining of *L. iners* is indeed a possible explanation for the paradox of the absence of the lactobacillar cell type in *L. iners*-dominated BV samples, this raises another paradox. Indeed, because low Nugent scores can be observed when *L. iners* is the sole predominant species, as is apparent from several metagenome studies, this means that *L. iners* can also stain Gram-positive with bacillar cell morphology. This is also illustrated the study of Srinivasan et al. [35], who found that women with almost exclusively *L. iners* had low Nugent scores (and absence of clinical symptoms), whereas women dominated by *L. iners*, but in conjunction with *G. vaginalis*, had high Nugent scores.

Also, the original description of *L. iners* mentions it as a Gram-positive bacillus [2], and the pictures presented by McMillan et al. [65] show a Gram-positive bacillus, the

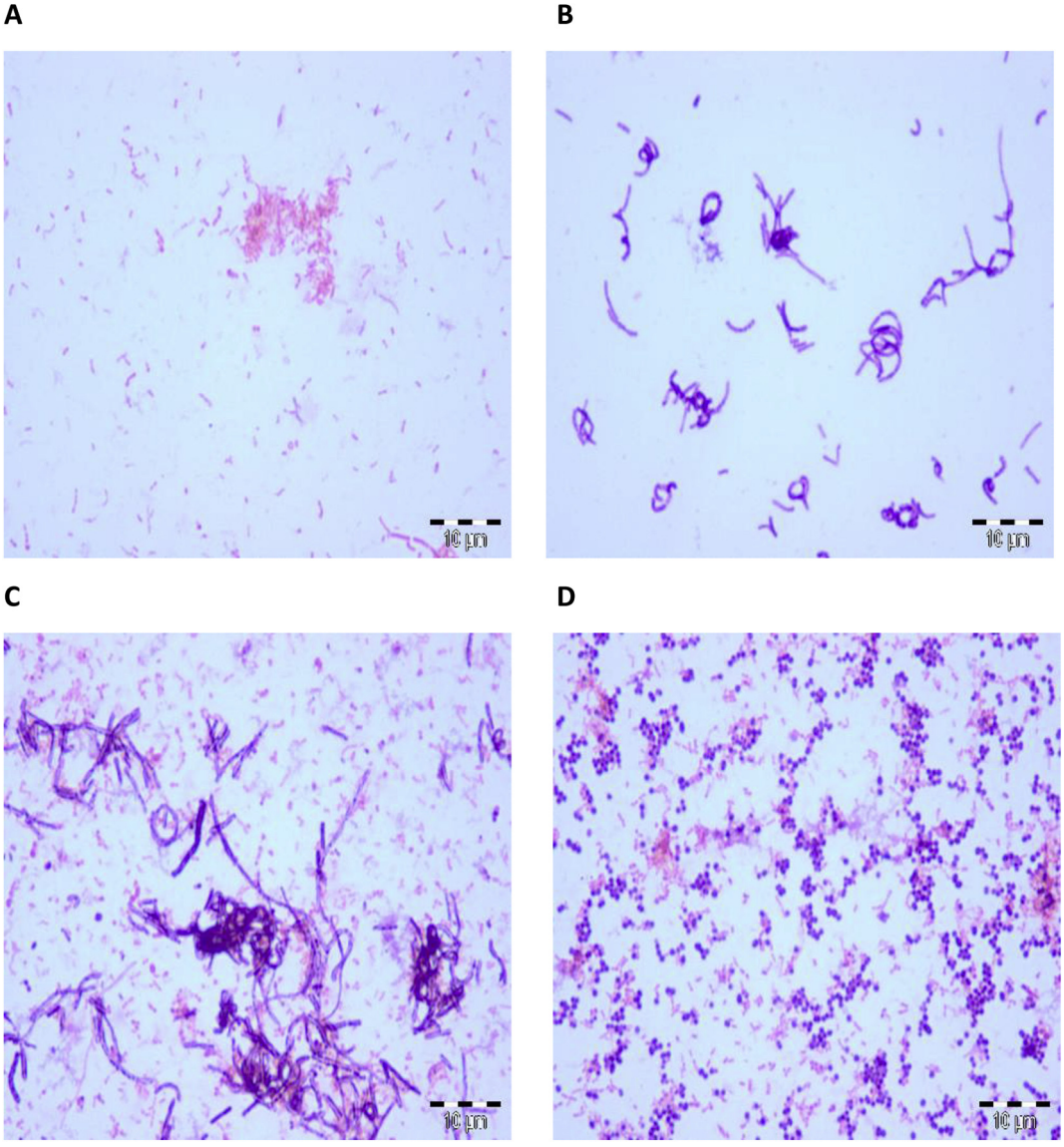

Fig. 1. Gram stain of A: *Lactobacillus iners*; B: *L. crispatus*; artificial mixtures of equal amounts of C: *L. iners* and *L. crispatus*; D: *L. iners* and *Streptococcus agalactiae*.

morphology of which is highly variable and dependent on, for example, co-incubation with fibronectin.

The suggestion that there might be lactobacilli recognizable by their different cellular morphology, with limited protective value, had previously been put forward [68].

It should be noted that the putative presence of a thinner peptidoglycane layer in *L. iners* compared to other lactobacilli, may lead to overestimation of the number of *L. iners* cells by nucleic-acid-based studies, since *L. iners* cells might be more easily lysed and DNA extraction may be more efficient from *L. iners* cells. For example, the study of Kusters et al. [21], reporting higher loads of *L. iners* compared to *L. crispatus* in low Nugent vaginal smears, did not perform extra cell wall lysis steps prior to the automated method, though the latter are needed to efficiently lyse (normal) Gram-positive cell walls.

3.3. Adherence to vaginal epithelial cells

L. iners has been claimed to be aberrant with regard to its adherence to vaginal epithelial cells. While *L. iners* lacks the adhesion molecules that are common to other *Lactobacillus* species, its genome encodes for a fibronectin-binding protein which, moreover, contains a protein A motif (designated FBPA) common to pathogenic strains of *Staphylococcus aureus* and central to the adherence to, and invasion of, human cells by *S. aureus* [46,65,69]. McMillan et al. [65] showed that *L. iners* strain AB-1 binds strongly to immobilized human fibronectin (Fn), even more so than *S. aureus* strain MN8, well-studied for its Fn-binding capacities, and that this process is protein-mediated. Vaginal strain ATCC 3800 of *L. crispatus* did not adhere [65], although it has been shown that the closely-related *L. acidophilus* (*sensu stricto*) strongly adheres to fibronectin via surface(S)-layer-associated proteins [e.g., Ref. [70]] and although Ojala et al. [71] concluded that the core genome of *L. crispatus* contains a functional *fbpa* gene, encoding the same putative fibronectin/fibrinogen binding protein described for *L. iners* by McMillan et al. [65]. Interestingly, the expression of fibronectin, a glycoprotein found in an insoluble form in the extracellular matrix of the vaginal epithelium and also attached to the surface of host cells, increases 20- to 30-fold during menses [65].

Although one in vitro study indicated that *L. iners* was able to displace *G. vaginalis* biofilm on glass slides [28], another study indicated that *L. iners* enhanced adhesion to cervical epithelial cells (HeLa) of BV-associated, but not of commensal *G. vaginalis*, in contrast to *L. crispatus*, which reduced adherence of both *G. vaginalis* strains more than tenfold [72]. Moreover, *G. vaginalis* displaced *L. crispatus*, but not *L. iners* from HeLa cells [72].

In summary, although some contradictory findings were reported, possibly explainable as the consequence of strain differences within the species *L. iners*, enhanced adhesion abilities of *L. iners* might help to explain its resilience during vaginal dysbiosis, whereas its ability to increase adhesion of certain types of *G. vaginalis* might help to understand its reduced protection against vaginal dysbiosis when compared to other vaginal lactobacilli. Considering the possible presence of *L. iners* in *G. vaginalis*-dominated biofilms (see 2.2), (some types of) *L. iners* might even contribute to the onset and/or recurrence of vaginal dysbiosis.

3.4. Vaginal pH and acid production

Gajer et al. [44] reported that *L. crispatus* and *L. iners*-dominated microbiomes have rather low median pH values of 4.0 and 4.4, respectively, compared to communities dominated by *L. jensenii* (5.0) and *L. gasseri* (4.7), but Srinivasan et al. [35] found that, in contrast to *L. crispatus*, which was consistently strongly associated with low pH, women with high *L. iners* levels could have either low or high pH. From their Fig. 1, it is apparent that high pH was present predominantly in those cases where *L. iners* was found in association with *G. vaginalis*, pointing again to a dual role for *L. iners*: when predominant and monopolizing the vaginal econiche, it may appear (3.2. Cell morphology) and behave (causing low pH) as a normal lactobacillus, but at the same time, the species is rather unable to resist overgrowth and pH increase when challenged by other (non-*Lactobacillus*) bacteria, although it may persist during such dysbiosis conditions.

As already mentioned, Macklaim et al. [46] found that *L. iners* differentially expressed over 10% of its genome under dysbiotic conditions compared to healthy states, with increased expression of inerolysin and of mucin and glycerol transport systems and related metabolic enzymes. Furthermore, these changes likely result in the production of succinate and other short-chain fatty acids, contributing to increased vaginal pH, whereby it should be kept in mind that a high ratio of succinate to lactic acid was the original marker of BV, and that high succinate concentrations distinguish BV from the conditions of aerobic vaginitis [46]. Again, considering this - in conjunction with other observations, such as its potential to enhance *G. vaginalis* adhesion, it cannot be excluded that (at least some strains of) *L. iners* actually contribute(s) to the onset of vaginal dysbiosis.

Analysis of the metabolite profiles by Gajer et al. [44] showed that high levels of lactate were maintained in some women even during menses, when the VMC shifted from *L. crispatus* domination to *L. iners* domination, but a previous metagenome study found that all *L. crispatus*-dominated communities had a pH below 4.5, while this was the case for only 74% of *L. iners*-dominated communities [13]. The latter is in closer agreement with the results of Witkin et al. [49], who showed that *L. iners* is a poor lactate producer, producing only L-lactate. These authors found that vaginal fluids from 12 women with *L. iners*-dominated microbiomes had the lowest median concentrations of D-lactic acid, *i.e.* 0.06 mM, at about the same level of that of *G. vaginalis* microbiomes (8 women, 0.07 mM), and far below that of *L. crispatus* (17 women, 1.32 mM – as indicated in a corrigendum), *L. jensenii* (2 women, 0.45 mM) and *L. gasseri* (2 women, 2.92 mM). Also, because of the almost complete lack of D-lactic acid, the L/D lactic acid ratio was highest in *L. iners* (3.15 compared to 0.48 for *L. crispatus*, 0.73 for *L. gasseri* and 2.43 for *L. jensenii*). Unfortunately, no pH values were reported. The

lactic acid measurements of Witkin et al. [49] correspond to genomic findings. Indeed, the genome sequences of the four principal species of *Lactobacillus* found in the vaginal microbiome show differences in their potential for producing D- and L-lactic acid based on the presence or absence of D- and L-lactate dehydrogenase, whereby the gene coding for D-lactate dehydrogenase is completely absent from the genome of *L. iners* UPII 60-B [40,49].

3.5. Genome size

L. iners has an unusually small genome, i.e., 1.28 Mbp on average, compared to 2.25 Mbp for *L. crispatus* [40], which is already in the lower range of genome sizes within the genus *Lactobacillus*, with genomes of 3 up to 4 Mbp. Correspondingly, the *L. crispatus* pangenome counts 4300 genes versus 2300 genes for that of *L. iners* [40]. Such a low genome size is strongly indicative of a more parasitic, host-dependent lifestyle [1]. Also, one is inclined to intuitively agree with France et al. [40] who state that a smaller genome size predicts higher vulnerability to environmental fluctuations, although this is contradicted by the resilient presence of *L. iners* under very different conditions in the vagina. France et al. [40] also conclude that genome size reduction occurs more frequently in host-associated species, that experience relatively constant environments, although the vaginal eco-niche should be considered as a rather dynamic and unstable environment [73].

3.6. Nutritional requirements, glycolysis and glycogen metabolism

In agreement with its small genome size, *L. iners* has more complex nutritional requirements compared to, for example, *L. crispatus*. Falsen et al. [2] used the epitheton 'iners', meaning 'inert', 'lazy', to indicate that, except for the production of acid from glucose (all strains) and from maltose (4 strains out of 11), the strains were asaccharolytic and did not produce acid from L-arabinose, D-arabitol, cyclodextrin, glycogen, N-acetylglucosamine, lactose, mannitol, melezitose, melibiose, methyl P-D-glucopyranoside, pullulan, raffinose, ribose, rhamnose, sorbitol, sucrose, tagatose, trehalose or D-xylose. Genome analysis also clearly indicated a higher dependence of *L. iners* on exogenous sources of amino acids [40]. Boyd et al. [74] used the API 50 CH carbohydrate fermentation identification system (bioMérieux, France) for identification of 97 strains of the species *L. crispatus*, *L. jensenii*, *L. gasseri*, *L. iners* and *L. vaginalis*, and found that all of the *L. iners* isolates (and three of the *L. vaginalis* isolates) were non-reactive in all of the tests (including glycogen). However, Macklaim et al. [46] reported that, compared to healthy conditions, *L. iners* genes for the uptake of mannose and maltose were strongly upregulated during BV, as well as genes for four glycosylases predicted to target α-1,6-glucocidic linkages that bridge the branching points in glycogen, i.e., genes for breakdown of glycogen and genes for mucin and glycerol transport systems.

Borgdorff et al. [47] identified four *L. iners*-specific peptides in cervicovaginal lavage supernatants of Rwandan women at high HIV risk. The *L. iners* enzymes glyceraldehyde-3-phosphate dehydrogenase (GAPDH), fructose-bisphosphate aldolase (ALDO), glucose-6-phosphate isomerase (GPI) and DNA starvation/stationary phase protection protein (DPS), were all significantly lowered in cases of elevated pH and/or vaginal dysbiosis, which was considered by the authors as an indication that these key metabolic enzymes play a role in the maintenance of lactobacillus dominance. However, the reduced expression of these enzymes might also be interpreted by the notion that they are no longer needed once *L. iners* finds itself in a multifunctional multi-species consortium.

High extracellular abundance of the − normally intracellular − *L. crispatus* glycolysis enzymes GAPDH and GPI was shown in previous in vitro studies [75,76]. Since, for all the *Lactobacillus* glycolysis proteins identified in the study of Borgdorff et al. [47], additional extracellular functions have been described in closely related bacterial species, the authors suggested that the increased abundance in CVL of these normally intracellular enzymes indicated extracellular roles, including (competitive) adhesion, plasminogen binding and immune modulation. It is also tempting to suggest that these enzymes might play a role in glycogen metabolism. The differing expression of these proteins is in line with the observation that *L. iners* may behave differently (and possibly look different) under differing vaginal conditions.

3.7. Nutrition: inerolysin, blood and iron

Intriguingly, *L. iners* has been shown to be the only *Lactobacillus* species known thus far to encode for a pore-forming cytolytic toxin, inerolysin, that requires cholesterol to be present in the membrane and that is inhibited by an excess of exogenous cholesterol [77,78]. Inerolysin has 68.4% sequence similarity to vaginolysin of *G. vaginalis*, from which species it probably has been acquired by horizontal gene transfer [40]. It is under strong positive selective pressure [40], which indicates that it plays an important role in the lifestyle of *L. iners*. It can lyse murine, ovine and human erythrocytes in a dose-dependent manner, in contrast to vaginolysin, which is human-specific [77]. Rampersaud [78] reported that inerolysin has maximal activity at a pH of 4.5 and is active across a pH range of 4.5−6.0, which led Macklaim et al. [46] to suggest that *L. iners* may have an unappreciated role in BV and might contribute to the pathogenesis of the condition, also because they established upregulation of this porin during conditions of BV.

Although qPCR in our hands was positive for inerolysin for all *L. iners* strains tested (unpublished data), the genomic data of Macklaim et al. [46] indicate that some strains are negative, which leaves room for the suggestion that the occurrence of this species under different conditions might be partially explained by the existence of inerolysin-positive strains that thrive best in conditions of vaginal dysbiosis. On the other hand, the finding of Macklaim et al. [46] that inerolysin is

strongly upregulated during BV indicates that an individual strain may adapt to conditions of vaginal dysbiosis, although the pH optimum of 4.5 for inerolysin might not be adapted to the elevated pH during BV [78].

It seems straightforward that cytotoxicity, i.e. lysis of cells, functions as a means to obtain cellular components such as iron, which are limiting factors for metabolism. *G. vaginalis* cannot grow in iron-limiting conditions, but can use iron sources such as hemoglobin for growth. This is consistent with the observation that *G. vaginalis* increases during menses and produces vaginolysin. Also, *L. iners* needs nutrient-rich media such as blood agar and thrives during the menses. We found that most *L. iners* isolates could grow on MRS upon addition of 1–5% sheep and human blood (unpublished data), and that most strains showed alpha hemolysis on TSA + 5% sheep blood. However, none of the strains tested showed hemolysis on TSA + 5% or 10% of human blood (blood group A+), which contradicts the observation of Rampersaud et al. [77] that inerolysin could lyse ovine and human erythrocytes. Also, we could not observe growth improvement upon addition of up to 50 μM $FeSO_4$ to MRS (unpublished data).

Macklaim et al. [69] found no apparent iron uptake systems in *L. iners*, but a ferrochelatase was detected that is not present in the other *Lactobacillus* species. However, an oxygen-independent coproporphyrinogen III oxidase (hemN) that could cooperate with ferrochelatase to break down heme for transport is lacking in *L. iners*, and no transport system has yet been characterized.

L. iners AB-1 encodes an iron–sulfur protein cluster (Fe–S), that is also present and even mainly detected in other vaginal *Lactobacillus* species including *L. crispatus* [69], and that is is hypothesized to act as an oxygen stress sensor.

It is unclear how these findings relate to the iron abstinence of many *Lactobacillus* species that has been suggested to provide lactobacilli with intrinsic resistance to oxygen stress, as discussed elsewhere in this issue [79].

3.8. L. iners *and vaginal immunity: an increased risk for preterm birth?*

Using 3-D vaginal epithelial cell aggregates that had been colonized with *L. iners*, Doerflinger et al. [80] showed increased expression of multiple transcription factors and pro-inflammatory cytokines related to pathogen recognition receptor-mediated signaling, although this induction of pro-inflammatory immune pathways did not result in proinflammatory cytokine secretion.

The presence of *L. crispatus* and *L. jensenii* has been associated with lowered IL-1β, even in cases with abnormal microflora and BV, whereas this was not the case for *L. iners* [30]. Anahtar et al. [82] reported that *L. iners*, unlike *L. crispatus*, induced moderate IL-8 secretion, although strong pro-inflammatory signals were associated with lactobacillus-devoid communities, comprising predominantly *Prevotella* and *Sneathia*.

Leizer et al. [81] determined differences in properties of vaginal epithelial cells and the composition of vaginal secretions when either *L. crispatus* or *L. iners* was numerically dominant in the vagina of pregnant women. The median epithelial levels of nucleoporin p62, a marker for autophagy, were 0.41 and 4.26 ng/mL in women with *L. crispatus* (n = 69) and *L. iners* (n = 23) dominance, respectively. The corresponding median heat shock protein (hsp) 70 levels were 4.24 and 14.50 ng/mL, respectively. The D-lactic acid concentration in vaginal fluid was highest in association with *L. crispatus* dominance, while all other vaginal fluid compounds, except for extracellular matrix metalloproteinase inducer (EMMPRIN), were highest when *L. iners* was dominant. The authors conclude that epithelial cells exhibit a higher level of autophagy, lower induction of stress-related hsp70, and release lower levels of mediators when *L. crispatus* is most abundant, compared to when *L. iners* dominates the vaginal econiche.

Possibly, the moderate increase in inflammation associated with *L. iners* colonization compared to *L. crispatus* colonization may partially explain the observed increased risk of preterm birth [56], although anti-inflammatory progesterone treatment of *L. iners*-colonized pregnant women did not improve the outcome [56].

The lack of H_2O_2 production by *L. iners* in relation to the possible role of this reactive oxygen species in maintenance of lactobacillar dominance and in relation to its putative anti-inflammatory role in vaginal immunity is addressed elsewhere in this issue [79].

4. Questions, paradoxes and future research

1. We previously [22] observed, and confirm here (Fig. 1), that *L. iners*, upon culture on TSA + 5% sheep blood agar, stains as a Gram-negative coccobacillus. Might this be the explanation for the fact that BV is described as 'overgrowth by Gram-negative strict anaerobes', whereas paradoxically in many cases of vaginal dysbiosis, the predominant species are the Gram-positive species *L. iners* and *G. vaginalis* (the latter also known to stain Gram-'variable')?
2. If this is indeed the correct explanation, then we need to explain another paradox: Large numbers of *L. iners* during BV do not result in large numbers of lactobacilli on Gram stain, whereas large numbers of (monopolizing) *L. iners*, i.e., not in association with BV species, are seen as numerous Gram-positive lactobacilli on Gram stain. Can *L. iners* change morphology depending on the condition? This would be in agreement with the observation that strains of this species can drastically change their expression patterns in case of BV [46] and have a different proteome during BV [47].
3. Do different strains of *L. iners* exist, namely beneficial/commensal genotypes and more pathogenic genotypes? If so, which markers would be useful? Might inerolysin positivity be useful as a marker to distinguish 'commensal' from 'pathogenic' strains?
4. How can we reconcile the knowledge that *L. iners* is a weak lactic acid producer (not producing D-lactic acid)

[49,81] with the observation in several reports of normal acidic pH in case of *L. iners* dominance? Should we pay attention primarily to D-lactic acid production and not as much to pH?

5. Why is *L. iners* rather incompatible with other vaginal lactobacilli, even during the menstrual cycle of a single subject, whereas it is more able than other lactobacilli to persist or thrive during conditions of dysbiosis, as occur after sexual intercourse, during menses and during BV, frequently in association with large numbers of cells of typical BV species such as *G. vaginalis* and *A. vaginae*?
6. Why is *L. iners* the only *Lactobacillus* (known thus far) that codes for a virulence characteristic, such as a cytotoxin (inerolysin) [77,78] which, moreover, is upregulated during BV [46]?
7. Why is *L. iners* more prevalent among black women? Because Beamer et al. [26] concluded that the higher prevalence of *L. iners* was due to higher prevalence of BV among black women, and because Ness et al. [27] refuted the explanation that the increased prevalence of BV among black women can be explained by behavioral practices, the conclusion seems to be that it is largely the genetic background of black women which predisposes to colonization with *L. iners* which, in turn, offers less protection against vaginal dysbiosis. Still, the question remains as to why a less protective species would be more prevalent among some populations.
8. Is *L. iners* part of the *G. vaginalis*-dominated biofilm in some/most cases?
9. Does *L. iners* increase the risk of preterm birth, as indicated by some studies [e.g., Ref. [53]], including a very recent one [56]? Should we consider BV (or still other dysbioses, see Ref. [57]) or rather the presence of *L. iners* when considering the risk of adverse pregnancy outcomes?

5. Conclusions

In conclusion, *L. iners* is a very intriguing bacterial species. Although it has been considered as the most prevalent and persistent commensal vaginal lactobacillus, which may even promote the onset of the restoration of a healthy microbiome, we are inclined to consider *L. iners* as an unusual suspect, i.e., a species that, although beyond suspicion because it belongs to a genus with many probiotic characteristics, thrives best in an environment such as that present during vaginal dysbiosis, and that might even contribute to the onset and maintenance of vaginal dysbiosis, as such predisposing to STI and APO. It remains to be elucidated whether the species contains commensal and more pathogenic genotypes, or whether individual strains behave differently according to the condition, which might explain several paradoxical observations, such as possible differing cell morphology under different conditions.

The overall picture at present indicates that *L. iners* may be the predominant vaginal lactobacillus, in association with low pH and low Nugent score. Although it may resemble a lactobacillus and acidify the environment like a typical lactobacillus, most studies indicate that *L. iners* offers overall less protection against vaginal colonization by other species, possibly because it produces less lactic acid and no D-lactic acid, and little or no hydrogen peroxide.

The similarity and relatedness of its cytotoxin (inerolysin) to that of *G. vaginalis* (vaginolysin), its small genome and its concurrent nutrient dependency do not exclude the possibility that *L. iners* plays a role in the onset of vaginal dysbiosis. Moreover, when *Gardnerella* biofilms are formed, *L. iners* might enhance *G. vaginalis* adherence [72] and might become part of the biofilm, which would explain *Lactobacillus*-positive signals that have been observed in *G. vaginalis* biofilms [36].

In summary, the high prevalence of *L. iners* during vaginal eubiosis and dysbiosis, together with several characteristics atypical of a lactobacillus, does not permit us to consider it simply as the most prevalent commensal of the vagina, but warrants its being viewed as a highly unusual suspect in the onset and maintenance of vaginal dysbiosis, and even in the risk of preterm delivery.

Conflict of interest

The author declares no conflict of interest.

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