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The human vaginal microbial community

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Abstract

Monopolization of the vaginal econiche by a limited number of *Lactobacillus* species, resulting in low pH of 3.5–4.5, has been shown to protect women against vaginal dysbiosis, sexually transmitted infections and adverse pregnancy outcomes. Still, controversy exists as to which characteristics of lactobacilli are most important with regard to colonization resistance and to providing protection. This review addresses the antimicrobial and anti-inflammatory roles of lactic acid (and low pH) and hydrogen peroxide (and oxidative stress) as means of lactobacilli to dominate the vaginal econiche.

Keywords: Vagina; Glycogen; Lactobacilli; Lactic acid; Hydrogen peroxide

1. Definition and dynamics of the normal vaginal microbial community

1.1. What is normal?

It has recently been claimed that almost any type of vaginal microbial community (VMC) can be considered as normal, since even in the absence of lactobacilli there is frequently a lack of symptoms, there is organic acid production and there is long-term stability of the VMC [1,2]. Witkin et al. [1], for example, stated that in black women, the enhanced prevalence of asymptomatic bacterial vaginosis might merely reflect an increased likelihood that bacteria other than lactobacilli predominate, fulfilling the same protective functions, in agreement with the common function hypothesis (see 1.4).

Although science usually proceeds most quickly by paradigm shifts, in this case it may be wise not to throw overboard knowledge that has been gathered during more than a century, starting with Döderlein [3]. Indeed, numerous epidemiological studies continue to confirm that it is especially hydrogen peroxide (H_2O_2)-producing lactobacilli that are protective against sexually transmitted infections (STIs) and adverse pregnancy outcome (APO). In other words, the mere absence of these probiotic bacteria from the vaginal econiche is a health risk factor for women, their partners and their unborn and new-born children. Moreover, it is becoming clear that it is especially lactic acid [4,5], and probably even more, D-lactic acid, as produced by only some vaginal lactobacilli [6], but not any organic acid, that are health-promoting. Also, it is rather odd to consider stability as a hallmark of a healthy VMC, because the normal VMC fluctuates during the menstrual cycle (see 1.5) and because the most stable VMC is observed in cases of chronic bacterial vaginosis (BV). The notion that 10–42% of women whose vaginal microbial communities lack appreciable numbers of lactobacilli are asymptomatic is in fact long-established knowledge [e.g., [7]].

With regard to H_2O_2 production, it is not clear whether this in itself is a health-promoting characteristic rather than a biomarker for the presence of certain species of lactobacilli [4], i.e., lactobacilli other than *Lactobacillus iners* [5](see also 4).

Interestingly, a recent report indicates that the presence of lactobacilli is not jeopardizing anti-HIV medication, whereas colonization with *Gardnerella vaginalis* did impede the efficacy of the treatment [8].

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For all these reasons, it seems reasonable to consider only lactobacilli-dominated VMC as normal or health-promoting (healthy).

1.2. The predominant lactobacilli

Lactobacilli are the most aciduric bacteria among the acidophilic lactic acid bacteria, thriving at pH of 4.0 (and below, according to [4]), at which other species cannot grow. This makes them the favored bacteria in fermented food production, such as yoghurt, sauerkraut and fermented meat, since they are the last survivors when pH has sharply decreased. The same principle applies in the human vagina, although we wonder why, among mammals, this seems to be the case only in our species (see 1.4).

Based on DNA homology studies, the previous species *Lactobacillus acidophilus* sensu lato has been divided into six DNA homology groups that could not be distinguished biochemically [9], but that later could be characterized as six distinct species: *L. acidophilus* (*sensu stricto*), *Lactobacillus amylovorus*, *Lactobacillus crispatus*, *Lactobacillus gallinarum*, *Lactobacillus gasseri* and *Lactobacillus johnsonii* [10]. The L. *acidophilus* group now comprises 25 species, including *L. iners* and *L. jensenii*. tRNA-intergenic spacer length polymorphism analysis is another molecular approach that was extremely useful in identifying cultured lactobacilli [11] and that was applied for the identification of vaginal lactobacilli [12].

Culture-based studies using DNA:DNA hybridization to identify the isolates revealed that *L. crispatus*, *L. gasseri*, *L. jensenii* and *Lactobacillus vaginalis* (more closely related to *L. fermentum* and *L. reuteri*) are the most common species found in the vagina [13–15], and confirmed an association between vaginal health and H_2O_2-producing species/strains as well [14,15]. Another species, *L. iners*, was first described in 1999 [16] and its presence (and its limited production of H_2O_2) was already established in the culture-based study of Antonio et al. [14], where it was listed as 'species L. 1086V' [Sharon Hillier, pers. comm.]. Another early study pointing to the prevalence of *L. iners* was the one by Tärnberg et al. [17].

Still, studies from around the world (using adequate identification methods) indicate that more species may be involved [18]. Predominant vaginal species were *L. plantarum* and *L. fermentum* in a Bulgarian study [19], *L. reuteri*, *L. fermentum* and *L. salivarius* in an Indian study [20] and *L. pentosus*, *L. fermentum*, *Enterococcus faecalis*, and also the strongly H_2O_2-producing *Weissella (Lactobacillus) viridescens* in a South African study [21].

It may be important to also consider rectal colonization with regard to the vaginal dominance of lactobacilli [22–24].

1.3. Is predominance of lactobacilli always indicative of health?

Cytolytic vaginosis and lactobacillosis, mostly considered together, also known as *Lactobacillus* overgrowth syndrome or Döderlein's cytolysis (and possibly as *Leptothrix vaginalis* [25]), are characterized by abundant growth of (frequently long and slender) lactobacilli in association with very low pH and numerous bare nuclei – easily confused with leukocytes, and possibly with debris cytoplasm – which may be confused with the type of bacterial cells that are characteristic of BV. Bare nuclei and debris cytoplasm result from the lysis of vaginal epithelial cells, which is generally assumed [e.g., [26]] to be due to the overgrowth of, and subsequent over-acidification by, lactobacilli. This assumption is corroborated by the cyclicity of the symptoms and their association with the luteal/secretory phase, when predominance of lactobacilli is usually most evident [27–29].

The prevalence of cytolytic vaginosis may be underestimated, because the clinical presentation is suggestive of vulvovaginal candidiasis (VVC) [30,31]. Cibley and Cibley [27] even state that most of the presumed chronic candidiasis cases seen at their practice were, in fact, cases of cytolytic vaginosis. A Bulgarian study, taking into account (and thus not biased by) confusion with VVC, reported 3.9% among 1152 women to present with cytolytic vaginosis [31].

Cytolytic vaginosis, despite the predominance of lactobacilli, cannot be considered as a healthy condition, because it frequently comes with symptoms, such as vaginal itching, burning and irritation [27,28], and thick or thin white cheesy vaginal discharge, pruritus, dyspareunia and vulvar dysuria [29], for which alkalinizing sodium bicarbonate treatment can be prescribed. The condition has even been linked to invasive carcinoma of the cervix [32] and as a risk factor for post-operative infection after first-trimester abortion [33].

1.4. The 'normal' human vaginal econiche is unique to humans and is highly abnormal when compared to other mammals

Under normal conditions, i.e., vaginal eubiosis, the vaginal econiche of humans is usually dominated by one or two out of only five major species of lactobacilli and characterized by a pH below 4.5. In fact, pH is as low as 3.5 [4]. It is mostly not realized that this situation of monopolization and concurrent low vaginal pH is extraordinary, considering that the vagina is not a sterile place, and moreover, that it is unique among mammals, because all available data on composition of VMCs in other mammals – including that of primates [e.g., [34–36]] – indicate the presence of a mixture of species, low levels of glycogen [35] and of lactic acid, and a neutral pH [37]. This was recently documented in more detail by Miller et al. [37], who found that, across 10 studies of human women, median vaginal pH was 4.5 (range = 4.0–4.9), while median vaginal pH across non-human mammals, including many primate species, was 6.8, with no species falling into the range of normal human pH. Furthermore, while the same *Lactobacillus* species as in humans were present, the average relative abundance of lactobacilli was only 1.1% in non-human mammals compared to 69.6% in human women.

Stumpf et al. [38] hypothesized that the specific protective role of lactobacilli in humans might have evolved because human females, in particular, may experience relatively high

risk of STI and obstetric complications due to prolonged intromission and due to continuous sexual receptivity throughout the menstrual cycle, pregnancy and the postpartum period. However, research regarding the presence and absence of penis bones in primates seems to contradict the generally accepted assumption that humans have prolonged intromission relative to other primates [39]. With regard to continuous sexual receptivity, there is consistent evidence of extended female sexuality in the rhesus monkey and chimpanzees, and especially in bonobos (*Pan paniscus*), which mate at all stages of the ovarian cycle [40]. However, available data for these species show the absence of low vaginal pH, of lactobacillar predominance and of high levels of lactic acid and glycogen. It would be of interest to obtain data on the VMC of bonobos, who have even more pronounced continuous female receptivity. Also, recently, Miller and colleagues [37] tested these infectious disease and obstetric risk hypotheses, but found no significant relationship between vaginal pH or lactobacilli relative abundance and multiple metrics of STIs or birth injury risk in different animals, including several primates.

Another 'hypothesis', i.e., the common function hypothesis, which simply states that in other mammal species the protective role of lactobacilli is taken over by other bacterial species, does not offer an answer as to why humans should have evolved a unique microbial solution to assumed relatively similar selective pressures.

Miller et al. [37] therefore put forward their own hypothesis, i.e. that the specific lactobacillar dominance in the human vagina may follow from a starch-rich diet, as became available since the cooking of food and the agricultural life-style of humans, and which may provide a glycogen-rich vaginal environment. Of course, it can be assumed that direct glycogen supply, from meat and/or seafood, might have the same effect. Moreover, if agricultural life-style were to be the cause, this would mean that such thorough evolutionary events should have taken place during the last 10,000 years. More data on the vaginal microbiology of females of the few remaining hunter-gatherer tribes might provide a test for this hypothesis.

I take the liberty and the opportunity of offering another suggestion, which starts from the assumption that, at one time in human evolution, the selective pressures were quite different when compared to that of other primates, instead of relatively similar, as assumed by e.g., Miller et al. [37], at once explaining why the solution turned out to be so very different and specific. Indeed, there are several strong arguments that our ancestors went through a semi-aquatic past since the split with the chimpanzees, probably most intensively during the last two to three million years [41,42], whereby our ancestors were shallow-diving, coastal, seafood-consuming primates. This view of human evolution has strong explanatory power for several of the many other very peculiar features of the human body, unique among primates, such as increased brain size, naked skin with a subcutaneous fat layer, voluntary breath control and an oropharyngeal cavity that can be completely locked. A semi-aquatic past may also explain characteristics more closely related to the many peculiarities of human female reproductivity, such as the presence of a hymen – absent in all primates, but present in several (semi-aquatic) mammals – the presence of permanent external vaginal labiae, the intriguing absence of placentophagy (present in most terrestrial mammals and all primates, including chimpanzees [43]) or the near absence of odor-related sexual arousal/attraction in humans [44] in relation to concealed ovulation. With regard to the explanatory power of a semi-aquatic past for the normal dominance of lactobacilli in the vagina, it may be hypothesized that the low pH that apparently results from monopolization by lactobacilli may – in conjunction with the hymen and the permanent external labiae – have contributed to protection against submersion in (salty) water, which might have increased the risk of infection.

Further adding to the uniqueness of the human vaginal econiche is a general abnormality with regard to the innate immune system of the human species, i.e., the absence of Neu5GC from the human sialome, whereas most other mammals have a mixture of Neu5Ac and Neu5Gc. Given the predominant role of sialic acid (neuraminic acid) biology in immunity [45], we still underappreciate the repercussions of this mutation upon colonization and infection of human mucosal tissues [46].

As a consequence, the profound differences in vaginal ecology between human females and the females of our closest relatives limit the value of primates as animal models for e.g., HIV infection in humans. Also the relevance of in vitro cell culture models may be limited because they lack cervical mucus flow, vaginal epithelial transudate, the immune component and/or lactobacilli.

1.5. Dynamics of the vaginal econiche

Although fluctuation of the numbers of lactobacilli during the menstrual cycle can be regarded as established knowledge, with least numbers during menstruation and largest numbers of lactobacilli during the secretory/luteal phase, many reports indicate that this is highly variable between women. van de Wijgert et al. [47] concluded that, although most studies that evaluated the influence of the menstrual cycle were small, they consistently suggest that high levels of estradiol (as is the case during the secretory/luteal phase) promote proliferation of lactobacilli, in particular, *L. crispatus* [2,48–50]. Eschenbach et al. [51] found that for all subjects, the rate of recovery of heavy growth of *Lactobacillus* increased over the menstrual cycle and, in contrast, the concentration of non-*Lactobacillus* species tended to be higher at menses. Brotman et al. [52], using Gram stain analysis of vaginal smears, observed rapid fluctuation of the VMC during the cycle and association of menses with high Nugent scores. Wilson et al. [53] made a strong case for the role of estrogen levels in the maintenance of a *Lactobacillus*-dominated VMC, since they determined that the mean estradiol level was 39 ng/L in case of BV, compared to 176 ng/L in case of normal VMC. Note that other studies indicate that there is, rather, a link with progesteron and increased glycogen levels in the vagina (see: 3). Interestingly, in case of cytolytic vaginosis, symptoms are most clear-cut during the luteal/secretory phase [29], when

lactobacilli are most predominant, supporting the existence of cyclic changes in *Lactobacillus* abundance.

van de Wijgert et al. [47] found that, in most studies, menses were the strongest disturbing factor, sometimes with large reductions in lactobacilli, shifts from *L. crispatus* to *L. iners* [50,54,55] or the appearance of BV-associated bacteria, streptococci or other Gram-positive cocci.

Perhaps we need to differentiate between putative 'physiological BV' and 'pathogenic BV'. Leppäluoto [56] has argued that BV-like VMCs, as can be observed after sexual intercourse [55], are a normal physiological state which may even be selectively advantageous because the concomitant increase in pH enhances sperm survival and thus fertilization success. We have also suggested that some or many cases of BV might be sexually enhanced rather than sexually transmitted [57]. In summary, 'physiological BV' would be a transient state, enhanced by transient disturbances such as menstruation and sexual activity, whereas 'pathogenic BV' is characterized by biofilm formation [58,59] and recurrence, and is possibly sexually transmitted by fragments of complex, multi-species biofilm [58].

However, many reports indicate that this fluctuation of lactobacillus numbers is not the case in many women. Keane et al. [60] found nine women with a continuous normal VMC, four with a continuous abnormal VMC and seven in whom the basically normal VMC underwent a change to either intermediate or BV, changes which occurred during the first 9 days (i.e., menses) of the cycle in five of these. Chaban et al. [61] reported overall stability during the menstrual cycle for most of their 27 women (15 Caucasian, 9 Asian), in the absence of BV, although the number of samples taken during menses was limited. Witkin et al. [1] even state that levels of vaginal lactobacilli appear to remain constant throughout the cycle.

Possibly, these conflicting data can be partially reconciled by the observation that large numbers of *L. iners* can replace *L. crispatus* during the menses [50,54,55].

Finally, it is also well-established that the VMC of pregnant women is more stable [62], possibly due to the absence of menses, coincident with further reduction of vaginal pH, whereby *L. crispatus* offers more protection than *L. gasseri* and *L. iners* against shifts to vaginal dysbiosis [63–65].

1.6. Terminology

Because of the confusion between and discussion about the validity of terms such as 'microbiome', 'microbiota', 'microflora', and in order to avoid grammatically incorrect usage of terms such as 'microbiotas', which is a double plural, we adopt here the general term 'vaginal microbial community', abbreviated as 'VMC'.

Also, the recently introduced term 'community state type' [2] will not be used for the following reasons: it is devoid of informative content, i.e., it does not refer to microbes nor to the vaginal econiche; it does not add much to existing knowledge regarding the different VMCs that prevail; and the numbering of the CSTs is somewhat odd. Moreover, introduction of new category terminology jeopardizes the link with established knowledge obtained by over a century of clinical studies in conjunction with microscopy, culture and numerous more recent non-culture-based molecular approaches. The same information might be contained in a more straightforward manner with terminology such as VMC-C for vaginal microbial communities dominated by *L. crispatus*, VMC-I for *L. iners*, VMC-J for *L. jensenii*, VMC-G for *L. gasseri* and VMC-O for communities without (dominance of) lactobacilli (meaning zero/nill ('0') or other ('O')), although the latter category, of course, lumps together several very different conditions (which is also the case with the CST-IV category of Gajer et al.) [2]. Possibly, other categories such as VMC-A for aerobic vaginitis orVMC-L for lactobacillosis/cytolytic vaginosis might be added.

1.7. Importance and drawbacks of non-culture-based approaches

van de Wijgert et al. [47] reviewed findings reported on the composition of VMCs by 63 studies published between January 2008 and November 2013 that used at least one nucleic-acid-sequence-based technique. They concluded that these approaches basically confirm established knowledge: lactobacilli-dominated VMCs are associated with a healthy vaginal environment, BV is best described as polybacterial dysbiosis with decreased load of lactobacilli, increased load and diversity of strictly anaerobic bacteria, yeasts (*Candida*) are more often present in lactobacilli-dominated VMC, *Trichomonas vaginalis* is more closely associated with BV and increased estradiol levels are associated with higher loads of lactobacilli. Most studies also confirmed that *L. crispatus* is predominantly found in BV-negative women, whereas *L. iners* (and, to a lesser extent, *L. gasseri*) is also found in women with intermediate VMC or BV. The latter observation is also reflected by the association of *L. iners* with BV-associated bacteria, but not with *L. crispatus* [55,66–68]. The ambiguous role of *L. iners* is addressed in more detail in this issue [5].

Still, exactly for the area where non-culture-based techniques could have brought clarification, i.e., the distinction between different categories of dysbiosis, the results are inconclusive and not very informative: van de Wijgert et al. [47] noted that most articles identified more than one cluster with mixed taxa, not differing significantly in the total number or type of bacterial taxa present, but only in their relative proportions, but they were not able to discern consistent patterns across studies.

Possibly most informative is the subclustering obtained by Dols et al. [69], who divided BV-negative women into two clusters, i.e., one *L. crispatus*-dominated and one *L. iners*-dominated and BV-positive women into three clusters, i.e., *Sneathia sanguinegens/Leptotrichia amnionii* (undistinguishable)-dominated (represented by an average of 22% of the reads), *G. vaginalis*-dominated (43% of the reads) and *Lachnospiraceae*-dominated (52% of the reads).

Also, remarkable for most of the high throughput sequencing (HTS) studies is the lack of reads for *Escherichia*

coli, *Staphylococcus aureus* and/or *Streptococcus agalactiae* (group B streptococci, GBS), i.e., the lack of species which have been shown to play an important role in prenatal, perinatal and postnatal morbidity and mortality and to be predominant in aerobic vaginitis, a condition which is found in 7–12% of women according to Donders et al. [70]. van de Wijgert et al. [47] could identify only three studies [67,71,72] reporting VMC clusters dominated by any of these three species. Finally, because only bacterial primers are mainly used in most nucleic-acid-based studies, important information regarding co-colonization with fungi (e.g., *Candida albicans*), unicellular protozoans (e.g., *T. vaginalis*) and viruses is often lacking. Further limitations and biases of nucleic-acid-based techniques have been outlined [73,74].

Hyman et al. [75] pointed out how differences in primer choice may influence the conclusions drawn from molecular studies. Ravel et al. [76] described three subcategories of non-lactobacillus-dominated VMCs, but *G. vaginalis* played no role in any of these, although – after long years of doubt, disagreement and debate – it is now clear that this species is frequently predominant and important in BV [59,77]. Possibly, the absence of *G. vaginalis* in all three BV subcategories in the study of Ravel et al., [2011] was due to underrepresentation of 16S rRNA gene reads of this species, which might be caused by mismatches for *G. vaginalis* in the frequently used 'universal' 16S rRNA gene primers, a problem also encountered in the study of Verhelst et al. [12].

It should also be noted that culture and HTS provide only (semi-)quantitative information, which moreover is often biased, e.g., due to the selectivity of the culture media or due to nucleic acid extraction techniques and primers, but does not reveal the structural organization of the VMCs, as can be seen when microscopic approaches are used.

Finally, metatranscriptomic studies (e.g., [78]), which reveal functionality rather than species composition of the different VMCs, might add more value to present knowledge than has been contributed by metagenomic studies.

2. Etiology of perturbation of the normal VMC

Although it is continuously repeated that we have little knowledge about the etiology of perturbation of the normal VMC, numerous possible and probable, but not mutually exclusive and even mutually reinforcing, reasons have been recognized: 1) changing estrogen/progesterone levels – in relation to (a) their influence on glycogen concentrations/vaginal acidity and (b) innate immunity – during life and during each cycle; 2) changing conditions due to menstruation, (a) reducing the acidity and (b) increasing nutrients and iron; 3) vaginal intercourse, (a) causing physical perturbance of mucosal integrity, (b) enhancing introduction of rectal microbes, (c) increasing aeroby/decreasing antioxidant capacity and (d) lowering acidity due to alkaline semen; 4) sexual transmission of (a) single infectious agents causing inflammation (STI), (b) BV biofilm consortia (pathogenic BV) or (c) *Lactobacillus* bacteriophages; 5) (a) drug abuse, (b) usage of antibiotics, or (c) administration of other medication/introduction of medical devices; 6) predisposing morbidity, such as diabetes/insulin resistance, which influence glycemia; 7) behavioral practices regarding vaginal hygiene; 8) genetic polymorphism among human hosts; and 9) different protective strengths of different *Lactobacillus* species.

Therefore, we might conclude that, in general, we do know the etiology of perturbation and no further research in this field is needed, but on the other hand, there are so many different and interwoven disturbing factors possible that it is almost impossible to know what causes the perturbation at a certain moment in a certain individual. For example, it is generally known that *L. iners* is less protective than *L. crispatus*, but perhaps colonization with *L. iners* vs *L. crispatus* depends itself on the genetic background of the host.

In conclusion, we might not need to look any further, because we have a long list of possible and probable causes and we might not bother too much about the lack of knowledge of what causes perturbation in a certain individual. This knowledge may have limited clinical relevance, except in cases where we can interfere with the cause, such as limiting the use of antibiotics or increasing the estradiol level or providing advice on behavior such as smoking and sexual activity. For example, Cools [79] came to the conclusion that limiting sexual activity during third-trimester pregnancy might be advisable to decrease the risk of *E. coli* colonization of the vagina. And smoking, for example, has been associated with increased risk of BV, which might be explained by the fact that the vaginal estradiol level of smoking women (119 ng/L) is well below that of non-smoking women (174 ng/L) [53]. For example, some studies point to a correlation between a fat-rich diet or high body mass index and increased risk of BV or prevalence of (less protective) *L. iners* (as reviewed in [5]).

3. Factors influencing/promoting the dominance of lactobacilli: glycogen and lactic acid

In fact, little is known about the way lactobacilli monopolize the vaginal econiche and recover from vaginal dysbiosis, although several mechanisms have been proposed: maintenance of low vaginal pH through production of lactic acid, production of antimicrobial substances such as H_2O_2 and bacteriocins, and competition for adherence to vaginal epithelial cells.

We will address the latter two only briefly, since data on bacteriocins and adherence competition are fragmentary and limited, and studies rarely address vaginal species.

3.1. Glycogen content, lactic acid and low vaginal pH

The knowledge that the vaginal epithelium is glycogen-rich dates back to at least 1897 (Menge and Krönig, cited by Cruickshank [80]). The concomitant presence of glycogen and lactobacilli during the first months of life (when the neonate vagina still contains maternal glycogen) and from puberty onwards, together with the absence of both glycogen and lactobacilli before puberty and after menopause, is a strong argument that vaginal glycogen accumulation favors colonization by lactobacilli, also because glycogen is believed to be the source of lactic acid after it has first been degraded to

maltose and glucose. The glycogen/lactobacilli/lactic acid correlation was confirmed by showing that vaginal samples with the highest free glycogen had the highest number of lactobacilli and that pH was significantly lower (pH 4.4) compared to samples with low glycogen (pH 5.8) [81]. Interestingly, high concentrations of glycogen corresponded to higher levels of *L. crispatus* and *L. jensenii*, but not *L. iners*.

Glycogen synthesis itself is believed to be stimulated by increasing estradiol levels. Wilson et al. [53] determined that the mean estradiol level was 39 ng/L in case of BV, compared to 176 ng/L in case of normal VMC. Glycogen content of the human vagina was determined as 0.2 μg glycogen/μg protein, compared to <0.001–0.004 μg in two species of *Macaca* monkeys [35]. Milwidsky et al. [82] showed that, in the endometrium, the glycogen content, as well as the activity of glycogen synthetase (GS, the rate-limiting enzyme in glycogen metabolism) and glycogen synthetase phosphatase (GSP, which activates GS through dephosphorylation), vary strongly during the menstrual cycle. During the proliferative phase (days 7–14), the activity of GSP is negligible and the glycogen concentration increases from less than 2 mg/g wet weight during days 1–15 to tenfold during the early part of the secretory phase, and the activity of GSP increases as well, from near absence to 20-fold during the secretory phase.

The synergistic effect of estrogen and progesterone on endometrial glycogen accumulation is well-known, according to Milwidsky et al. [82]; in other mammals as well, increased estrogen is related to lower pH, but never as low as in humans [37]. However, Wrenn et al. [83] did not observe any influence of progesterone, alone or in combination with estradiol, on glycogenesis. Recently, Mirmonsef et al. [84] were unable to establish a relationship between estrogen and free glycogen, but they did, instead, between progesterone and free glycogen, coinciding with the findings of Milwidsky et al. [82], since progesterone levels are highest at the mid-secretory phase.

3.2. The conversion of glycogen into lactic acid by lactobacilli

The debate on whether glycogen can be metabolized to glucose directly by lactobacilli, i.e., without prior degradation by a human enzyme such as alpha-amylase, dates back to at least 1908 [85] and is still not resolved. Contradictory but mostly negative previous findings were reviewed by Cruickshank [80], who, however, provided strong indications in favor of glycogen degradation by lactobacilli by studying in detail the development of vaginal acidity in newborn girls. Although vaginal pH was already lowered at birth (around 5.7) before colonization with 'Döderlein's bacilli', the pH plummeted to 4–4.5 in each child immediately and only upon colonization with vaginal lactobacilli, which occurred between 1 and 4 days after birth [80]. Part of the discrepancy among these reports might be due to the fact that several of the negative results were obtained with oyster glycogen, shown to be less accessible for degradation by lactobacilli [86,87].

The first report was in favor of glycogen degradation to glucose by vaginal lactobacilli [85]. Stewart-Tull [88] found that none of 36 vaginal/cervical *Lactobacillus* isolates of pregnant women could metabolize glycogen. Wylie and Henderson [86] found that only two out of 11 vaginal '*L. acidophilus*' strains from pregnant women were able to produce acid from human glycogen, and Martin et al. [89] did not observe acidification of 0.5% glycogen (source not specified) added to de Man Rogosa Sharpe agar for any of the 21 *L. crispatus*, 17 *L. jensenii* or six *L. gasseri* strains. Recently, Spear et al. [90] were unable to establish growth of vaginal *Lactobacillus* species (although *L. crispatus* and *L. iners* were not tested) on glycogen-containing resources. On the other hand, Cato et al. [91] found 9/14 *L. crispatus* strains to ferment glycogen and Boyd et al. [92] reported 8 out of 18 *L. crispatus* strains to be positive when tested with API CH50, unlike *L. jensenii* (n = 19), *L. gasseri* (20) and *L. iners* (20), which were all negative.

Contradictory findings exist as well at the level of genomic studies: Goh and Klaenhammer [93] identified a glycogen phosphorylase (glgP), which sequentially releases glucose moieties from the non-reducing ends of glycogen and forms glucose-1-phosphate in the genome of *L. crispatus* strain EM-LC1; Macklaim et al. [78] reported that, during BV compared to healthy conditions, *L. iners* strongly upregulated genes for the uptake of mannose and maltose, as well as genes for four glycosylases predicted to target α-1,6-glucosidic linkages that bridge the branching points in glycogen, i.e., genes for breakdown of glycogen and genes for mucin and glycerol transport systems. Still, more recently, France et al. [94] concluded that enzymes necessary to degrade glycogen are not present in the core genomes of *L. iners* and *L. crispatus*.

It is clear that the capacity for glycogen breakdown and usage by vaginal lactobacilli needs systematic study, and it should be noted that even the presence of genes necessary for the synthesis of glycogen has been described for the closely-related *L. acidophilus* [95].

A solution to the glycogen-usage question is possibly provided by Spear et al. [90,96], who found that vaginal (human) α-amylase is present that can degrade glycogen into dimers, trimers or tetramers of glucose, i.e., maltose, trimaltose and/or tetramaltose. However, this raises the question as to how these simpler saccharides can selectively favor lactobacilli, as they will stimulate growth of most bacterial species.

Nasioudis et al. [97] found that the median vaginal fluid α-amylase level was 1.83 mU/mL in 62 control women, compared to 1.07 mU/mL in 43 women with BV. Vaginal levels of α-amylase were further correlated with D-lactic acid, but not with L-lactic acid, and with hyaluronidase-1, matrix metalloproteinase 8 (MMP-8), neutrophil gelatinase-associated lipocalin (NGAL) and secretory leukocyte protease inhibitor (SLPI). The authors hypothesized that hyaluronidase-1 and MMP-8 increased the exfoliation of glycogen-rich epithelial cells into the vaginal lumen, leading to increased glycogen availability, and also promoted α-amylase activity, whereby a combination of both effects increased maltose availability, favoring proliferation of lactobacilli, explaining the observed increase in D-lactic acid and, finally, the lowered pH in the

vagina. Concomitant production of NGAL, a bacteriostatic agent that interferes with siderophore-mediated iron acquisition, and SLPI might retard growth of BV-related bacteria, which could contribute to a selective advantage for the (iron-independent; see 4.5) lactobacilli. Indeed, amylase activity leading to increased availability of simple saccharides would promote growth of most bacteria and therefore would not in itself provide a selective advantage for lactobacilli, unless lactobacilli are much more efficient in saccharide uptake and metabolism, or unless increased amylase activity is linked to concomitant production of substances that inhibit bacterial species other than lactobacilli.

3.3. Conclusions

It is rather amazing that more than a century after establishing a strong relation between glycogen, lactobacilli and lactic acid in the vagina, we are still ignorant about the ability of lactobacilli to metabolize glycogen. Nunn and Forney [98], assuming that lactobacilli cannot use glycogen directly, recently suggested that the association between abundant glycogen and abundant lactobacilli might stem from the combined inability of lactobacilli to use glycogen and their ability to suppress amylase-producing and glycogen-consuming other species.

4. Factors influencing/promoting dominance of the lactobacilli: hydrogen peroxide (H_2O_2)

4.1. Epidemiological data on the protective role of H_2O_2-producing lactobacilli

The protective role of H_2O_2-producing lactobacilli for the maintenance of eubiosis, as well as for the prevention of APO, has been well-documented by the groups of Holmes, Eschenbach, Hillier and Klebanoff [14,22,99–103] and the potential usefulness of H_2O_2 as an antimicrobial agent in the vagina was already recognized and clinically tested as early as in 1949 [104]. For example, in the study of Eschenbach et al. [99], 96% of women with normal VMC harbored H_2O_2-generating lactobacilli, whereas only 6% of women with bacterial vaginosis harbored H_2O_2-producing lactobacilli, and 36% harbored lactobacilli which did not generate H_2O_2.

Antonio et al. [22] found that *L. crispatus* (16%), *L. jensenii* (10%) and *L. gasseri* (10%) were the prevalent lactobacilli colonizing the rectums of 290 females. Only 13 (9%) of 147 females colonized by *L. crispatus* or *L. jensenii* vaginally and/or rectally had bacterial vaginosis (BV), compared with 12 (44%) of 27 females colonized by other lactobacilli. Co-colonization of the vagina and rectum by H_2O_2-producing lactobacilli was associated with the lowest prevalence of BV (5%), whereas females colonized only vaginally, only rectally or at neither site had a successively increased risk of BV. *L. jensenii* was the strongest producer, followed by *L. crispatus* [105].

It should be mentioned that not all studies are unambiguous with regard to the protective effects of H_20_2-producing lactobacilli, as reviewed by Pybus and Onderdonk [106].

4.2. In vitro data on the antimicrobial role of H_2O_2-producing lactobacilli

Although H_2O_2-producing lactobacilli have been shown to inactivate HIV-1, herpes simplex virus type 2 (HSV-2), *E. coli*, *G. vaginalis*, *Prevotella bivia* and *T. vaginalis* in vitro, Tachedjian et al. [4] question the direct role of H_2O_2 as a protective agent, a role that has been defended by Klebanoff [102,103], and they suggest that H_2O_2 production is merely a biomarker for probiotic lactobacilli, which are health-promoting for other reasons. For example, the (mostly) H_2O_2-negative *L. iners* is known to be less protective [5], but this might be due to other characteristics such as the lack of D-lactic acid production or the production of inerolysin [5].

Klebanoff et al. [102] found that lactobacilli at high concentrations were toxic to *G. vaginalis.*

When the concentration of lactobacilli was lowered to a level where it was ineffective alone, the addition of myeloperoxidase (MPO) (see 4.3) and chloride re-instituted toxicity. Moreover, since toxicity was inhibited by catalase and was not seen when H_2O_2-negative lactobacilli were used, and because lactobacilli could be replaced by H_2O_2 and chloride by iodide, bromide or thiocyanate, they concluded that H_2O_2 was the toxic molecule.

Further indications of the microbicidal role of H_2O_2 come from inhibition of *S. aureus* by *L. crispatus*, which can be abolished by addition of catalase [107], and inhibition of gonococci in both acidic and neutral pH conditions by *L. crispatus* and *L. jensenii*, that could be neutralized by bovine catalase [108].

However, convincing arguments exist against the ability of H_2O_2 to act as a bactericidal agent under conditions that prevail in the healthy vaginal econiche [4,109,110]. O'Hanlon et al. [109] demonstrated that addition of only 1% of cervicovaginal fluid and semen blocks the activity of 1 M H_2O_2, and that physiological concentrations of H_2O_2 below 100 μM fail to inactivate any of the 17 tested BV-associated bacterial species, even in the presence of MPO. Only supra-physiological concentrations of exogenous H_2O_2 (100 mM) were sufficient to inactivate BV-associated bacteria at which concentration, however, lactobacilli were more potently inactivated. A concentration of 100 mM H_2O_2 is approximately 50-fold-higher than lactobacilli are capable of producing even under optimal aerobic, low-antioxidant conditions, and approximately 5000-fold higher than the estimated H_2O_2 concentration in vivo. Indeed, Strus et al. [111] found that H_2O_2 reached concentrations of 0.05–1.0 mM for cultured lactobacilli which, under intensive aeration, increased up to 1.8 mM; Ocana et al. [106] found that the levels of H_2O_2 in *L. crispatus* supernatant were at a maximum at the early stationary phase (3.29 mM) under aerated conditions, i.e., in agitated cultures, but that there were no detectable levels of H_2O_2 in non-agitated cultures. Hertzberger et al. [112] reported up to 1 mM H_2O_2 upon aeration.

Possible explanations may be that cervicovaginal fluid and semen contain proteins, glycoproteins, polysaccharides, lipids and other molecules with the potential to react with and

inactivate H_2O_2. In addition, the vagina is hypoxic most of the time, whereas lactobacilli require oxygen to produce H_2O_2. Indeed, there is a general problem regarding the limited rate of H_2O_2 production in the absence of molecular oxygen, as is the case in the anaerobic conditions of the vagina, whereas most studies indicate increase in H_2O_2 production only upon oxygen exposure (see above). When lactobacilli are grown in an aerobic environment, NADH oxidases and pyruvate oxidases compete with lactate dehydrogenase for NADH and favor the production of H_2O_2 rather than lactate. But, assuming a vaginal low-level oxygen tension, the lactobacilli would be expected to favor fermentation, i.e., lactacte dehydrogenase activity, producing lactate rather than H_2O_2 as the major metabolic end product.

On the other hand, Whittenbury et al. [113] reported H_2O_2 production in both aerobic and anaerobic conditions, and Hertzberger et al. [112] established constitutive H_2O_2 production, also in anaerobic conditions (although H_2O_2 production was strongly increased when oxygen was present). Moreover, it has been noted that even in the gut oxygen gradients exist near the mucosa [110]. Moreover, sexual intercourse/sexual stimulation [114] or introduction of vaginal devices (tampons, rings) may cause temporary oxygenation, exactly at moments when H_2O_2 production might be needed as an additional protection against dysbiosis.

4.3. The mechanisms of antimicrobial activity of reactive oxygen species (ROS)

The presence of atmospheric oxygen (dioxygen, O_2) results in the generation of highly reactive oxygen species, predominantly superoxide (O_2^-) and hydrogen peroxide (H_2O_2), which attack any oxidizable group, e.g., sulfhydryl groups, iron–sulfur (Fe–S) centers, sulfur-ether groups and Fe-heme groups, and as a consequence, there may be a loss of microbial membrane transport and interruption of the membrane electron transport chain. Dismutation of superoxide yields H_2O_2, and Klebanoff [103] showed how human MPO, in the presence of halides (such as Cl^-), can also convert the H_2O_2 to hypochlorite (HOCl) during the neutrophil's respiratory burst. At low pH, HOCl predominates, and it can react with excess chloride to form molecular chlorine (Cl_2). MPO also oxidizes tyrosine to tyrosyl radicals using H_2O_2 as an oxidizing agent. All of these products are highly reactive short-lived oxidizing agents that are used by neutrophils to kill pathogens. MPO needs heme which causes the green color of pus (rich in neutrophils). This was dubbed the MPO-H_2O_2-chloride antimicrobial system.

Klebanoff et al. [101,102] established that vaginal fluids of 17 out of 21 women contained adequate amounts of MPO, and that amounts of MPO in uterine mucus are increased during ovulation. However, inhibition of bacteria occurred at an optimum pH of 5.0–6.0 [102]. Klebanoff [103] concluded that the finding that lactobacilli can serve as a source of H_2O_2 for the MPO-mediated antimicrobial system is pertinent to the female genital tract.

McLean and McGroarty [115] were able to confirm the results of Klebanoff et al. [102] that incubation of lactobacilli and *G. vaginalis* in the presence of MPO (75 mU/ml) and NaCl (0.01 M) under aerobic conditions and with agitation reduced the number of *G. vaginalis* cells by up to 2000-fold at the 'optimum' pH of 5–6 (but see Atassi and Servin [116], below). However, under conditions more closely resembling those of the vagina, i.e., static and anaerobic incubation, *G. vaginalis* was not killed. McLean and McGroarty [115] found that at a low pH (±4), lactic acid accounted for 60–95% of inhibitory activity of different *Lactobacillus* species on *G. vaginalis* isolates, and H_2O_2 for only 0–30%. These findings raise the question as to how important this system would be in control of *G. vaginalis* growth in vivo, given the low-level oxygen tension and the low pH of the healthy vagina, and they suggest that the production of acids such as lactic acid and a low pH are of more significance in the vaginal ecosystem. Also, O'Hanlon et al. [109,110] showed that, under optimal anaerobic growth conditions, physiological concentrations of lactic acid inactivated BV-associated pathogens without affecting the vaginal lactobacilli.

To further add to the controversy, it should be noted that, in contradiction with an optimum activity of H_2O_2 at elevated pH, as established by Klebanoff et al. [102], Atassi and Servin [116] reported increased bactericidal effects of H_2O_2 in the presence of lactic acid, i.e., at low pH (see also 4.6).

4.4. The sources of vaginal H_2O_2

4.4.1. Superoxide as an antimicrobial agent

Superoxide is an anion that is the product of a one-electron reduction of O_2, i.e., of adding one electron to O_2, and can be the result of oxygen entering the reducing atmosphere inside bacterial cells or of active production as an antibacterial product by the eukaryotic NAD(P)H oxidases, present in leukocytes and vascular cells [103]. When two superoxide molecules interact, superoxide dismutation occurs, whereby one O_2^- is oxidized with the formation of O_2 and the other is reduced with the formation of H_2O_2, another ROS. This reaction can occur spontaneously or can be catalyzed by superoxide dismutase (SOD). Spontaneous dismutation occurs optimally at pH 4.8 at a rate constant approaching that of SOD-catalyzed dismutation [103].

Considering the presence of superoxide molecules both produced by leukocytes and/or present in the vaginal transudate, and the colonization of the vagina with lactic-acid-producing lactobacilli, one can hypothesize that the H_2O_2 concentration might increase as a consequence of dismutation of superoxides which occurs spontaneously under conditions of low pH.

4.4.2. H_2O_2 as the result of the inability of anaerobic lactobacilli to neutralize H_2O_2

In aerobes, further reduction of H_2O_2 can be achieved by catalases, which convert two peroxides into H_2O and O_2, or by peroxidases, i.e., peroxide reductases which use, for example, NADH as the electron donor to reduce H_2O_2 to H_2O, with production of an oxidized substrate such as NAD^+ that can be re-used in glycolysis.

However, enzymes to convert O_2^- to e.g., H_2O_2 (SOD) and to convert H_2O_2 to H_2O (catalases and peroxidases) are largely lacking in many anaerobes, which evolved to live in oxygen-free environments. Especially in the case of vaginal lactobacilli, H_2O_2 accumulates in the medium because they seem to lack (most of all lactobacilli) efficient H_2O_2-scavenging mechanisms such as catalase (Fe-heme-dependent as well as Mn-nonheme-dependent) [105], NADH peroxidase and manganese antioxidant complexes [117].

4.4.3. H_2O_2 as the result of active production by lactobacilli

France et al. [94] found that *L. crispatus* encodes for a pyruvate oxidase, leading to production of H_2O_2. Condon [116] indicated that H_2O_2 can also be generated from O_2 during glycerol metabolism by, for example, glycerol-3-phosphate oxidase. However, Hertzberger et al. [112] found that H_2O_2 production is not dependent on predicted pyruvate oxidase-, lactate oxidase- or NADH oxidase-encoding genes. Probably, most H_2O_2-production results from the iron-free lifestyle that lactobacilli have developed, not only by using mangano-catalases instead of Fe-heme-containing catalases and by using Mn-SOD instead of Fe-SOD, but also by replacing the Fe-heme-containing cytochrome *c* system for terminal oxidation by flavoproteins [103,118]. Ironically, carrying out terminal oxidation by means of an Fe-dependent cytochrome system results in the production of water from oxygen, and doing so by means of flavoproteins results in production of H_2O_2 from oxygen [112].

Hertzberger et al. [112] found that a newly identified constitutive NADH-dependent flavin mononucleotide reductase of *L. johnsonii*, also present in most *L. acidophilus* group species, is the primary source of lactobacillar H_2O_2.

Indeed, flavoprotein oxidases had been previously recognized as the enzymes responsible for H_2O_2 production in lactic acid bacteria [118].

4.5. How do lactobacilli protect themselves against H_2O_2? Iron abstinence

Lactobacilli, together with other lactic acid bacteria such as streptococci, are notorious for their vitamin requirements, which are even more extensive than those of humans. For example, they cannot produce heme. On the other hand, lactobacilli are exceptional because they do not require iron for growth [119], instead relying largely on manganese and cobalt in the active centers of their redox enzymes. So, despite their nutritional demands, their iron abstinence might put them at an advantage in iron-deprived environments, as is the case in animal bodies.

The outcome of nearly all encounters of iron-dependent microbial pathogens with vertebrate hosts is determined in part by the ability of the pathogen to extract iron from the host and the ability of the host to withhold the metal from the pathogen [120]. While the host tries to reduce concentrations of free iron to limit bacterial growth, low iron concentrations may also induce bacterial virulence characteristics, whereby the bacteria try to gain access to the cellular iron, e.g., by invading or lysing host cells. Perhaps lactobacilli rarely cause cellular damage and infection and earned their status as probiotic bacteria in part because they are not that much in need of iron.

The limited metabolic capacities of lactobacilli may put them at a disadvantage in iron-rich environments, where they become quickly overgrown by other, no longer limited bacteria. Is this the explanation as to why lactobacilli – except for the (inerolysin-positive!) *L. iners* – disappear during menses, when plenty of iron-rich heme from lysed/lysable red blood cells is supplied? On the other hand, H_2O_2 becomes a more potent antimicrobial in iron-rich environments due to Fenton chemistry (see below).

Both iron and manganese can serve as cofactors for enzymes that remove ROS (mainly SODs and catalases), but the oxidative damage caused by both metals differs dramatically. Iron is well known for its reactivity with ROS, generating the highly reactive hydroxyl radical (OH) through so-called Fenton chemistry, whereby ferric acid (Fe^{3+}) is reduced by superoxide, producing ferrous acid (Fe^{2+}) and H_2O_2, with the latter reoxidizing ferrous iron to ferric ions with production of more oxygen radicals. Similarly, nitric oxide is not hazardous to lactobacilli because metabolic dysfunctions induced by nitric oxide mainly involve its combination with iron in various heme- and non-heme enzymes [120].

Manganese is less prone to such chemistry due to a higher reduction potential. Iron often acts as a pro-oxidant under situations in which manganese is an anti-oxidant. Without the deleterious side effects of Fenton chemistry, manganese can safely operate as a cofactor for superoxide dismutase (Mn-SOD) and non-heme-based, i.e., non-iron based, mangano-catalase, and furthermore provide oxidative stress resistance through formation of non-proteinaceous manganese-based anti-oxidants, i.e., Mn-rich complexes [117].

Although many or most vaginal lactobacilli lack SOD, the enzyme used by most bacteria to detoxify superoxide, this may be less of a problem in an acidic environment, as is the case in the vagina, because of spontaneous dismutation of superoxides that occurs at low pH [103]. For further reduction of H_2O_2, some lactobacilli produce mangano-catalases, and in one study [113], lactobacilli could develop catalase activity, but only when cultured on lysed blood cells, indicating that they could produce the apo-enzyme but not the heme co-factor, hence their vitamin dependency. However, catalase activity seems to be largely absent from vaginal lactobacilli.

Besides their innate reduced vulnerability to the strong oxidative stress which arises from their iron abstinence, lactobacilli can produce SOD and accumulate high levels of Mn^{2+}, growing equally well aerobically as anaerobically; they can produce only (Mn-)SOD, still growing well aerobically, or they contain neither, becoming oxygen-intolerant, which is defined as 'strictly anaerobic', although Condon [118] correctly points out that this terminology is not appropriate in the case of lactic acid bacteria.

Some lactobacilli can accumulate up to 7000 μM of H_20_2 without loss of viability, whereas the LD50 of H_20_2 has been shown to be only 10 μM for *S. aureus* [120]. Aguirre and

Culotta [117] showed that *L. plantarum*, lacking Mn-SOD, accumulates up to 20 mM of Mn, compared to the low μM concentrations in other organisms, but the '*L. acidophilus*' isolates studied were the ones that were not accumulating Mn.

Although most strains of *L. crispatus* grow well aerobically, it is unfortunate for our efforts to explain how vaginal lactobacilli might tolerate their own production of H_2O_2, that they seem to belong to the oxygen-intolerants, lacking SOD, catalase and manganese antioxidant complexes. Thus, despite their iron abstinence, the limited ROS-neutralizing capacity of vaginal lactobacilli might render them especially vulnerable to their own ROS production. Indeed, in vitro growth of lactobacilli is inhibited by their own H_2O_2 production, since addition of catalase to aerated cultures of *L. johnsonii*, accumulating up to 1 mM H_2O_2, abolished growth stagnation [112]. This was also the outcome of the study by O'Hanlon et al. [109], who showed that in vitro (at neutral pH!), H_2O_2 was more toxic toward vaginal lactobacilli than toward BV-associated bacteria (see also 4).

Interestingly, Hertzberger et al. [112] suggest that oxidative stress generated by the NADH-dependent flavin mononucleotide reductase (Nfr) of *L. johnsonii*, shown to be primarily responsible for production of endogenous H_2O_2, may, in fact, enable *L. johnsonii* to prevent or reduce oxidative stress. Because cytoplasmatic flavins may be the most readily oxidized parts in the cytoplasm, they may most effectively capture oxygen and, as such, prevent other, more damaging effects of oxygen, such as direct oxidation of iron–sulfur clusters or formation of semiquinones. In summary, NADH-dependent reduction of oxidized flavins by Nfr may restore their oxygen-capturing capacity, of use in terminal oxidation and, as such, bypassing the need for iron-containing heme groups of the cytochrome-based respiration systems and resulting production of H_2O_2 (instead of H_2O, as in cytochrome-systems), may be preferred over other uncontrollable effects that might result from oxygen.

Indeed, the *nfr* deletion derivative failed to produce H_2O_2 upon exposure to O_2 and reduced aerotolerance compared to its wild-type counterpart, confirming that the reaction catalyzed by this enzyme may enable *L. johnsonii* to prevent or reduce oxidative stress.

4.6. Lactic acid or H_2O_2 as the predominant antimicrobial

There is much controversy regarding the importance of H_2O_2 in vaginal colonization resistance provided by lactobacilli [4,109,110]; rather, the bactericidal effects of lactobacilli are believed by several research groups to be due to the production of lactic acid. Strus et al. [111] found that the most potent inhibitory activity against bacteria and yeasts was achieved by supernatants of cultures from H_2O_2-producing lactobacilli, followed by the non-producing strains, and only thereafter by pure H_2O_2. They concluded that the antimicrobial activity of lactobacilli is a summation of various inhibitory mechanisms in which H_2O_2 plays an important but not crucial role.

Possibly, however, this is not a matter of 'lactic acid or H_2O_2' rather than both lactic acid and H_2O_2.

Atassi and Servin [116] conclude that there is synergism between H_2O_2 and lactic acid, which may be the consequence of at least two possible pathways. First, lactic acid can induce the acid tolerance response, which causes H_2O_2 sensitivity in e.g., *Salmonella typhimurium* via downregulation of the OxyR regulon. A second mechanism could result from the permeabilizing effect of lactic acid on the Gram-negative bacterial outer membrane, thus facilitating the passage of molecules across the membrane, increasing the effects of antimicrobial compounds.

Organic acids may also contribute to lactobacillar ROS tolerance because the low pH promotes spontaneous superoxide dismutation and because lactate can chelate Mn^{3+} and thus stabilize Mn^{3+} in solution [121], also increasing H_2O_2 tolerance through Mn-dependent anti-oxidant activity. Moreover, although Mn^{2+} is in itself a weak scavenger of superoxide, this ability is strongly enhanced by its interaction with e.g., lactate [116,121], abundantly present in lactobacillar cells.

We need to learn more about the pivotal roles played by Fe and Mn with regard to increasing the toxicity of ROS toward vaginal microbes, as well as increasing tolerance of vaginal lactobacilli to ROS.

4.7. Conclusion

Although several studies indicate that vaginal lactobacilli do not easily tolerate exogenous H_2O_2, the answer to resistance to endogenous H_2O_2 production may be offered by the realization that their abstinence from iron provides innate reduction of vulnerability to H_2O_2, because of the absence of Fenton chemistry which generates oxidizing agents in other bacterial species [120]. At the same time, the use of flavoproteins instead of Fe-heme-containing cytochromes in respiratory metabolisms, which contributes to their iron independence [112,118], leads to increased generation of endogenous H_2O_2, but this is a lower price to pay than uncontrolled oxygen damage, which is prevented by the fact that these flavins are most readily oxidizable, as such taking away the ROS burden from other molecules. Moreover, the activity of flavins is readily restored by Nfr, such that their role in respiration is not jeopardized.

The high prevalence of women colonized by (mostly) H_2O_2-negative *L. iners* indicates that H_2O_2 production is not needed to monopolize the vaginal econiche, but at the same time, it is now well-established that women colonized with *L. iners* are at higher risk of vaginal dysbiosis and even adverse pregnancy outcome [5], indicating that H_2O_2 production might play an important role in protection, although it is also possible that H_2O_2 production is merely a marker for lactobacilli that are more protective for other reasons.

The importance of iron in vaginal microbiology may have some implications for the administration of lactobacilli as vaginal probiotics. Weinberg [120] suggests that vaginal probiotics, to our knowledge already considered in 1920 [122] and addressed in more detail elsewhere in this issue [4],

should be administered together with iron-sequestering agents (such as lactoferrin). Indeed, we might think further and consider what simply decreasing iron and increasing manganese might do for promoting lactobacillar growth, even in the absence of probiotically administered strains.

5. Factors influencing/promoting the dominance of the lactobacilli: bacteriocins

Besides lactic acid and H_2O_2, bacteriocins are usually mentioned as antimicrobial compounds produced by lactobacilli. These have been reviewed elsewhere [123–125], but data are fragmentary and we found only one broad study regarding bacteriocins of vaginal lactobacilli [125].

6. Factors influencing/promoting the dominance of the lactobacilli: adherence

It remains to be considered to what extent adherence competition may play a role in lactobacillar dominance and vaginal colonization resistance in vivo, given an estimated microscopy-based average density of five lactobacillar cells per epithelial cell. Still, several in vitro studies show that lactobacilli can prevent/inhibit adherence of other bacterial species. The extracellular protein surface layer (S-layer) of *L. crispatus*, well-preserved in most other *L. acidophilus* group species [126], can inhibit adherence of *E. coli* O157:H7 to HeLa cells [127,128]. S-layers may also play roles in enhancing the bactericidal activity of bacteriocins, as synergism was shown between nisin and the *L. acidophilus* S-layer protein [129].

Adherence of L. *crispatus* may also proceed by means of 'moonlighting proteins', such as glutamine synthetase, glucose-6-phosphate isomerase, enolase and glyceraldehyde-3-phosphate dehydrogenase, which are bound to the bacterial surface at acidic pH [130].

7. Interference of lactobacilli with vaginal immunity

7.1. Anti-inflammatory effects of lactic acid and H_2O_2

Possibly, beneficial effects in general, and colonization resistance offered by H_2O_2 production of lactobacilli might result from the anti-inflammatory, non-microbicidal effects of H_2O_2. For example, Mitchell et al. [131] found that H_2O_2-producing lactobacilli are associated with lower levels of pro-inflammatory vaginal interleukin-1 β, independently of bacterial vaginosis, although here too, a direct link with H_2O_2 remains to be established.

It has been suggested that H_2O_2 can contribute to an anti-inflammatory effect through its influence on the peroxisome proliferator activated receptor γ (PPAR-γ), which plays a central role in regulation of intestinal inflammation and homeostasis. Expression of PPAR-γ has been shown to be induced in vivo and in vitro by the presence of *L. crispatus*, and to be inhibited by the addition of either catalase or glutathione, pointing to H_2O_2 as the responsible factor for the observed induction [132].

The S-layer protein of *L. acidophilus* has been shown to be involved in regulation of immature dendritic cell and T-cell functions [133]. Somewhat surprisingly, *L. crispatus* has been shown to enhance activation of the proteolytic plasminogen (Plg)/plasmin system, which is also engaged by a number of pathogenic microbial species to increase tissue invasiveness and to obtain nutrients [134].

7.2. The importance of host polymorphism in vaginal innate immunity and mucosal make-up

Spear et al. [90] observed that the vaginal fluids of some women contain an amylase that is also active at lower pH, and perhaps these women might be more prone to glycogen depletion and loss of lactobacilli. The frequency of many gene polymorphisms varies in different racial groups, and this may also relate to population differences in the composition of the vaginal ecosystem. The group of Witkin has extensively studied host gene polymorphism for different markers of (innate) immunity, showing that polymorphism in genes such as the anti-inflammatory mediator interleukin-1 receptor antagonist or TLR-4 (the cell surface receptor for innate immune recognition of Gram-negative bacteria) influences the bacterial composition of the vagina [1]. The same group showed that extracellular hsp70, produced in response to abnormal vaginal microflora, induces the release of nitric oxide in the vagina [135]. Women deficient in mannose-binding lectin production have been shown to be more susceptible to developing recurrent vulvovaginal candidiasis [136].

8. Conclusions

In summary, it may be advisable to consider only H_2O_2-producing vaginal lactobacilli as the normal colonizers of the human vaginal econiche, because of their well-established health-promoting capacities that are of importance to women, their partners and their unborn and newborn children. Many well-known causes can disturb the normal VMC, and the most important question lies in the mechanism(s) by which the unique condition of lactobacillar dominance and low vaginal pH, unseen in any other mammal, is maintained. There is a clear connection between estrogen/progesterone production and glycogen production/storage by vaginal epithelial cells, leading to glycogen degradation, with some controversy as to whether lactobacilli are able to do so or depend on human enzymes such as alpha-amylase. Subsequent glycolysis by the bacteria of glucose (-oligomers) into pyruvate and fermentation of pyruvate into D- and L-lactic acid results in reduction of the vaginal pH, which is important for its direct bactericidal activity, for enhancing the bactericidal activity of H_2O_2, for deactivation of bacterial virulence enzymes, and for its anti-inflammatory properties. The extent to which these depend on low pH rather than on specific production of D-lactic acid compared to L-lactic acid is a new venue of research. Also, whether H_2O_2 is important in preserving the predominance of the lactobacilli or is merely a marker of lactobacilli that can do so remains a matter of debate. With regard to the role of H_2O_2,

we need to learn more about the extent to which iron abstinence is developed in vaginal lactobacilli, as this plays a role in H_2O_2 production and in resistance to H_2O_2.

It is rather surprising that, after more than a century of research, there is apparently still much cause for debate, even on essential issues, and that controversial reports continue to be published. Because of the huge impact of vaginal microbiology on women's health and on pregnancy outcome, it is important to resolve these controversies to obtain better insight into vaginal microbiology and its interplay with vaginal immunity, to further improve vaginal probiotics and strategies for maintaining and/or (re-) installing lactobacillus dominance.

Conflict of interest

The author declares no conflict of interest.

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