Biosynthesis and degradation of H2O2 by vaginal Lactobacilli

Rebeca Martín, Juan E. Suárez

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 2010, p. 400–405 Vol. 76, No. 2
0099-2240/10/$12.00 doi:10.1128/AEM.01631-09

Biosynthesis and Degradation of H_2O_2 by Vaginal Lactobacilli[▽]

Rebeca Martín[1] and Juan E. Suárez[1,2]*

Area de Microbiología, Instituto Universitario de Biotecnología, Universidad de Oviedo, Oviedo,[1] and Instituto de Productos Lácteos de Asturias (CSIC), Villaviciosa,[2] Spain

Received 10 July 2009/Accepted 15 November 2009

Hydrogen peroxide production by vaginal lactobacilli represents one of the most important defense mechanisms against vaginal colonization by undesirable microorganisms. To quantify the ability of a collection of 45 vaginal *Lactobacillus* strains to generate H_2O_2, we first compared three published colorimetric methods. It was found that the use of DA-64 as a substrate rendered the highest sensitivity, while tetramethyl-benzidine (TMB) maintained its linearity from nanomolar to millimolar H_2O_2 concentrations. Generation of H_2O_2 was found to be especially common and strong for *L. jensenii* strains, while it was variable among *L. crispatus* and *L. gasseri* strains. Biosynthesis of H_2O_2 only occurred upon agitation of the cultures, but the H_2O_2-producing machinery was already present in them before aeration started. Calcium, magnesium, manganese, and zinc ions did not affect H_2O_2 production, while Cu^{2+} inhibited the growth of *Lactobacillus jensenii* CECT 4306, which was chosen as a model strain. Cultures with Fe^{3+}, hemin, and hemoglobin did not accumulate H_2O_2. Fe^{3+} activated an extracellular peroxidase that destroyed the H_2O_2 being produced by the cultures. This protected the lactobacilli against its antimicrobial effect. The production of the enzyme appears to be constitutive, the Fe^{3+} ions being a necessary cofactor of the reaction.

The vaginal microbiota of healthy, fertile women is dominated by lactobacilli. Their proportion in this habitat is consistently higher than 70%, in some cases being practically exclusive (8, 20, 26). This abundance contrasts with their scarcity on other mucosae; for example, they never account for more than 1% of the intestinal microbiota and, in many cases, they are not found in feces (35).

Upon first isolation, the vaginal lactobacilli behave as fastidious microorganisms, whose recovery and growth are improved by the addition of a heme source, such as hemin or hemoglobin, to the medium and by incubation under an atmosphere enriched to 10% CO_2 or even under anaerobic conditions. The identification of newly isolated strains leads to their ascription to *Lactobacillus acidophilus* or *L. fermentum* when culture-dependent methods are used (1, 30). However, genotyping reveals a predominance of *L. crispatus*, *L. gasseri*, and *L. jensenii*, with *L. iners* and *L. vaginalis* also being found (5, 6, 34).

The dominance of the lactobacilli in the vagina suggests that they play a fundamental role in the protection of the cavity against the generation of pathological conditions. Among them are those caused by the excessive proliferation of indigenous microorganisms, such as *Gardnerella vaginalis*, whose dominance may lead to bacterial vaginosis, and colonization by pathogens, such as *Candida* spp. and *Trichomonas vaginalis*, which may induce vaginitis or, less frequently, cervicitis and other regional and systemic problems (15, 24, 28).

The antagonistic properties of the vaginal microbiota have two complementary aims: promotion of specific adherence to the epithelial cells and production of antimicrobial substances. Of these, the generation of organic acids and hydrogen peroxide is of paramount importance. Lactobacilli present a strictly fermentative metabolism of sugars, with production of mainly lactic acid (4). As a consequence, the physiological pH of the vagina is around 4, which is inhibitory to the growth of many potential colonizers. In fact, a differential symptom of bacterial vaginosis is an exudate with a pH close to neutral (24, 28).

Hydrogen peroxide generation appears to be common among vaginal lactobacilli, while it is exceptional in strains that usually live in the intestines or in the external environment, such as *L. plantarum* and *L. paracasei*, even if they are isolated from the vagina (29). Hawes et al. (13) have shown that the frequency of vaginosis is lower among women that harbor H_2O_2-producing lactobacilli (3%) than among those colonized by H_2O_2-negative strains (25%) or by other bacterial species (46%). However, not all H_2O_2-positive lactobacilli appear to be equally protective, with the most efficient strains belonging to the species *L. crispatus* and *L. jensenii* (2).

The bactericidal effect of H_2O_2 operates through the generation of oxidizing metabolites, such as the radical OH^- that introduces breaks in the DNA of the cell. This effect is notably enhanced in the acidic vaginal environment by the presence of myeloperoxidase and halides, which are very abundant in the uterine mucus, especially during ovulation (17, 18).

Given the apparent importance of hydrogen peroxide in the maintenance of vaginal homeostasis, we wanted to know the capacity for generation of this compound of a collection of vaginal lactobacilli, as part of selecting those with the best probiotic properties (19). Previously, we compared the sensitivity and range of measurable concentrations provided by three published H_2O_2 quantification methods (11, 22, 25). We have also studied the influence of a series of environmental conditions on the production and persistence of H_2O_2 in the cultures and how H_2O_2 affects the viability of the lactobacilli producing it.

* Corresponding author. Mailing address: Area de Microbiología, Facultad de Medicina, Universidad de Oviedo, Julián Clavería 6, 33006 Oviedo, Spain. Phone: 34 985103559. Fax: 34 985103148. E-mail: evaristo@uniovi.es.

▽ Published ahead of print on 30 November 2009.

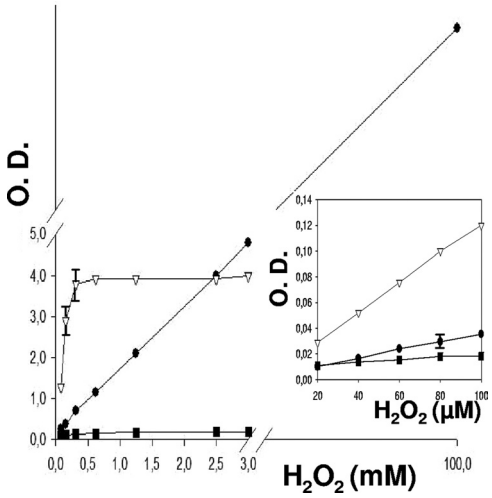

FIG. 1. Standard curves of the relation between the concentration of hydrogen peroxide and the intensity of color produced by its reaction with the chromophores tetramethylbenzidine (●), *o*-dianisidine (■), and DA-64 (▽), catalyzed by horseradish peroxidase (the wavelengths used were 620, 405, and 727 nm, respectively). DA-64 was the most sensitive at low concentrations of hydrogen peroxide (inset). Error bars show standard deviations. O.D., optical density.

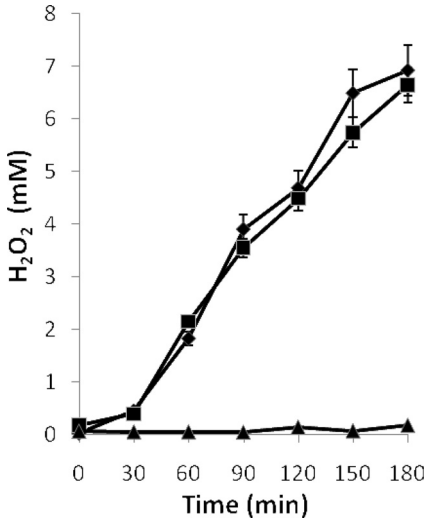

FIG. 2. Production of hydrogen peroxide by exponential-phase cultures of *L. jensenii* CECT 4306 (8 h of incubation) subjected to aeration (■) or maintained in static conditions (▲). The same culture was treated with chloramphenicol 30 min before the start of aeration (◆). Error bars show standard deviations.

MATERIALS AND METHODS

Microorganisms and culture conditions. In this study, 45 vaginal isolates of *Lactobacillus*, obtained from healthy premenopausal women, were used. They were previously classified as *L. jensenii* (17 strains), *L. crispatus* (21 strains), *L. gasseri* (6 strains), and *L. plantarum* (one strain) (19). *L. jensenii* CECT 4306 was used as a positive control for H_2O_2 generation. The bacteria were grown on MRS agar (Oxoid) supplemented with 1% hemoglobin (Sigma) and incubated at 37°C under a 10% CO_2 atmosphere. Liquid cultures were performed in MRS medium and incubated under the same conditions without agitation. Inhibition of protein synthesis was performed with 100 μg/ml chloramphenicol (it was previously determined that the MIC was 1.5 μg/ml).

Quantitative hydrogen peroxide determination tests. The concentration of H_2O_2 was determined colorimetrically through detection of the absorbance induced with the following chromophores: *N*-(carboxymethylaminocarbonyl)-4,4′-bis(dimethylamino)-diphenylamine sodium salt (DA-64) (Wako), 3,3′,5,5′-tetramethylbenzidine (TMB) (Sigma), and *o*-dianisidine (Sigma) (11, 22, 25). The reaction mixtures were set up as follows: a commercial H_2O_2 stock solution (30%, corresponding to 9.812 M) was diluted with 100 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES) buffer (pH 6.5) or MRS broth to 1 M. The working concentrations were prepared by diluting this solution with PIPES buffer by a factor of at least 10 (to avoid any color interference by the MRS broth), and 100-μl amounts of the resulting solutions were mixed with 100 μl of the corresponding chromophore (10 mM, final concentration) and 2 μl of a horseradish peroxidase solution (1 mg/ml). After incubation for 10 min at 16°C, the absorbance was measured at 405, 620, and 727 nm for *o*-dianisidine, tetramethylbenzidine, and DA-64, respectively.

H_2O_2 generation by the *Lactobacillus* cultures. Cultures were set up in 10 ml of liquid MRS medium by inoculation with 1 ml of a 48-h-old culture. Incubation proceeded for several time periods (usually 8 or 16 h) at 37°C under a 10% CO_2 atmosphere or anaerobically, without agitation. The following cofactors (2 mM, final concentration) were added to the cultures to determine their influence on H_2O_2 production: $MgCl_2$, $MnSO_4$, $FeCl_3$, $CaCl_2$, $ZnCl_2$, and $CuSO_4$ (Sigma). Hemoglobin and hemin (1% and 30 mM final concentration, respectively) (Sigma) were also tested. At the end of the incubation period, H_2O_2 generation was induced by placing the cultures in 100-ml Erlenmeyer flasks, which were subjected to agitation at 300 rpm for up to 3 h at 37°C. Samples (220 μl) were taken every 30 min and centrifuged for 2 min at 12,000 rpm, and the supernatants stored at 4°C until the end of the experiment. The concentration of H_2O_2 was immediately determined as described in the previous paragraph, using at least three replicates from each sample.

H_2O_2 degradation measurements. Cultures of *L. jensenii* CECT 4306 were set in liquid MRS medium with or without 2 mM $FeCl_3$. After incubation for 16 h at 37°C, the culture grown without added Fe cations was divided into two aliquots and one of them was adjusted to 2 mM Fe^{3+}. Fifteen minutes later, H_2O_2 (2.5 mM final concentration) was added to the three cultures and incubation continued at 37°C for 3 h. Samples were taken every 15 min, and the residual H_2O_2 concentration was quantified with tetramethylbenzidine.

RESULTS

Comparison of H_2O_2 determination tests. H_2O_2 in solution was quantified by measuring the degree of color generation by oxidation of 3,3′,5,5′-tetramethylbenzidine (TMB), *o*-dianisidine, and DA-64 in the presence of horseradish peroxidase (Fig. 1).

The procedure using DA-64 turned out to be the most sensitive at low concentrations of hydrogen peroxide (Fig. 1, inset). However, the system became saturated at relatively low H_2O_2 concentrations. In contrast, the reaction mixtures set with TMB kept the linearity of the determination up to at least 100 mM, irrespective of whether the determination was made in PIPES or in 1/10 volume of MRS (diluted in PIPES).

H_2O_2 generation in response to aeration by vaginal lactobacilli. As part of a previous communication, the isolation of 45 strains of lactobacilli from the vaginas of an equal number of healthy, fertile women was reported (19). Liquid cultures of all these strains were taken during the exponential and stationary phases and subjected to strong agitation under a normal aerobic atmosphere for 3 h, and the generation of H_2O_2 was quantified at 30-min intervals, using DA-64 and TMB to cover the whole range of production shown by the strains.

The kinetics of production was similar for all producing strains, i.e., H_2O_2 started to accumulate after 30 min of aeration, with no production in static cultures (Fig. 2).

The data obtained indicate that the majority of strong producers were *L. jensenii* strains, followed by *L. crispatus* isolates (Table 1). The cultures of strains that achieved a high H_2O_2 concentration at 8 h of incubation (exponential phase) remained so or with slightly lower values at 16 h (stationary

TABLE 1. Classification of the 45 strains of vaginal lactobacilli studied as a function of their H_2O_2 generation ability

Species	Amt of H_2O_2 (mM) generated at indicated time[a]								Total no. of strains
	<0.3		0.3 to 1		1 to 2		>2		
	8 h	16 h	8 h	16 h	8 h	16 h	8 h	16 h	
L. crispatus	18	16	3	5	0	0	0	0	21
L. gasseri	5	4	0	2	1	0	0	0	6
L. jensenii	6	5	6	7	4	2	1	3	17
L. plantarum	1	0	0	0	0	0	0	0	1

[a] Samples were taken from liquid MRS cultures incubated for 8 and 16 h at 37°C under a 10% CO_2 atmosphere.

phase). Some others reached levels of 0.5 mM or more only after 16 h of incubation.

Cultures of *L. jensenii* CECT 4306 (a strong H_2O_2 producer, which was chosen for further work) treated with chloramphenicol prior to the agitation period produced concentrations of H_2O_2 similar to those in the untreated cultures (Fig. 2), indicating that the specific H_2O_2-generating system was already present in the cells before the culture was subjected to aeration and, consequently, that this treatment did not act as an inducer of the system.

Effect of cofactors on H_2O_2 biosynthesis. To determine whether H_2O_2 generation could be improved, different cofactors were added to cultures of *L. jensenii* CECT 4306 at the beginning of the incubation. Supplementation with Mg^{2+}, Mn^{2+}, Ca^{2+}, and Zn^{2+} affected the production of H_2O_2 only slightly with respect to the levels in cultures grown in MRS without additions (Fig. 3). Cu^{2+} inhibited bacterial growth. Surprisingly, cultures containing Fe^{3+}, hemin, or hemoglobin did not accumulate H_2O_2 upon agitation (Fig. 3), while the growth of the strain was generally slightly improved (data not shown, but see Fig. 7).

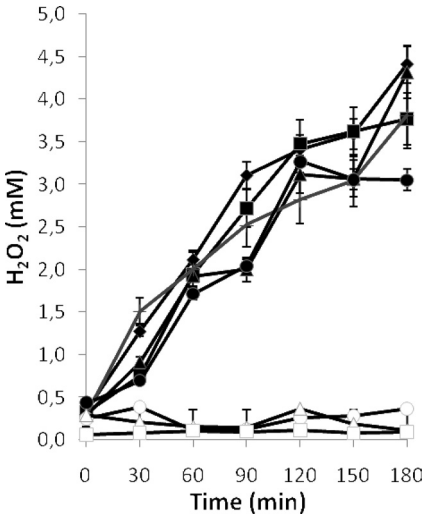

FIG. 3. Effect of the addition of cations (2 mM) and other cofactors on H_2O_2 generation by cultures of *L. jensenii* CECT 4306 grown at 37°C for 16 h and subjected afterwards to aeration. ◆, No additions; ▲, Mg^{3+}; ■, Ca^{3+}; ●, Zn^{3+}; + (gray line), Mn^{3+}; ○, Fe^{3+}; □, 30 mM hemin; △, 1% hemoglobin. Error bars show standard deviations.

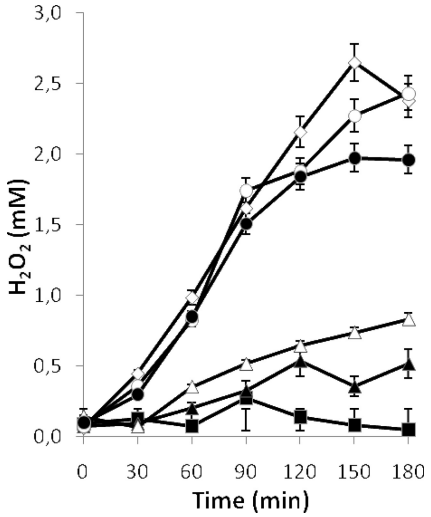

FIG. 4. Effects of different concentrations of Fe^{3+} added to cultures of *L. jensenii* CECT 4306 on H_2O_2 generation upon aeration. ◇, Control culture without added Fe^{3+}; ○, 25 nM; ●, 50 nM; △, 0.1 mM; ▲, 0.2 mM; ■, 2 mM. Error bars show standard deviations.

Fe^{3+} induces degradation of H_2O_2 by lactobacilli. The lack of H_2O_2 in the supernatants of cultures grown in the presence of Fe^{3+} might be due to inability to generate the compound or to its subsequent destruction. To discriminate between these possibilities, two complementary experiments were performed. In the first, cultures were set up with increasing iron concentrations (from 25 nM to 2 mM), and their capacity to generate H_2O_2 after 16 h of incubation was tested. An inverse relationship was found between the concentrations of Fe^{3+} added to the cultures and those of H_2O_2 recovered (Fig. 4). In the second, cultures were set up and H_2O_2 was added to them after growth for 16 h. The culture medium with or without Fe^{3+} and the cultures grown without an excess of the cation did not destroy H_2O_2. Conversely, Fe^{3+}-containing cultures eliminated the exogenously added H_2O_2, as did the cultures grown in the absence of added Fe^{3+} upon addition of the cation (Fig. 5).

To determine whether Fe^{3+} was an inducer of the synthesis of a peroxidase or simply acted as a cofactor for a preformed enzyme, chloramphenicol was added to aliquots of 16-h-old cultures grown in MRS medium with or without Fe^{3+}, to inhibit their protein synthesis. After 30 min, Fe^{3+} was added to the latter cultures and incubation continued for 30 min before the cells were subjected to forced aeration. The cultures with Fe^{3+} did not accumulate H_2O_2, irrespective of the moment of its addition and whether chloramphenicol was present or not, while parallel controls without Fe^{3+} did. This indicates that iron was a necessary cofactor for the enzymatic activity of a constitutively synthesized peroxidase (Fig. 6).

Protective role of Fe^{3+} against H_2O_2-driven growth inhibition of lactobacilli. Cultures of *L. jensenii* were inoculated into liquid MRS medium with or without Fe^{3+}, containing increasing concentrations of H_2O_2. Incubation proceeded for 6 h at 37°C. The data obtained (Fig. 7) indicate that Fe^{3+} slightly improved the growth of the strain (cultures without H_2O_2) and that this compound, at concentrations equal to or greater than 2.5 mM, completely inhibited the development of *L. jensenii*.

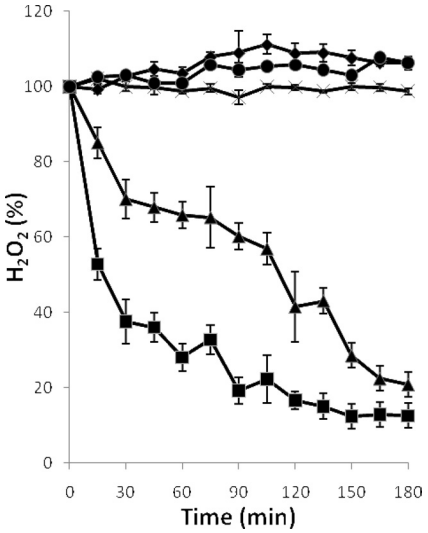

FIG. 5. Degradation of H_2O_2 by cultures of *L. jensenii* CECT 4306. Cultures were grown for 16 h in MRS medium prior to the addition of H_2O_2 (2.5 mM). Results shown are for replicate cultures that were supplemented with 2 mM Fe^{3+} (■) or grown without supplements (×), the same unsupplemented cultures to which 2 mM Fe^{3+} and H_2O_2 were added with a 30-min interval (▲), and uninoculated MRS medium with (◆) and without (●) added Fe^{3+}. Error bars show standard deviations.

However, complete inhibition of growth was observed at 1.25 mM H_2O_2 in the cultures devoid of Fe^{3+}, while those containing the cation were only marginally inhibited.

DISCUSSION

Usually, determination of the ability of vaginal lactobacilli to generate H_2O_2 is performed by semiquantitative methods based mainly on the growth of the strains under anaerobiosis on the surface of solid medium containing a chromophore and

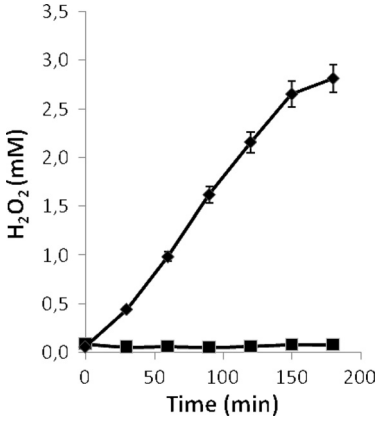

FIG. 6. Effect of inhibition of protein synthesis on H_2O_2 accumulation by aerated cultures of *L. jensenii* CECT 4306. Results shown are for replicate control cultures grown without Fe^{3+} (◆) and replicate cultures grown without Fe^{3+} and subjected successively to 100 μg/ml chloramphenicol, 2 mM Fe^{3+}, and aeration at 30-min intervals (■). Similar data for lack of H_2O_2 accumulation were obtained from cultures grown in the presence of Fe^{3+} and the same cultures treated with chloramphenicol. Error bars show standard deviations.

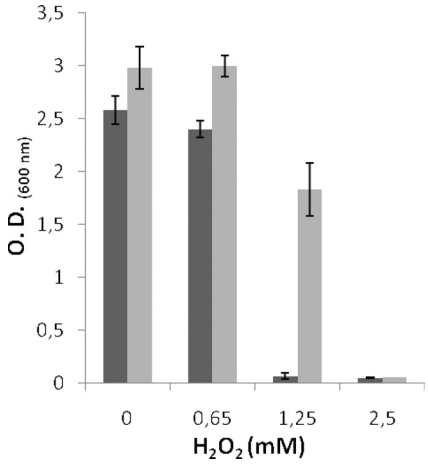

FIG. 7. Effect of Fe^{3+} on the growth of *L. jensenii* CECT 4306 cultures in the presence of increasing H_2O_2 concentrations. The absorbance of the cultures after 6 h of incubation is represented. Light gray bars, cultures with added Fe^{3+}; dark gray bars, cultures without added Fe^{3+}. Error bars show standard deviations. O.D., optical density.

a peroxidase (8, 25). The resulting colonies are exposed to air to allow color development by the positive strains. The intensity of the color is the clue for classification of the lactobacilli as non-, weak, or strong H_2O_2 producers. However, a more precise determination is desirable to allow for the selection of probiotic precursor strains with the most significant properties for protection of the vaginal environment. In this respect, it must be taken into account that H_2O_2-producing strains appear to be more stable in the vaginal environment (32) and to effectively antagonize undesirable microorganisms (3, 8, 17, 32), including those that produce sexually transmitted infections, such as *Neisseria gonorrhoeae* (31).

We chose to test the three selected H_2O_2 quantification methods because they use similar technologies, thus allowing an easy comparison. Other methods, such as iodination (10) and oxidation of 4-amino-antipyrine (12), were discarded because they were considered to have a low sensitivity (11) or because there was some interference with components of MRS medium (21), the medium used almost universally to grow lactobacilli. The data obtained make the use of both the DA-64- and the TMB-based procedures advisable. The first will allow a precise quantification at low H_2O_2 concentrations due to its extreme sensitivity, while the second maintains its usefulness through a wide range of concentrations, allowing discrimination between high producers.

The data obtained by applying these procedures to our 45 strains were only partially coincident with those reported after testing H_2O_2 production on TMB-plus solid medium (19, 25). In both cases, *L. jensenii* accounted for the highest proportion of producing strains, but some that were considered to be strong producers on TMB-plus medium behaved differently after growth in liquid medium and vice versa.

The application of these methods also enabled the study of the characteristics of H_2O_2 generation and degradation by *L. jensenii* CECT 4306, a vaginal isolate that was chosen as a model.

Hydrogen peroxide starts to be produced as a consequence

of the aeration of the cultures, presumably because the static liquid cultures are virtually anaerobic, i.e., the limiting factor is the absence of O_2 rather than the lack of the responsible enzymes. In fact, the H_2O_2 generation system was already present in the cultures prior to aeration, as was determined with cultures whose protein synthesis was inhibited before aeration started. This suggests that the production of H_2O_2 is constitutive upon exposure of the cells to oxygen. Synthesis of H_2O_2 in response to aeration is common not only among vaginal lactobacilli but in other lactic acid bacteria as well. However, instead of being considered a positive trait, it is perceived as detrimental for starters of food fermentation because it may inhibit their growth (21, 27, 33) and/or affect the sensory quality of the products; for example, H_2O_2 induces fat rancidity and discoloration of cured meats (23, 33).

Most probably, H_2O_2 generation proceeds through oxidation of NADH and/or pyruvate by the corresponding dehydrogenases, as happens in *Lactobacillus delbrueckii* (21) and *Bifidobacterium bifidum* (16). Furthermore, in the sequenced genomes of two strains of *Lactobacillus acidophilus* and *L. gasseri*, two species closely related to *L. jensenii*, there are genes that encode putative pyruvate dehydrogenases, and the first harbors an NADH oxidase as well. In addition, neither encodes sequence-recognizable superoxide dismutase determinants (GenBank accession numbers NC_006814 and NC_008530).

H_2O_2, either produced by *L. jensenii* or incorporated into the medium, was degraded when the cultures contained Fe^{3+} ions, added as part of an inorganic salt or as an iron-containing metabolite, such as hemin or hemoglobin. Even cultures grown without added Fe^{3+} showed this ability upon addition of the cation. This Fe^{3+}-dependent, H_2O_2-degrading activity could be a consequence of a Fenton reaction (9) or an enzymatic process catalyzed by a peroxidase. We favor the second hypothesis because liquid MRS medium with Fe^{3+} did not destroy H_2O_2 (Fig. 5), indicating that the reaction is a consequence of the growth of the lactobacilli. Besides, when grown cultures were diluted and Fe^{3+} was subsequently added to them, the ability to destroy H_2O_2 decreased as a function of the dilution factor (data not shown). Finally, the activity became completely lost upon heating of the cultures to 60°C for 10 min. These findings suggest that the reaction is catalyzed by a heat-susceptible peroxidase that uses Fe^{3+} as a cofactor. Heme-dependent peroxidases have been found previously in *Lactobacillus plantarum* (14), *Lactobacillus sakei* (7), and other environmental, aerotolerant lactobacilli (33) but not in species that are normal inhabitants of the vagina, although it is known that their growth is improved by the addition of a heme source to the medium under partially aerobic conditions (19). However, a second, heme-independent catalase activity that is present in *L. plantarum* but not in other environmental species (14, 27) appears to also be absent in vaginal lactobacilli.

The addition of an iron source to the cultures did not induce the synthesis of the peroxidase, because cultures grown without added Fe^{3+}, in which the protein synthesis was inhibited, destroyed exogenously added H_2O_2 upon addition of the cation. However, it cannot be ruled out that the iron included in the culture medium *per se* might induce the synthesis of the apoenzyme. In a previous study, it was found that *Lactobacillus pentosus* cells suspended in phosphate buffer degraded H_2O_2 following the addition of hematin (33). Our data indicate that under these nonproliferating conditions, hematin acted as an iron source to be used as a cofactor by a preexisting peroxidase.

The results obtained indicate that the processes of H_2O_2 generation and degradation may be part of the same detoxification mechanism and that this might occur on the vaginal mucosa. On one hand, the cavity is exposed to atmospheric oxygen, which might mediate deleterious oxidation of essential cell components, possibly explaining why most of the lactobacilli used in this work do not grow on the surface of solid media under aerobiosis (19). Besides that, the NADH oxidase activity postulated as the H_2O_2-generating system is energetically neutral (21), leaving detoxification as the only benefit of the reaction to the cell. On the other hand, the vaginal surface presumably contains an elevated concentration of Fe^{3+}, which would be provided, among other sources, by menstruation. The benefit to the cell of the resulting H_2O_2-degrading activity is quite clear after having observed that the growth of the producing *Lactobacillus* is inhibited by H_2O_2. Thus, the MIC for *L. jensenii* became doubled in the presence of added Fe^{3+} with respect to MICs of cultures without any additions. It appears, then, that the protection provided by H_2O_2 against colonization by undesirable microorganisms would result from the equilibrium between its generation and destruction by the resident lactobacilli, to avoid the toxicity of O_2 and of H_2O_2 in excess.

ACKNOWLEDGMENTS

This work was supported by CICYT grants SAF2004-0033 and BFU2007-65781 from the Ministry of Science and Technology (Spain) and the FEDER Plan. R.M. is the holder of a scholarship from FICYT (Principado de Asturias).

REFERENCES

1. **Álvarez-Olmos, M. I., M. M. Barousse, L. Rajan, B. J. Van Der Pol, D. Fortenberry, D. Orr, and P. L. Fidel.** 2004. Vaginal lactobacilli in adolescents: presence and relationship to local and systemic immunity, and to bacterial vaginosis. Sex. Transm. Dis. **31:**393–400.
2. **Antonio, M. A., L. K. Rabe, and S. L. Hillier.** 2005. Colonization of the rectum by *Lactobacillus* species and decreased risk of bacterial vaginosis. J. Infect. Dis. **192:**394–398.
3. **Boris, S., J. E. Suarez, F. Vazquez, and C. Barbes.** 1998. Adherence of human vaginal lactobacilli to vaginal epithelial cells and interaction with uropathogens. Infect. Immun. **66:**1985–1989.
4. **Boskey, E. R., R. A. Cone, K. J. Whaley, and T. R. Moench.** 2001. Origins of vaginal acidity: high D/L lactate ratio is consistent with bacteria being the primary source. Hum. Reprod. **16:**1809–1813.
5. **Boyd, M. A., M. A. Antonio, and S. L. Hillier.** 2005. Comparison of API 50 CH strips to whole-chromosomal DNA probes for identification of *Lactobacillus* species. J. Clin. Microbiol. **43:**5309–5311.
6. **Burton, J. P., P. A. Cadieux, and G. Reid.** 2003. Improved understanding of the bacterial vaginal microbiota of women before and after probiotic instillation. Appl. Environ. Microbiol. **69:**97–101.
7. **Chaillou, S., M. C. Champomier-Vergès, A. M. Cornet, M. Crutz-Le Coq, A. M. Dudez, V. Martin, S. Beaufils, E. Darbon-Rongère, R. Bossy, V. Loux, and M. Zagorec.** 2005. The complete genome sequence of the meat-borne lactic acid bacterium *Lactobacillus sakei* 23K. Nat. Biotechnol. **23:**1527–1533.
8. **Eschenbach, D. A., P. R. Davick, B. L. Williams, S. J. Klebanoff, K. Young-Smith, and C. M. Critchlow.** 1989. Prevalence of hydrogen peroxide-producing *Lactobacillus* species in normal women and women with bacterial vaginosis. J. Clin. Microbiol. **27:**251–256.
9. **Fenton, H. J. H.** 1894. Oxidation of tartaric acid in presence of iron. J. Chem. Soc. Trans. **65:**899–911.
10. **Fontaine, E. A., and D. Taylor-Robinson.** 1990. Comparison of quantitative and qualitative methods of detecting hydrogen peroxide produced by human vaginal strains of lactobacilli. J. Appl. Bacteriol. **69:**326–331.
11. **Gilliland, S. E.** 1968. Enzymatic determination of residual hydrogen peroxide in milk. J. Dairy Sci. **52:**321–324.
12. **Green, M. J., and H. A. O. Hill.** 1984. Chemistry of dioxygen. Meth. Enzymol. **105:**3–22.
13. **Hawes, S. E., S. L. Hillier, J. Benedetti, C. E. Stevens, L. A. Koutsky, P.**

Wolner-Hanssen, and K. K. Holmes. 1996. Hydrogen peroxide-producing lactobacilli and acquisition of vaginal infections. J. Infect. Dis. **174:**1058–1063.
14. **Johnston, M. A., and E. A. Delwiche.** 1965. Distribution and characteristics of the catalases of *Lactobacillaceae*. J. Bacteriol. **90:**347–351.
15. **Johnston, V. J., and D. C. Mabey.** 2008. Global epidemiology and control of *Trichomonas vaginalis*. Curr. Opin. Infect. Dis. **21:**56–64.
16. **Kawasaki, S., T. Satoh, M. Todoroki, and Y. Niimura.** 2009. *b*-Type dihydroorotate dehydrogenase is purified as a H_2O_2-forming NADH oxidase from *Bifidobacterium bifidum*. Appl. Environ. Microbiol. **75:**629–636.
17. **Klebanoff, S., and M. Belding.** 1974. Virucidal activity of H_2O_2-generating bacteria: requirement for peroxidase and halide. J. Infect. Dis. **129:**345–348.
18. **Lepargneur, J. P., and V. Rousseau.** 2002. Protective role of the Doderleïn flora. J. Gynecol. Obstet. Biol. Reprod. **31:**485–494.
19. **Martin, R., N. Soberon, M. Vaneechoutte, A. B. Florez, F. Vazquez, and J. E. Suarez.** 2008. Characterization of indigenous vaginal lactobacilli from healthy women as probiotic candidates. Int. Microbiol. **11:**261–266.
20. **Martin, R., N. Soberon, F. Vazquez, and J. E. Suarez.** 2008. Vaginal microbiota: composition, protective role, associated pathologies, and therapeutic perspectives. Enferm. Infecc. Microbiol. Clin. **26:**160–167. (In Spanish.)
21. **Marty-Teysset, C., F. de la Torre, and J. Garel.** 2000. Increased production of hydrogen peroxide by *Lactobacillus delbrueckii* subsp. *bulgaricus* upon aeration: involvement of an NADH oxidase in oxidative stress. Appl. Environ. Microbiol. **66:**262–267.
22. **Miyata, K., M. Miyashita, R. Nose, Y. Otake, and H. Miyagawa.** 2006. Development of a colorimetric assay for determining the amount of H_2O_2 generated in tobacco cells in response to elicitors and its application to study of the structure-activity relationship of flagellin-derived peptides. Biosci. Biotechnol. Biochem. **70:**2138–2144.
23. **Niven, C. F., and J. B. Evans.** 1957. *Lactobacillus viridescens* nov. spec., a heterofermentative species that produces a green discoloration of cured meat pigments. J. Bacteriol. **73:**758–759.
24. **Nyirjesy, P.** 2008. Vulvovaginal candidiasis and bacterial vaginosis. Infect. Dis. Clin. North Am. **22:**637–652.
25. **Rabe, L. K., and S. L. Hillier.** 2003. Optimization of media for detection of hydrogen peroxide production by *Lactobacillus* species. J. Clin. Microbiol. **41:**3260–3264.
26. **Redondo-Lopez, V., R. L. Cook, and J. D. Sobel.** 1990. Emerging role of lactobacilli in the control and maintenance of the vaginal bacterial microflora. Rev. Infect. Dis. **12:**856–872.
27. **Rochat, T., J. J. Gratadoux, A. Gruss, G. Corthier, E. Maguin, P. Langella, and M. van de Guchte.** 2006. Production of a heterologous nonheme catalase by *Lactobacillus casei*: an efficient tool for removal of H_2O_2 and protection of *Lactobacillus bulgaricus* from oxidative stress in milk. Appl. Environ. Microbiol. **72:**5143–5149.
28. **Sobel, J. D.** 2000. Bacterial vaginosis. Annu. Rev. Med. **51:**349–356.
29. **Song, Y. L., N. Kato, Y. Matsumiya, C. X. Liu, H. Kato, and K. Watanabe.** 1999. Identification of and hydrogen peroxide production by fecal and vaginal lactobacilli isolated from Japanese women and newborn infants. J. Clin. Microbiol. **37:**3062–3064.
30. **Song, Y. L., N. Kato, Y. Matsumiya, C. X. Liu, H. Kato, and K. Watanabe.** 1999. Identification of *Lactobacillus* species of human origin by a commercial kit, API50CHL. Rinsho Biseibutshu Jinsoku Shindan Kenkyukai Shi **10:**77–82.
31. **St. Amant, D. C., I. E. Valentin-Bon, and A. E. Jerse.** 2002. Inhibition of *Neisseria gonorrhoeae* by *Lactobacillus* species that are commonly isolated from the female genital tract. Infect. Immun. **70:**7169–7171.
32. **Vallor, A. C., M. A. Antonio, S. E. Hawes, and S. L. Hillier.** 2001. Factors associated with acquisition of, or persistent colonization by, vaginal lactobacilli: role of hydrogen peroxide production. J. Infect. Dis. **184:**1431–1436.
33. **Wolf, G., A. Strahl, J. Meisel, and W. P. Hammes.** 1991. Heme-dependent catalase activity of lactobacilli. Int. J. Food Microbiol. **12:**133–140.
34. **Zhou, X., S. Bent, M. Schneider, C. Davis, M. Islam, and L. Forney.** 2004. Characterization of vaginal microbial communities in adult healthy women using cultivation-independent methods. Microbiology **150:**2565–2573.
35. **Zoetendal, E. G., E. E. Vaughan, and W. M. De Vos.** 2006. A microbial world within us. Mol. Microbiol. **59:**1639–1650.