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Selection of Vaginal H_2O_2-Generating *Lactobacillus* Species for Probiotic Use

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Abstract. Lactobacilli are believed to contribute to the control of the vaginal microflora by different mechanisms such as production of antagonistic substances like lactic acid, bacteriocins, and H_2O_2. This paper describes the selection of H_2O_2-generating lactobacilli among 35 hydrophobic isolates from the human vagina. *Lactobacillus crispatus* F117, which generated the highest H_2O_2 level, was chosen to study: (a) the kinetics of H_2O_2 production considering different culture conditions, and (b) the effect of this metabolite on the growth of urogenital tract pathogens. The levels of H_2O_2 in *L. crispatus* supernatant increased during its growth and were maximum at the early stationary phase (3.29 mmol $H_2O_2L^{-1}$) under aerated conditions (agitated cultures). In nonagitated cultures there were no detectable levels of H_2O_2. *L. crispatus* F117 spent supernatant inhibited *Staphylococcus aureus* growth in plaque assay. Inhibition was due to H_2O_2 since catalase treatment of the supernatant suppressed inhibition. In mixed cultures performed with *L. crispatus* and *S. aureus* a significant decrease in pathogen growth was observed. The inhibitory effect depended on the initial inoculum of *S. aureus*. Further evaluation of the properties of *L. crispatus* F117 will be performed to consider its inclusion in a probiotic for local use in the vaginal tract.

Lactobacilli have been long considered to constitute the primary microbiological barrier to infection by urogenital pathogens. They exert a protective role mainly by a combination of steric exclusion and inhibitory substance production [12]. Among these substances, lactic acid produced from carbohydrates helps to maintain a low vaginal pH [11]. In addition, different *Lactobacillus* species produce H_2O_2 and bacteriocin-like substances that may affect undesirable or pathogenic strains in even lactic acid bacteria themselves [18]. The H_2O_2-generating lactobacilli are predominant in the vaginal tract of healthy women [10]. Absence of *Lactobacillus* strains with this characteristic is related to a high incidence of infections, mainly caused by *Gardnerella vaginalis* [3, 6]. Redondo-López et al. [18] have concluded that the production of this metabolite might represent a nonspecific antimicrobial defence mechanism of a healthy vaginal ecosystem. Furthermore, production of H_2O_2 may be one of the responsible factors in the dominance of lactobacilli in the vaginal tract.

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The term *probiotics* was redefined by Havenaar et al. [5] as ''a viable mono or mixed culture of microorganisms which, applied to animal or man, beneficially affects the host by improving the properties of the indigenous microflora.'' The characteristics of lactobacilli for probiotic use in the human female urogenital tract have been described previously [12]. Our objective is the selection of *Lactobacillus* species for probiotic use in the human vagina as a barrier to infections and as an alternative to long-term use of antibiotics. Previously, 134 lactobacilli were isolated from premenopausal women. They were identified and their surface characteristics studied [15]. In the present work the capability to generate H_2O_2 of 35 hydrophobic lactobacilli was tested. *Lactobacillus crispatus* F117 was selected to study the kinetic of H_2O_2 production and the inhibitory effect against *Staphylococcus aureus* pathogen related with toxic shock syndrome.

Materials and Methods

Strains, culture media, and growth conditions. Samples were obtained from vaginal swabs from premenopausal women. Lactobacilli were isolated and identified by standard biochemical tests [9] and API

CH50 system (Biomerieux, France) as described previously [15]. Microorganisms were isolated from vaginal swabs and identified by morphological and biochemical standard methods. *Streptococcus agalactiae* ATCC 1022 (from American Type Culture Collection) was also studied. All strains were stored in milk-yeast extract at −70°C.

H_2O_2 production by vaginal lactobacilli. H_2O_2 production was studied for *Lactobacillus* strains that showed good adhesion properties (tested previously) [15]. Levels of this metabolite in lactobacilli spent supernatant fluid were determined spectrophotometrically by the *o*-dianisidine horseradish peroxidase method [14], modified by increasing the peroxidase concentration to 120 μg ml^{-1}. Microorganisms were grown twice in LAPTg broth [17] without agitation and a third time under agitation (New Brunswick gyratory shaker water bath, model g 76, speed 5, heat high). After 15 h of incubation at 37°C, supernatant fluids were separated by centrifugation, and H_2O_2 was quantified by using an H_2O_2 standard curve.

Kinetics of H_2O_2 production. *L. crispatus* F117 (*L. crispatus* CRL 1266 from Cerela Culture Collection; F117 accounts for the internal nomenclature), the highest H_2O_2-generating strain, was selected to study the production kinetics of H_2O_2. Samples from *L. crispatus* cultures with and without agitation (both in LAPTg broth at 37°C) were taken at different times during the growth of lactobacilli. Supernatants were separated by centrifugation, and H_2O_2 was determined as described above. The plate dilution method using LAPTg agar was applied to determine CFU ml^{-1}. Growth parameters were calculated for each case.

Effect of *L. crispatus* supernatant on uropathogen growth. The plate diffusion technique was employed according to standard methods [13] to detect the effect of *L. crispatus* supernatant fluid on uropathogen growth. LAPTg agar plates of *S. aureus*, *Enterococcus* sp., Group B *Streptococcus* sp., *Streptococcus agalactiae* ATCC 1022, *Escherichia coli*, *Klebsiella* sp., and *Candida* sp. were prepared from cultures of these microorganisms at early stationary phase to achieve a final concentration of 10^6–10^7 CFU ml^{-1}. The supernatant fluid of *L. crispatus* F117 was filter sterilized (Millipore, 25 μm) and aliquots were neutralized with NaOH (2 mol L^{-1}) or treated with catalase (1,000 U ml^{-1}). Nontreated and treated supernatant aliquots were assayed on uropathogen plates into which 4-mm holes had been punched and which were filled with the supernatant. The plates were incubated for 5 h at room temperature and then 24 h at 37°C. The diameter of the inhibition halos was measured. Control assays of medium (LAPTg, pH 6.5), pH (LAPTg, pH 4), and catalase (1,000 U/ml in LAPTg, pH 7) were also performed.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of H_2O_2 on *S. aureus*. MIC was determined by the plate diffusion technique: *S. aureus* was inoculated on LAPTg agar plates; holes were made in the agar and filled with 25 μl of different H_2O_2 concentrations (from 4.4 mmol L^{-1} to 0.88 mmol L^{-1}). The plaques were incubated for 5 h at room temperature and then 24 h at 37°C. MIC was defined as lowest H_2O_2 concentration able to produce *S. aureus* growth inhibition halos.

To determine the bactericidal concentration of H_2O_2 on *S. aureus* growth, tubes containing decreasing H_2O_2 concentrations (from 880 mmol L^{-1} to 0.88 mmol L^{-1}) were prepared. They were inoculated with *S. aureus* at 5% from a 5×10^5 CFU ml^{-1} culture and incubated at 37°C during 48 h. After that time, samples were taken from the different tubes, inoculated on LAPTg agar plates and incubated for 48 h. After incubation CFU ml^{-1} was determined for each tube. The lowest concentration able to kill all cells was defined as MBC.

Mixed cultures of *L. crispatus* and *S. aureus*. Mixed cultures of *L. crispatus* and *S. aureus* were performed in LAPTg broth under agitation at 37°C. The inocula were between 10^4–10^7 CFU ml^{-1} for *S. aureus* and 10^6–10^7 CFU ml^{-1} for lactobacilli. Viable cells count was determined by the plate dilution method by using selective culture media: Mannitol Salt Agar (MSA) for *S. aureus* and *Lactobacillus* Selective Medium (LBS) [2] for lactobacilli. MSA and LBS plates were incubated microaerophily at 37°C for 48 h. H_2O_2 produced during the growth of the microorganisms was determined by the modified *o*-dianisidine method.

Statistical evaluation. All experiments were performed in triplicate. The means of the data are represented in the graphs.

Results

H_2O_2 production by vaginal lactobacilli. The levels of H_2O_2 produced by hydrophobic *Lactobacillus* strains are shown in Table 1. There is a wide range of values, and no correlation was found between the capability to generate H_2O_2 and a particular *Lactobacillus* species or metabolic group. The highest producer was *L. crispatus* F117, and for this reason it was selected to study the kinetics of H_2O_2 production and its effect on the growth of other microorganisms. Three of the tested strains did not produce detectable concentrations of H_2O_2.

Effect of *L. crispatus* supernatant on uropathogen growth. *L crispatus* F117 supernatant fluid inhibited *S. aureus* growth, but other pathogens were not affected (Table 2). The inhibitory effect of *L. crispatus* on *S. aureus* growth is shown in Fig. 1. This effect can be attributed to H_2O_2 because growth was not affected when the solution was neutralized, but inhibition was lost after treatment with catalase.

Kinetics of H_2O_2 production. The levels of H_2O_2 generated by *L. crispatus* in Laptg broth in agitated and nonagitated cultures are shown in Fig. 2. In agitated cultures, H_2O_2 was produced at higher levels; there was no detectable production of this metabolite without O_2 supply (in nonagitated cultures). Levels of H_2O_2 increased simultaneously with the growth of the microorganisms. The highest concentration was reached at the early stationary growth phase with 3.29 mmol L^{-1} or 222 nmol $(10^7$ viable cells$)^{-1}$ of *L. crispatus* F117, produced after 12 h of incubation at 37°C. Even though in nonagitated cultures the growth rate was lower than in agitated ones, 0.49 h^{-1} and 0.98 h^{-1}, respectively, population density was higher under former conditions (1.30×10^9 CFU ml^{-1} and 1.50×10^8 CFU ml^{-1}).

MIC and MBC of H_2O_2 on *S. aureus*. The MIC of H_2O_2 on *S. aureus* growth determined by the plate diffusion method was 0.80 mmol L^{-1}. The MBC, evaluated in LAPTg broth containing decreasing concentrations of H_2O_2, was 88 mmol L^{-1}. Incubation of the pathogen for 24 h or 48 h in these media produced no significant differences in the CFU ml^{-1} (data not shown).

Table 1. H_2O_2 production by isolates belonging to different metabolic groups of the genus *Lactobacillus*

	H_2O_2 (mmol L^{-1})
Obligated homofermentative	
L. acidophilus F25[a]	0.10
L. acidophilus F51	0.10
L. acidophilus F15	0.16
L. acidophilus F58	0.00
L. crispatus F117	2.80
L. delbrueckii ssp. *bulgaricus* F37	0.32
L. delbrueckii ssp. *delbrueckii* F29	0.92
L. delbrueckii ssp. *delbrueckii* F32	0.04
L. delbrueckii ssp. *delbrueckii* F33	0.31
L. delbrueckii ssp. *delbrueckii* F65	0.29
L. delbrueckii ssp. *delbrueckii* F83	0.15
L. delbrueckii ssp. *delbrueckii* F86	1.13
L. delbrueckii ssp. *delbrueckii* F80	0.19
L. delbrueckii ssp. *delbrueckii* F6	0.00
L. delbrueckii ssp. *delbrueckii* F91	0.89
L. gasseri F85	0.38
L. gasseri F104	0.20
L. gasseri F106	0.02
L. salivarius F35	0.00
L. salivarius F71	0.03
Facultative heterofermentative	
L. agilis F54	1.06
L. casei ssp. *casei* F79	0.51
L. casei ssp. *tolerans* F3	0.77
L. coryniformis F39	0.06
L. paracasei ssp. *paracasei* F12	0.29
L. paracasei ssp. *paracasei* F57	0.14
L. paracasei ssp. *paracasei* F31	0.06
L. paracasei ssp. *paracasei* F2	2.17
L. paracasei ssp. *paracasei* F28	1.84
L. homohiochi F60	0.00
L. plantarum F47	0.48
Obligated heterofermentative	
L. brevis F18	0.04
L. brevis F66	0.11
L. brevis F69	0.00
L. reuterii F26	0.99

[a] F accounts for the internal nomenclature. The highest H_2O_2 producers are indicated in gray rows.

Mixed cultures of *L. crispatus* and *S. aureus*. In mixed cultures, growth of *S. aureus* was inhibited by *L. crispatus*. The decrease in *S. aureus* viable cells count depended on the initial inoculum of the pathogen. Figure 3A–C shows the $\log_{10}$ CFU ml^{-1} when *S. aureus* was inoculated at different concentrations (1.20×10^4 CFU ml^{-1}, 2.80×10^5 CFU ml^{-1}, and 2.50×10^6 CFU ml^{-1}, respectively). A decrease in CFU ml^{-1} of *S. aureus* was observed in all cases; inhibition occurred slower at higher initial pathogen inocula. At an initial inoculum of the pathogen of 10^6 CFU ml^{-1}, however, no complete inhibition was observed; after 24 h, 10^2 cells ml^{-1} of *S. aureus* remained viable.

Table 2. Strains tested for *L. crispatus* supernatant inhibition

Microorganism	Inhibition halo (mm)
Candida sp.	—
Escherichia coli	—
Enterococcus sp.	—
Klebsiella sp.	—
Staphylococcus aureus	10[a]
Streptococcus agalactiae ATCC[b] 1022	—
Streptococcus sp. (Group B)	—

[a] Diameter of the halo determined by the plate diffusion method.
[b] ATCC (American Type Culture Collection).

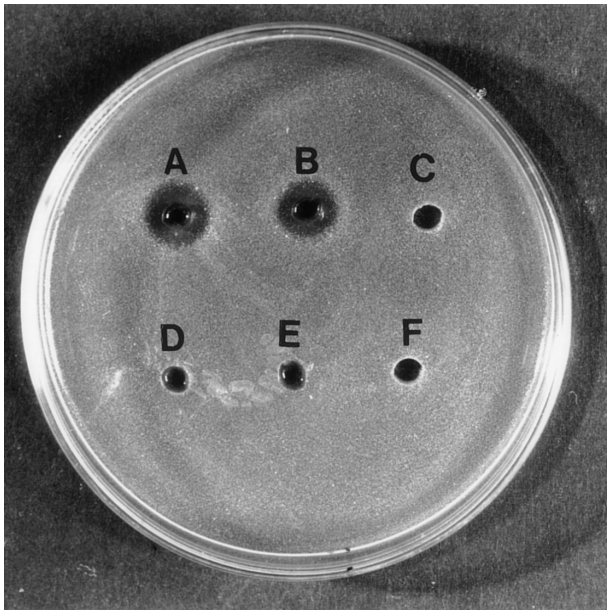

Fig. 1. Inhibition of *Staphylococcus aureus* by *Lactobacillus crispatus*. Supernatants untreated (A), neutralized (B), and treated with catalase (C). Controls of culture medium (pH 6.5), acidified medim (pH 4), and catalase (D, E, and F, respectively).

The levels of H_2O_2 determined in mixed cultures were very similar to those of pure lactobacilli cultures (data not shown). At an initial inoculum of *S. aureus* of 10^5 and 10^6 CFU ml^{-1}, the number of CFU ml^{-1} of *L. crispatus* decreased. However no inhibitory substance against *L. crispatus* was detected in neutralized supernatants of *S. aureus* by using the plate diffusion method (data not shown).

Discussion

Lactobacillus species are the predominant microorganisms isolated from the vagina of healthy women. They

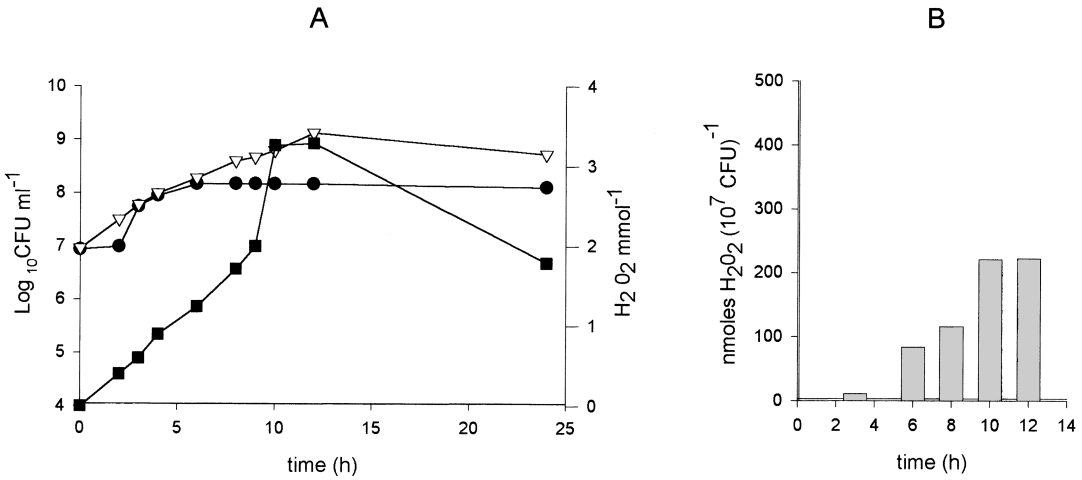

Fig. 2. (A) Kinetics of H_2O_2 production by *Lactobacillus crispatus* F117. Growing of lactobacilli under agitated (●) and nonagitated (▽) conditions, levels of H_2O_2 produced during the growth in agitated cultures (■). (B) Levels of H_2O_2 expresed per 10^7 viable cells.

exert a protective role against pathogen colonization by steric exclusion and inhibitory substance production, such as lactic acid, H_2O_2, and bacteriocins [7, 12]. Klebanoff et al. [10] have studied the control of the vaginal microflora by H_2O_2-generating lactobacilli and expressed that these microorganisms contribute to nonspecific antimicrobial host defense, probably through interaction with peroxidase and a halide. McGroarty et al. [13] have shown the direct correlation between H_2O_2-producing lactobacilli and their increased sensitivity to nonoxynol-9, suggesting that spermicide users could be depleted of these lactobacilli and therefore be more susceptible to infections.

As our objective is the selection of *Lactobacillus* strains with beneficial properties for probiotic use in the vaginal tract, 134 *Lactobacillus* isolates from the vaginas of 200 women were isolated and identified. Different techniques to predict adhesion capability were performed [15]. In this paper a study of the levels of H_2O_2 produced by *Lactobacillus* strains with good adhesion properties was carried out. Selection of H_2O_2-generating lactobacilli able to adhere to tissues is a first step in the study of potential probiotic strains.

H_2O_2 is generated by certain microorganisms growing aerobically. In aerobic cultures, oxygen is used as an alternative electron acceptor and is reduced to H_2O_2 or H_2O. Not possessing heme, lactobacilli (as well as other lactic acid bacteria) do not utilize the cytochrome system (which reduces O_2 to H_2O) for terminal oxidation during their respiratory processes [16]. Whittenbury [22] suggested that these organisms contain flavoprotein oxidases, which catalyze the production of H_2O_2. Among the strains tested for the production of this metabolite, *L. crispatus* F117 produced the highest level. Other H_2O_2 producers were *L. paracasei* subsp. *paracasei* F2 and F28, *L.delbrueckii* F86 and *L. agilis* F54, which reached concentrations higher than 1 mmol L^{-1}. Production kinetics studies showed a decrease in H_2O_2 concentration in the late stationary phase of *L. crispatus* F117. It is possible that the H_2O_2 concentrations reported in Table 1 were not maximum because for the screening they were determined in cultures after 15 h of incubation.

Studies of kinetics of H_2O_2 production showed that O_2 must be supplied through agitation to detect this metabolite in spent culture supernatant fluids. Conditions under which H_2O_2 is generated in vivo in the vaginal tract, where the concentration of oxygen is low, are not yet known. However, numerous studies have demonstrated that lactobacilli which produce H_2O_2 in vitro are those found in healthy women [3]. As shown in Fig. 1, H_2O_2 concentration was highest during the early stationary phase. *L. crispatus* F 117 produced 3.29 mmol L^{-1} (222 nmol $[10^7$ CFU$]^{-1}$) of H_2O_2 after 12 h. Similar concentrations have been reported for different genera of bacteria from diverse environments. Ryan and Kleinberg [20] observed that the production of H_2O_2 by *Streptococcus sanguis* and *Streptococcus mitor* was between 2.2 and 9.8 mmol L^{-1} when they were grown aerobically. A *Leuconostoc oenos* strain [19] from dairy origin produced between 0.23 and 1.38 mmol L^{-1} in aerobic cultures.

Inhibition of uropathogens by *L. crispatus* F117 spent supernatant fluid was studied. Only *S. aureus* growth was affected. The inhibitory effect of *L. crispatus* F117 on *S. aureus* was studied in mixed cultures. *S. aureus* was selected for the present study because of its association with toxic shock syndrome in women using tampons. This syndrome is caused by an *S. aureus* toxin, toxic shock syndrome toxin 1 (TSST-1) and has potentially serious consequences. Mixed cultures of lactobacilli and *S. aureus* showed inhibition of the pathogen

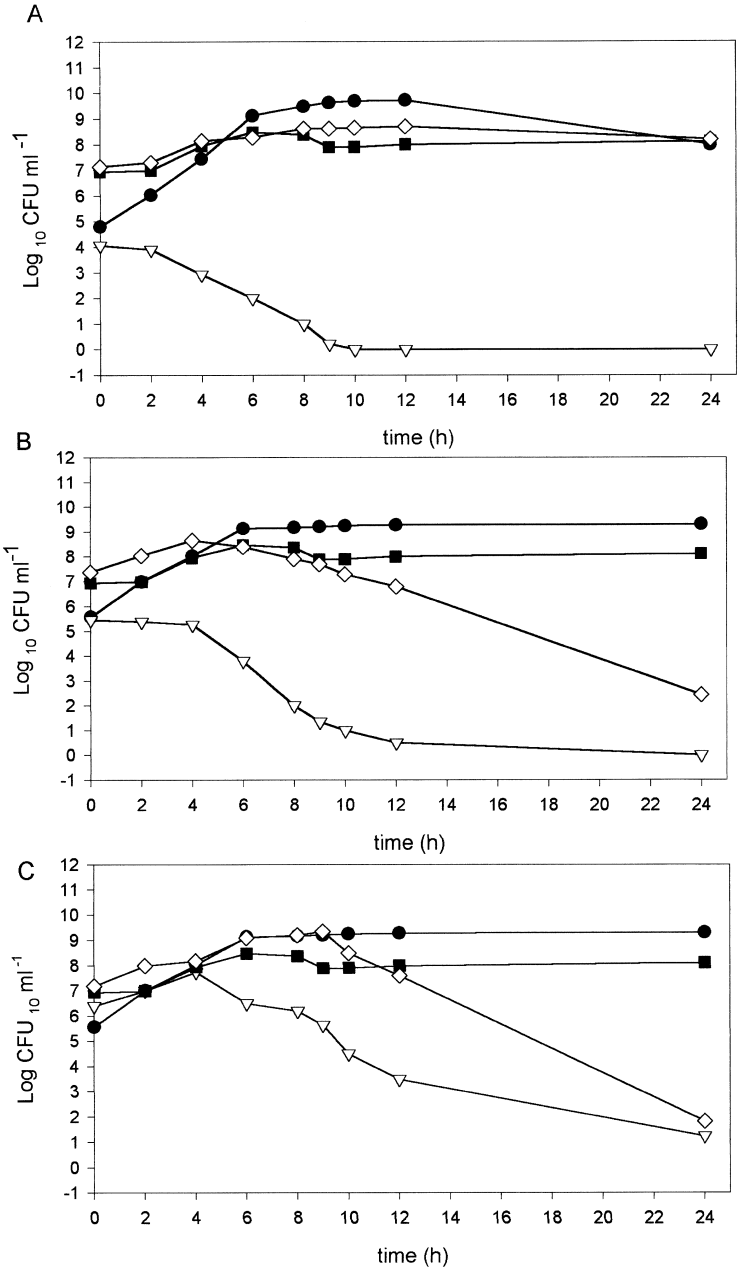

Fig. 3. Mixed cultures of *Lactocbacillus crispatus* and *Staphylococcus aureus*. Different initial concentrations of *S. aureus*: (A) 1.20×10^4 CFU ml^{-1}, (B) 2.8×10^5 CFU ml^{-1}, and (C) 2.5×10^6 CFU ml^{-1}. Viable cells of *S. aureus* in pure (●) and mixed (▽) cultures and of *L. crispatus* in pure (■) and mixed (◇) cultures. The method employed is described in the text.

depending on the initial inoculum of the latter. The lower inhibition observed when *S. aureus* inoculum was higher (10^6 CFU ml^{-1}) could be explained by an insufficient concentration of H_2O_2 for the number of cells. In these cases growth inhibition of *L. crispatus* was observed. As no inhibitory substance against *L. crispatus* F117 in *S. aureus* supernatant was detected by plate diffusion assay, a possible explanation for the lactobacilli inhibition in mixed cultures could be nutrient competition.

The toxicity of H_2O_2 is due to an oxidative effect exerted by the molecule itself or by its metabolites ($OH^{\cdot}$, O_2^{-}). Conversion of H_2O_2 into these cytotoxic compounds may be potentiated by reducing agents and by peroxidases present in the vaginal fluid [11]. These products cause cellular death by their action on nucleic acids, proteins, and other biomolecules [8]. Two main mechanisms of action for H_2O_2 activity may be distinguished: one leading to a bacteriostatic effect, the other being bactericidal [16]. The H_2O_2 MBC on *S. aureus* determined in this work was 88 mM. Lower concentrations produced a bacteriostatic effect (data not shown). Inhibition of different microorganisms by H_2O_2-generating lactobacilli has been demonstrated by several authors. Haines and Hamon [4] have reported that certain lactoba-

cilli inhibited growth of *S. aureus* at H_2O_2 concentrations of 0.18 mmol L^{-1}. At this concentration H_2O_2 acted as bacteriostatic, and it became bactericidal at concentrations of 0.6 to 1.0 mmol L^{-1}. Collins and Aramaki [1] have shown that some *L. acidophilus* from dairy origin were able to inhibit *Pseudomonas* species by producing 1.18 to 1.62 mmol L^{-1} (40 to 55 μg ml^{-1}) H_2O_2 in agitated cultures. Significant bactericidal activity was observed when 0.88 mol L^{-1} of H_2O_2 was applied to *Micrococcus* sp. or *Staphylococcus epidermidis* [21].

From this study, we conclude that H_2O_2-producing *L. crispatus* F117 inhibits growth of *S. aureus* depending on factors such as the O_2 provided and the initial inoculum of the pathogen. *L. crispatus* F117 has been selected for further studies of its probiotic properties and technological aspects considering the possibility to include this microorganism in a formula for local application in the vaginal tract.

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