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INHIBITION OF HERPES SIMPLEX VIRUS TYPE 2 BY VAGINAL LACTOBACILLI

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The protective effect of selected vaginal *Lactobacillus* strains (*L. brevis* CD2, *L. salivarius* FV2, *L. plantarum* FV9) towards herpes simplex virus type 2 (HSV-2) infection *in vitro* has been analyzed. Living bacterial cells affect different steps of virus multiplication. The effect on the early phases of virus infection appeared related to the bacterial adhesive potential to the cell membrane while all the strains strongly reduced intracellular events of virus multiplication. The anti HSV-2 activity was not mediated by a virucidal effect. Instead it was exerted through bacterial soluble factors able to down regulate the production of infective virions. In fact HSV-2 yield was significantly reduced in infected cells fed with cell-free supernatants of lactobacilli grown in cell culture medium. Purified lactic acid and H₂O₂, *Lactobacillus* metabolites with known antimicrobial activity, produced a dose-dependent virucidal effect. Lactic acid successfully interfered with viral intracellular antigen synthesis and both the virucidal activity and the inhibition of replication were correlated to acidic pH values. *L. brevis* CD2, the most active strain, does not produce H₂O₂ and neutralized lactic acid had no effect, thus indicating that factors other than H₂O₂ and lactic acid could be responsible for the antiviral effect.

Key words: *herpes simplex virus type 2, vaginal lactobacilli, antiviral activity, lactic acid, hydrogen peroxide*

INTRODUCTION

The cervico-vaginal mucosa represents a portal of entry for different pathogenic microorganisms in women. In healthy women of child-bearing age, the protective mucosa in the vagina is populated with microflora typically dominated by lactobacilli and their dominance over pathogenic anaerobes is positively associated with vaginal health (1). The most common vaginal disorder among reproductive age women involving a strong reduction in the number of vaginal lactobacilli is bacterial vaginosis (BV). BV is not caused by one specific pathogenic microorganism, but rather by an imbalance of the vaginal microbial flora. In BV, lactobacilli are reduced or absent or lacking specific antimicrobial properties (*i.e.* production of H₂O₂) and are replaced by *Gardnerella vaginalis* and other anaerobic microbiota.

Increasing data now indicate that abnormal vaginal flora lacking lactobacilli facilitates the acquisition of viral sexually transmitted diseases. The first clinical studies suggesting an association between BV and a viral sexually transmitted infection were reported for HIV. HIV seropositivity was significantly correlated with BV, independently of other behavioral variables (2, 3). More recent prospective studies demonstrated an association between alterations of vaginal flora and acquisition of HIV infection (1, 4). Lack of a *Lactobacillus*-predominant vaginal flora was identified as a risk factor for herpes simplex virus type 2 (HSV-2) and human papillomavirus (HPV) infections (5, 6). In addition, acquisition of HSV-2 and HPV infections have both been associated with BV (6, 7). Recently abnormal vaginal flora has been identified as a risk factor for genital tract shedding of cytomegalovirus and HSV-2 in women (8, 9). Moreover, female

genital-tract HIV load correlates inversely with vaginal lactobacilli bacterial counts (10). Therefore lactobacilli exert *in vivo* an important role in the epidemiology of sexually transmitted viral infections both in relation to the protection of female health as well as by reducing the risk of virus transmission from an infected woman to a healthy man.

In spite of the protective effect of vaginal lactobacilli as indicated by the epidemiological studies, the antiviral activity of probiotic bacteria has not yet been studied in detail in cell cultures. Klebanoff and Coon demonstrated that hydrogen peroxide produced by a strain of *L. acidophilus* displays virucidal effect on HIV-1, particularly in the presence of peroxidase and chloride (11). Recently it has been reported that the infectivity of vesicular stomatitis virus was reduced after preincubation with different *Lactobacillus* strains (12) and that cell-free filtrates of two *Lactobacillus* cultures inhibited the replication of HSV-2 (13). Therefore the mechanism of action of lactobacilli towards viral infections is still poorly understood.

Possible mechanisms to account for the protection exerted by vaginal lactobacilli include inactivation of pathogens by different metabolic *Lactobacillus* products (lactic acid, H₂O₂, bacteriocins), competition for epithelial cell attachment sites, preservation of mucin gel coating the vaginal/cervical epithelium through inhibition of glycosidase-producing anaerobes, and maintenance of appropriate innate immune response (14-16).

The purpose of this research was to evaluate the protective activity of vaginal lactobacilli towards HSV-2 infection in cell culture and to identify the possible mechanism of action. Worldwide, HSV-2, the primary cause of genital herpes, is one of the most prevalent sexually transmitted infections. Infection is considered life-long, as the virus becomes latent in sacral nerve

ganglia, and may result in recurrent genital lesions. Genital herpes plays a major role in increasing the risk for sexual acquisition and transmission of HIV (17) and HSV-2 infection cannot be prevented by a vaccine.

Three species of vaginal lactobacilli (*Lactobacillus brevis*, *Lactobacillus salivarius* and *Lactobacillus plantarum*) with different biochemical characteristics and adhesion capacity to cells have been compared for their antiviral activity. The strains have been characterized and selected for the prophylaxis and treatment of vaginal infections (18). *L. salivarius* and *L. plantarum* strains produce anti-infective agents including hydrogen peroxide. *L. plantarum* and *L. brevis* strains are able to adhere at high levels to human epithelial cells displacing vaginal pathogens (19). All the strains were able to temporarily colonize the human vagina after a 5 day-treatment (20).

MATERIALS AND METHODS

Bacterial strains and growth conditions

Lactobacillus brevis (strain CD2), *Lactobacillus salivarius* subsp. *salicinius* (strain FV2), *Lactobacillus plantarum* (strain FV9) were stored as a stock culture at -70°C in 90% de Man-Rogosa-Sharpe (MRS) broth (Oxoid) and 10% glycerol. Lactobacilli were inoculated from frozen vials onto MRS broth and cultured overnight at 37°C under anaerobic conditions in Anaerogen system (Oxoid).

Correlation between optical density and colony forming units

Lactobacilli were thawed-out directly performing 10-fold dilutions in MRS broth up to 10⁻⁶ to obtain mid-logarithmic-phase organisms after overnight incubation. One ml of the bacterial suspensions was washed twice in Phosphate Buffered Saline (PBS, pH 7.2) at 5000 g, 4°C, for 10 min and the optical density assessed by spectrophotometry at 600 nm. Viable microorganisms were determined by plating serial 10-fold dilutions of lactobacilli onto MRS-agar plates. Tests were performed in triplicate. Colony counts were carried out after 48 hrs incubation. Correlation between optical density and CFU was established.

Cells

Vero African green monkey kidney cells were cultured at 37°C in a 5% CO₂ atmosphere in Eagle's Minimum Essential Medium (MEM, HyClone) containing 1.2 mg/ml NaHCO₃ and supplemented with 6% (v/v) foetal bovine serum, 2 mM glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin. For cell maintenance the serum concentration was lowered to 2% (maintenance MEM).

Bacterial culture supernatant (CS) preparation

Exponentially growing cultures of lactobacilli in MRS broth, were washed thrice with PBS and resuspended in cell maintenance MEM without antibiotics. *Lactobacillus* CSs were obtained growing bacteria in six-well plates in the presence or absence of Vero cells in maintenance medium without antibiotics for 16 h at 37°C in 5% CO₂ atmosphere. Bacteria were removed by centrifugation at 5000 g for 10 min and supernatants immediately used in the antiviral assays.

HSV-2 and viral plaque assay

The P1 strain of HSV-2, a clinical isolate from the Sapienza Virology Laboratory, was grown on Vero cells in maintenance

medium. Subconfluent cell monolayers were inoculated with virus at a multiplicity of infection of 0.1 PFU/cell and incubated at 37°C for 48 h. After three cycles of freezing and thawing, the cultures were centrifuged at 1000 g for 20 min (4°C) to remove cellular debris, and clarified supernatants stored at -80°C.

Virus titre was determined by a standard plaque assay. Serial ten-fold dilutions of virus in maintenance medium were inoculated on confluent Vero cell monolayers in 6-well plates. After a 1 hrs adsorption period at 37°C, the inoculum was removed and cells were washed three times with PBS before being overlaid with MEM containing 0.4% (w/v) agar (Oxoid). After 3 days incubation at 37°C, plaques were stained with 0.1% crystal violet solution.

Immunofluorescence assay

Vero cells were grown in micro-tissue chamber slides at a concentration of 4×10⁴ cells/well for 24 hrs in 5% CO₂ at 37°C. Cell monolayers were infected with 1 PFU/cell of HSV-2 for 1 h at 37°C. After incubation for 16 hrs at 37°C, the percentage of infected cells was determined by a direct immunofluorescence assay using FITC-conjugated rabbit anti-herpes simplex virus type 2 immunoglobulins (Dako). The antibody reacts with all the major glycoproteins present in the viral envelope and at least one core protein as determined by crossed immunoelectrophoresis.

Virucidal effect

Living bacterial cells, bacterial culture supernatants, H₂O₂ and lactic acid were incubated with HSV-2 (9×10⁶ PFU/ml) for 1 h or 16 hrs at 37°C in sterile screw-cap microtubes. The tubes were centrifuged at 5000 g for 10 min to sediment bacteria where necessary and residual virus infectivity was determined by plaque assay.

Virus yield reduction assay

For antiviral assays, confluent monolayers of Vero cells in 24-well plates were inoculated with HSV-2 (1 PFU/cell). The infection was synchronized by allowing the virus to adsorb to the cells in the cold (4°C). After 1 h, the inoculum was removed by washing thrice with PBS. Then, the temperature was raised to 37°C to permit internalization. Single-cycle conditions were achieved by incubating the cells at 37°C for 16 hrs post infection. The cultures were freeze-thawed three times, cell debris removed by low speed centrifugation and supernatants titrated by plaque assay on Vero cell monolayers.

Determination of H₂O₂ production

The measurement of H₂O₂ produced by *Lactobacillus* strains was an adaptation of the technique described by Pick and Mizel (21). Briefly, Vero cells were incubated in phenol red-free maintenance MEM at 37°C in a 5% CO₂ atmosphere in the absence or presence of lactobacilli (1000 CFU/cell) for different time intervals: 4, 8, 12 and 16 hrs. Five hundred µl of culture supernatants obtained after centrifugation for 10 min at 5000 g were mixed with an equal volume of PBS containing 1.12 mM phenol red (Sigma) and 19 U horseradish peroxidase (Sigma). After 1 h incubation at 37°C, the samples were brought to pH 12.5 by the addition of 5 µl NaOH 2 N and the absorbance was read at 610 nm against a blank containing phenol red-free maintenance MEM. Standard curves were made using H₂O₂ solutions in phenol red-free maintenance MEM to result in final concentrations of 0.5, 1, 2, 4, 8 and 16 µM. The results, after deduction of control uninfected cell values, were expressed in µM H₂O₂.

Lactic acid determination

Lactic acid production by lactobacilli was evaluated with a commercial kit for the determination of D- and L-lactic acid (Test-Combination, UV-method; Boehringer Mannheim/R-Biopharm, Darmstadt, Germany). The NADH increase, stoichiometric to the amount of D- and L-lactic acid, was determined by absorbance at 340 nm.

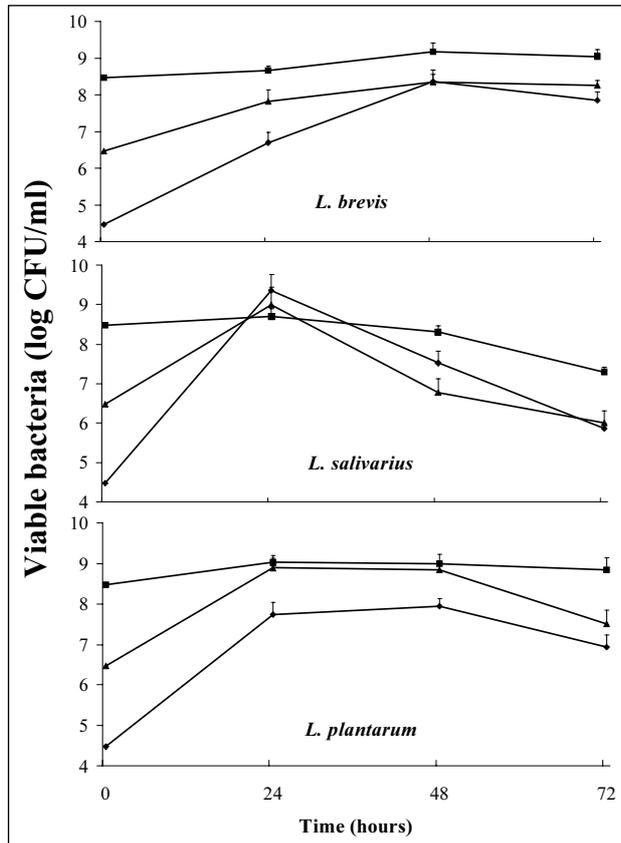


Fig. 1. Growth of lactobacilli on Vero cells. Bacteria were inoculated on cell monolayers in antibiotic-free maintenance MEM in 5% CO₂ atmosphere. CFU were determined on MRS agar after different times of incubation. ■ 1000 CFU/cell; ▲ 10 CFU/cell; ◆ 0.1 CFU/cell.

RESULTS

Co-culture of lactobacilli and Vero cells

The interaction between lactobacilli and cultured eukaryotic cells was analyzed incubating subconfluent cell monolayers with bacteria in different growth phase conditions. Fig. 1 shows the ability of microaerophilic strains of lactobacilli to survive and proliferate in the presence of Vero cells in aerobic atmosphere and antibiotic-free cell culture medium. After 24 h incubation all the *Lactobacillus* strains inoculated at 1000 CFU/cell, corresponding to the physiological concentration of lactobacilli in vaginal environment and to a late logarithmic growth phase, were viable maintaining the initial cell number of approximately 10⁸ CFU/ml. Instead, lactobacilli inoculated in exponential growth conditions, corresponding to a 10 and 0.1 CFU/cell inoculum, increased their number by 2 to 4 logs. In the following 48 hrs, the cell number of the faster growing strains (*L. salivarius* and *L. plantarum*) showed a significant decline. The pH value of cell culture medium after growth of lactobacilli was not modified in comparison to control at 24 and 48 hrs and only slightly lowered (from 0.3 to 0.4 units) after 72 hrs incubation. Microscopy inspection of cell monolayers did not show any change in cell morphology. Also Vero cell viability was unaffected by lactobacilli as verified by the uptake of the vital dye neutral red.

Inhibition of HSV-2 infection by lactobacilli

The antiviral potential of lactobacilli towards HSV-2 infection in cell cultures was studied measuring the virus yield after a single cycle of multiplication (Fig. 2). The effect of bacteria on different steps of HSV-2 multiplication was analyzed by evaluating their ability to prevent virus infection when bound to the cell surface, to compete with virus adsorption to the cell membrane, and to reduce virus multiplication when present during virus replication. The activity was studied at different bacteria/cell ratio. Pretreatment of cell monolayers with lactobacilli for 1 h before virus adsorption resulted in very low inhibition of HSV-2 multiplication. A similar effect was observed after exposure of Vero cells to living lactobacilli for 24 hrs before infection (not shown). On the contrary, a strain-dependent inhibition of virus yield was observed when bacteria were present during virus adsorption (1 h, 4°C), and removed before incubation at 37°C in antibiotic-containing medium to

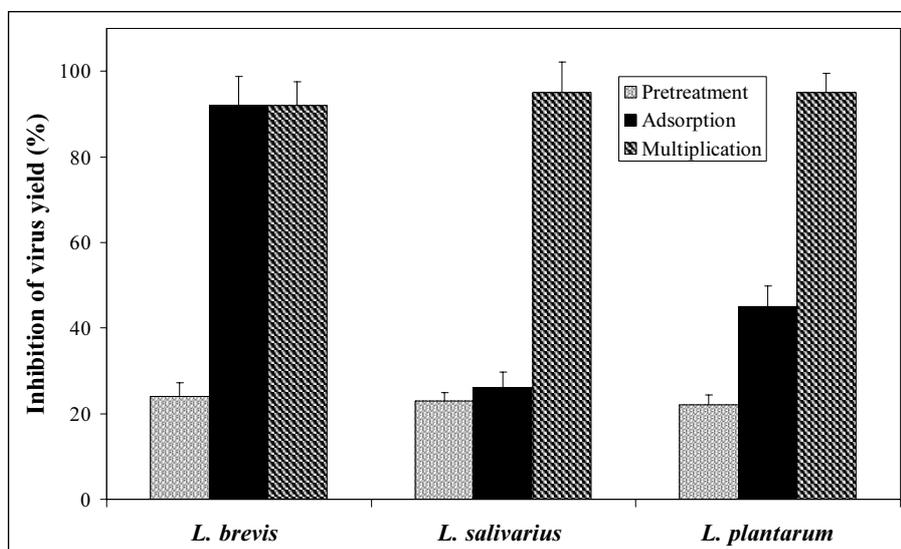


Fig. 2. Inhibition of HSV-2 multiplication by lactobacilli. Vero cells were incubated with bacteria (1000 CFU/cell) for 1 h at 37°C in antibiotic-free maintenance MEM before virus infection (▨), during HSV-2 adsorption for 1 h at 4°C (■), or for 16 hrs at 37°C after virus adsorption (▩). Antibiotics were added to pretreatment and adsorption samples during virus multiplication. Results are expressed as % of PFU of control virus in the same experimental conditions and each value is the mean±SD obtained from triplicate wells of two independent experiments. Control virus titre was ~6×10⁶ PFU/ml.

kill residual bacteria. The presence of *L. brevis* during virus binding to cell membrane receptors inhibited HSV-2 multiplication by more than 90%. Approximately 50% inhibition was exerted by *L. plantarum*, whereas low inhibition was shown by *L. salivarius*. To evaluate the effect of living lactobacilli on HSV-2 multiplication, bacteria were added immediately after the virus adsorption step and maintained during the virus replication cycle in antibiotic-free medium. Metabolically active lactobacilli showed good antiviral properties reducing virus yield by more than 90%. The three strains resulted equally effective towards HSV-2. The inhibitory effects were dependent on the bacteria/cell ratio. When a hundred-fold lower ratio (10 CFU/cell) was used only *L. brevis* produced a minor inhibition during HSV-2 adsorption (30% reduction) and multiplication (40% reduction). No inhibition was observed at 0.1 CFU/cell (data not shown).

Effect of lactobacilli and their culture supernatants (CSs) on virus infectivity

The inhibition exerted by lactobacilli during virus multiplication could be related to a direct effect on virus particles released from infected cells or on some intracellular event of virus replication. The effect on free virus particles was investigated titrating residual virus infectivity after incubation of high titre HSV-2 with bacterial cells or bacterial products. Virus titre was unaffected after preincubation of HSV-2 with each *Lactobacillus* strain at a PFU/CFU ratio of 1/1000 (data not shown). To test the effect of overall *Lactobacillus* metabolites on HSV-2 particles, antibiotic-free cell culture medium (MEM) from 16 h bacterial cultures in aerobic atmosphere were utilized. Culture supernatants obtained under these conditions (CSs) had

a neutral pH and did not modify the infectivity of HSV-2 virions (data not shown).

Effect of CSs on HSV-2 multiplication

Given that the anti-HSV-2 activity of lactobacilli was not exerted on virus particles, experiments were designed to verify whether the antiviral activity was exerted on intracellular events of virus multiplication. To this end, the overall effect of *Lactobacillus* products was evaluated using CSs (pH 7.2) as cell culture medium during HSV-2 multiplication. Virus yield was significantly reduced by all CSs although to a different extent. *L. brevis*-CS was the most effective causing more than 2 logs reduction (Fig. 3). To verify whether additional soluble antiviral factors could be also released by Vero cells upon incubation with *Lactobacillus* strains, parallel experiments were performed using culture supernatants of lactobacilli grown in the presence of cell monolayers (Cell-CSs). A similar level of HSV-2 inhibition was observed indicating that the antiviral activity is not mediated by a cell-released product.

Candidate antiviral components in CSs

The data previously reported seem to indicate that soluble factors released from lactobacilli inhibit intracellular HSV-2 multiplication. Hydrogen peroxide and lactic acid represent important *Lactobacillus* products with known antimicrobial activity towards several bacteria and mycetes. To verify the involvement of these metabolites in the above reported anti-HSV-2 activity of lactobacilli, we first investigated the production of H₂O₂ and lactic acid in the experimental conditions used in the antiviral assay. Unlike *L. brevis*, both

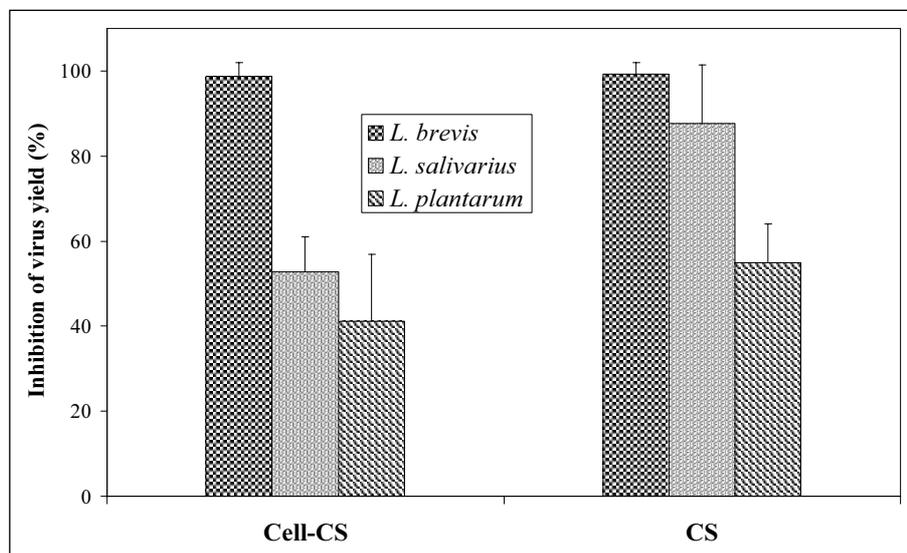


Fig. 3. Inhibition of HSV-2 multiplication by culture supernatants of lactobacilli. Supernatants of lactobacilli (4×10^8 CFU/ml, 1000 CFU/cell) grown overnight in MEM, in the presence (Cell-CS) or absence (CS) of Vero cells, were added to HSV-2 infected monolayers during the whole virus multiplication cycle. Virus yield was determined by plaque assay. Results are expressed as % of PFU of control virus grown in the same experimental conditions and each value is the mean \pm SD obtained from triplicate wells of two independent experiments. Control virus titre was $\sim 2 \times 10^6$ PFU/ml.

Table 1 Production of lactic acid by lactobacilli in different culture conditions

Lactic acid was measured after 16 h incubation of lactobacilli in maintenance MEM in aerobic atmosphere (final concentration $\sim 10^8$ CFU/ml) or MRS broth in anaerobic atmosphere (final concentration $\sim 5 \times 10^9$ CFU/ml).

Growth medium	MEM						MRS		
	Cells present [†]			Cells absent			D-	L-	TOTAL
Lactic acid	D-	L-	TOTAL	D-	L-	TOTAL	D-	L-	TOTAL
<i>L. brevis</i>	0.9	2.8 (0.1)	3.7 (1.0)	1.1	2.4	3.5	19.4	26.2	45.6
<i>L. salivarius</i>	0.5	6.5 (3.8)	7.0 (4.3)	0.6	8.8	9.4	9.3	147.4	156.7
<i>L. plantarum</i>	4.3	2.6 (0)	6.9 (4.3)	4.9	4.7	9.6	96.7	75.6	172.3

[†] Values in brackets are those obtained after subtracting the amount of L-lactic acid produced in control uninfected cells (2.7 mM).

strains of *L. salivarius* and *L. plantarum* resulted good H₂O₂ producers in bacterial growth medium (MRS) under anaerobic atmosphere (19). In the antiviral test conditions, H₂O₂ was detected in μ molar concentration and the amount measured at 4 h intervals remained constant for each strain during the 16 h period (*L. salivarius* 2.2 μ M; *L. plantarum* 0.7 μ M). The quantity of D- and L-lactic acid measured in culture medium (MEM) after incubation of lactobacilli in the absence or in the presence of Vero cells is reported in Table 1. All the *Lactobacillus* strains released substantial amounts of lactic acid (from 3.5 to 9.6 mM), although at different ratios of the two isomers. *L. brevis* and *L. plantarum* produced D- and L-lactic acid at approximately equimolar concentrations, whereas *L. salivarius* produced a fifteen-fold lower amount of the D isomer. The quantity of L-lactic acid measured in the presence of Vero cells and high producer *Lactobacillus* strains (*L. salivarius* and *L. plantarum*) was lower than expected considering the overall amounts produced by cells and lactobacilli. Indeed L-lactic acid concentration was lower than that produced by lactobacilli alone indicating a cell-mediated effect, probably due to cell metabolism. Altogether in cell culture medium and aerobic atmosphere lactic acid was produced in significantly lower concentration in comparison to that produced in the optimal atmosphere and growth medium (MRS) specific for lactobacilli (Table 1).

The antiviral potential of purified H₂O₂ and lactic acid towards HSV-2 was studied by evaluating both the effect on

virions or the activity during virus multiplication in one cycle of virus growth (1 PFU/cell, 16 h virus multiplication). Hydrogen peroxide is known to degrade in aqueous medium particularly in the presence of cultured cells (22). Therefore, before studying the effect on virus multiplication, we determined the rate of H₂O₂ degradation in our experimental conditions. Micromolar amounts of H₂O₂ were reduced by 50% after 4 h incubation at 37°C with medium alone and to one tenth after 2 hrs in the presence of Vero cells (data not shown). These results indicate that H₂O₂ is metabolized promptly in the cell environment and that it is not possible to maintain constant amounts of H₂O₂ on cell culture. Therefore only the virucidal effect of H₂O₂ was studied. HSV-2 was incubated with H₂O₂ or lactic acid in maintenance MEM to verify the role of both metabolites in the inhibition exerted by living *Lactobacillus* cells or CSs during virus multiplication. Hydrogen peroxide showed a dose-dependent virucidal effect with a 50% inhibition at 184 μ M after 1 h incubation (Fig. 4A). Lactic acid showed a time- and dose-dependent virucidal effect that was directly related to acidic pH (Fig. 4B). Exposure of herpes simplex virus to lactic acid amounts giving a pH 5.0 or lower for 1 h inactivated HSV and reduced virus titre by nearly 100% (3 and 5 log inhibition for 22 and 44 mM, respectively). Viral inactivation was less effective at pH 6.5 (11 mM lactic acid), which resulted in 1 log inhibition of infection and required exposure to the acidic environment for 16 hrs. Exposure to 5.5 mM lactic acid (pH 7.0) had little or no effect. Incubation of

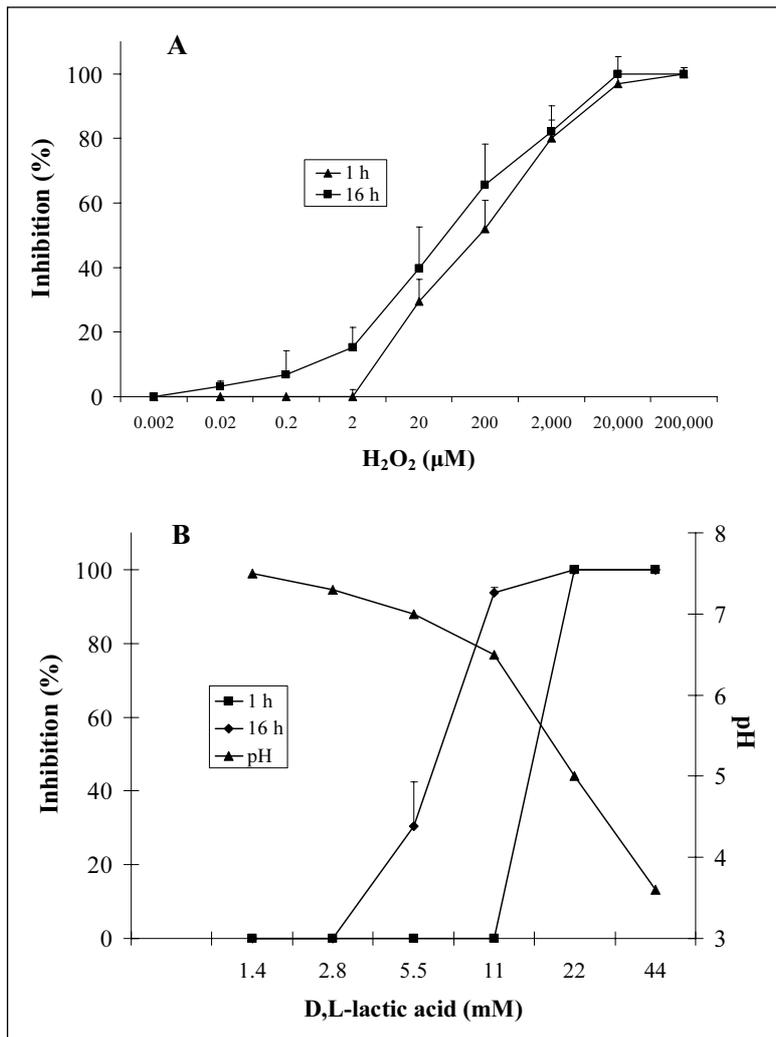


Fig. 4. Virucidal effect of H₂O₂ (A) and lactic acid (B). High titre HSV-2 (9×10^6 PFU/ml) was incubated in screw-cap tubes with different concentrations of compounds in maintenance MEM for 1 h or 16 hrs at 37°C before titration by plaque assay. Results are expressed as % of PFU of untreated control in the same experimental conditions and each value is the mean \pm SD obtained from triplicate wells of three independent experiments.

HSV in cell culture medium brought to identical acidic pH values with HCl, an unrelated mineral acid, produced the same levels of inhibition (data not shown). Moreover, when lactic acid solutions up to 44 mM were brought to pH 7.5 by NaOH addition before incubation with virus no virucidal effect was observed. The inactivation of HSV by lactic acid was irreversible since infectivity was not restored by neutralization of the lactic acid-treated virus preparation (not shown).

The antiviral activity of lactic acid towards intracellular events of virus multiplication was verified evaluating the viral antigen production by immunofluorescence (Fig. 5) in HSV-2-

infected Vero cells incubated in lactic acid containing medium. HSV-2 antigen synthesis was reduced by more than 80% at 22 mM lactic acid (pH 5.8) and by approximately 40% at 11 mM (pH 6.9), while no inhibition was observed at 5.5 mM (pH 7.4). The pH values reported were those of infected cell culture medium after 16 hrs incubation in CO₂ atmosphere. Neutralization of lactic acid containing medium suppressed the inhibitory activity.

DISCUSSION

Lactic acid-producing bacilli are part of the normal bacterial microbiota of the vagina and have a physiological role in maintaining a low pH (≤ 4.5) and protecting against invasion by other microorganisms. The mean vaginal lactic acid concentration of healthy women is 9.66 mM (from 4.7 to 17.7 mM) and lower lactate concentrations indicate severe depression of lactobacillary function (23). Indeed, women with BV have a mean vaginal lactic acid amount of 2.8 mM (23) and a higher risk of acquiring HSV-2 infection (7). The clinical observation of the inverse correlation between vaginal *Lactobacillus* load and genital herpes was not supported until now by *in vitro* studies confirming the role of lactic acid in the antiviral protection. The results presented here indicate that HSV-2 is irreversibly inactivated by concentrations of lactic acid giving pH values corresponding to that observed in the healthy human vagina. However, it must be noticed that in cell culture medium a pH value of ~ 4.5 is obtained using a lactic acid amount 3 fold higher than vaginal physiological concentration. This suggests that the buffering capacity of the vaginal milieu is different from that of culture medium or that the contribution of lactic acid to the vaginal acidic pH is only partial. The antiviral effect of lactic acid we observed is directly related to acidic pH values and to the time of exposure. Nicola *et al.* (24) demonstrated that HSV entry into Vero cells occurs *via* fusion at the plasma membrane and is inhibited by acid pretreatment of virions (pHs 4.7 to 6.0 in HEPES buffer), therefore it is possible that adequate concentrations of vaginal lactic acid are able to block the early stages of HSV-2 infection. Moreover, our study showed that intracellular events of virus multiplication are impaired in the presence of lactic acid amounts corresponding to the physiological concentrations of the compound in the vaginal environment. Vero cells exposed to lactic acid concentrations giving acidic pH bindi after HSV-2 resulted less effective in viral protein synthesis and in the production of infective virions. How this mechanism could act in the vagina is open to speculation. In fact, epithelial cells of the genital mucosal surface, that represent the initial target of HSV-2 infection, are covered by cervical mucus that provides a protective coating for the vaginal and cervical epithelium (25). However in healthy women the actual pH of the vaginal cell surface beneath the mucus layer, after removal of mucus with sterile cotton swabs, is 5.2-5.5 (C. Midulla, unpublished observations). Therefore it is possible that, in the presence of lactic acid producing vaginal microbiota, vaginal cells have a lower efficiency in supporting HSV-2 replication.

It has been suggested that H₂O₂ produced by some *Lactobacillus* species may play some role, though not a crucial one, in controlling vaginal microbiota (26). Although it is known that a H₂O₂ gas plasma sterilization process inactivates herpes simplex virus type 1 (27), no data are available on the virucidal effect of H₂O₂ on herpes simplex virus type 2. Our results demonstrated that H₂O₂ treatment impairs the infection capacity of HSV-2 virions. It is not possible to predict if such an activity could be effective in the vagina, since, to our knowledge, the vaginal concentration of H₂O₂ in women with H₂O₂-producing

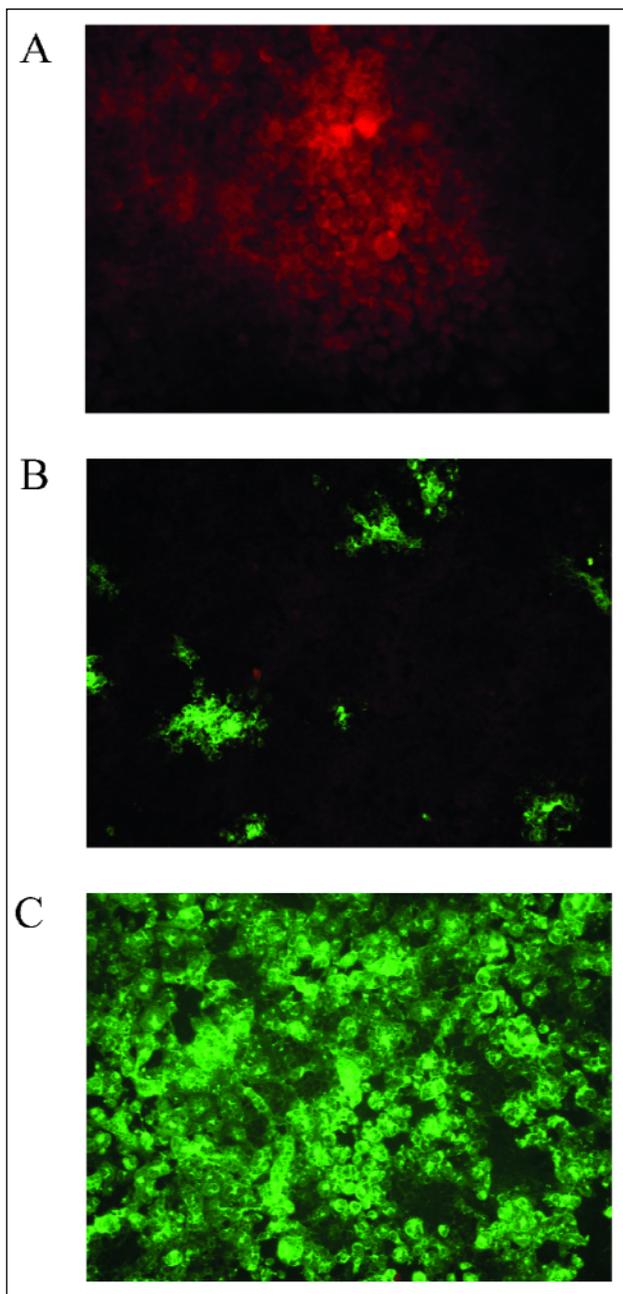


Fig. 5. Effect of lactic acid on HSV-2 antigen synthesis in Vero cells. Lactic acid containing medium was added to infected cells during the whole virus replication cycle. A: uninfected; B: 22 mM lactic acid; C: control virus. Viral antigens were labelled using FITC-conjugated rabbit anti-HSV-2 immunoglobulins that react with all the major viral envelope glycoproteins and at least one core protein.

lactobacilli has never been determined. Moreover an effect of H₂O₂ on intracellular virus replication cannot be ruled out. Our results demonstrated that some *Lactobacillus* strains produce H₂O₂ in cell culture giving micromolar amounts of the compound during the whole virus replication cycle. It is known that micromolar concentrations of H₂O₂ can produce different biological effects. Treatment with exogenous H₂O₂ (1 µM) result in protein phosphorylation in U937-neo cells (28) and oxidative stress induced by 5 µM H₂O₂ in PC12 cells significantly up-regulates neutral sphingomyelinase activity (29).

The results of the present study demonstrate that, beside the production of antiviral molecules, lactobacilli can protect susceptible cells from HSV-2 infection by other mechanisms. Vaginal lactobacilli were able to inhibit the first step of herpes virus infection. The antiviral activity exerted by the presence of lactobacilli during HSV-2 binding to the cell membrane was strain-dependent and appeared directly related to the adhesion capacity of *Lactobacillus* strains (19). In fact, *L. brevis* CD2, highly adhesive, was strongly inhibitory during HSV-2 binding, *L. plantarum* FV9 showed intermediate adhesiveness and inhibition, whereas *L. salivarius* FV2 adhering at low levels to the cell surface resulted a very poor inhibitor. Lactobacilli were unable to inhibit virus infection after preincubation with virions or cells before the adsorption step suggesting that bacterial strains did not bind to viral or cell surface molecules involved in the binding. Since the adsorption was performed at 4°C to allow binding but not entry of virus, we hypothesize that the simultaneous presence of virus and a great number of lactobacilli on the cell membrane impairs the fusion between viral envelope and the cell surface leading to an inhibition of virus entry into cells.

Infection was significantly reduced if HSV-2 was cultured in the presence of living lactobacilli. The inhibition does not seem to be related to the presence of *Lactobacillus* cells since virus replication is also inhibited if HSV-2 is cultured in cells fed with neutral pH culture supernatants of lactobacilli. It is unlikely that the inhibitory product in the CSs could be hydrogen peroxide or lactic acid since *L. brevis* CD2, the most active strain, does not produce H₂O₂ and neutralized lactic acid had no effect. The detected anti-HSV-2 activity of CSs, once the putative effects of lactic acid and H₂O₂ have been ruled out, raises the question of the chemical nature of active molecules. The identification of these compounds could give important contributes to the knowledge of natural defence mechanisms of the healthy human vagina against sexually transmitted viral infections.

In conclusion, numerous mechanisms may be involved in the antiviral effect of lactobacilli towards HSV-2: interference with early steps of virus infection (binding/entry), production of metabolites with a direct antiviral effect (lactic acid, hydrogen peroxide) and production of compounds able to inhibit intracellular events of virus replication.

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