Journal of Medical Microbiology (2003), **52**, 1117–1124 DOI 10.1099/jmm.0.05155-0

Growth and lactic acid production by vaginal *Lactobacillus acidophilus* CRL 1259, and inhibition of uropathogenic *Escherichia coli*

María Silvina Juárez Tomás,[1] Virginia S. Ocaña,[1] Birgitt Wiese[2] and María E. Nader-Macías[1]

[1]CERELA-CONICET (Centro de Referencia para Lactobacilos), Chacabuco 145, 4000, Tucumán, Argentina

[2]Institute of Biometrics, University Hospital, Hannover, Germany

Correspondence
María E. Nader-Macías
fnader@cerela.org.ar

Received 16 December 2002
Accepted 5 September 2003

Lactic acid-producing lactobacilli were selected from 134 human vaginal isolates by testing their capability to inhibit the growth of different pathogenic micro-organisms. *Lactobacillus acidophilus* CRL 1259 (from the CERELA Culture Collection) was selected to study the effects of temperature, pH and culture medium on growth and lactic acid production. Growth parameters were estimated by using the model of Gompertz. Kinetics of inhibition of uropathogenic *Escherichia coli* were evaluated in mixed cultures of the pathogen and *L. acidophilus*. Optimal conditions for growth and lactic acid production by *L. acidophilus* were pH 6·5 or 8·0 and 37 °C. Under these conditions, growth was higher in LAPTg (yeast extract/peptone/tryptone/Tween 80/glucose) broth than in MRS (De Man–Rogosa–Sharpe) broth. However, lactic acid production was more efficient in MRS broth. Under optimal conditions for lactic acid production, *L. acidophilus* inhibited the growth of *E. coli*. These results suggest that inclusion of *L. acidophilus* CRL 1259 in probiotic products for vaginal application would be beneficial.

INTRODUCTION

In the vaginal tract, high levels of oestrogens stimulate the deposit of glycogen in the epithelia, which is then fermented to acetic and lactic acids by epithelial cells and/or vaginal flora (Paavonen, 1983). Recent studies support the hypothesis that vaginal bacteria are the primary source of lactic acid in the vagina (Boskey *et al.*, 1999, 2001). Lactobacilli have been recognized as the predominant microflora of the healthy human vagina to maintain a pH of $< 4{\cdot}5$ (Redondo-López *et al.*, 1990). This low pH reduces the risk of colonization by pathogens (Stamey & Kaufman, 1975; Stamey & Timothy, 1975; Hanna *et al.*, 1985; Tevi-Bénissan *et al.*, 1997). Bacterial vaginosis, the most common vaginal pathology worldwide, is characterized by a vaginal pH of $> 4{\cdot}5$ and by an overgrowth of anaerobic bacteria (Eschenbach, 1993). An increase in vaginal pH is detrimental to the survival of lactobacilli; therefore, local acidification with lactic acid or lactobacilli is useful for restoration of the vaginal ecosystem (Melis *et al.*, 2000).

The characteristics of lactobacilli, i.e. their ability to colonize different hosts (Kotarski & Savage, 1979), led to the isolation of strains from the human vagina (Ocaña *et al.*, 1999a) and their use in vaginal probiotic products (Ocaña *et al.*, 1999b, c, d). Optimal culture conditions to obtain the highest growth of the selected micro-organisms (Juárez Tomás *et al.*, 2002a), as well as a higher degree of bacteriocins (Juárez Tomás *et al.*, 2002b), were reported.

Lactic acid production by lactobacilli that are used by food industries has been studied extensively (Passos *et al.*, 1994; Kylä-Nikkilä *et al.*, 2000). However, there are only a few reports concerning the growth and lactic acid production by vaginal lactobacilli (Boskey *et al.*, 1999, 2001). In this paper, the capability of autochthonous strains of vaginal lactobacilli to inhibit growth of different pathogenic micro-organisms was analysed. *Lactobacillus acidophilus* CRL 1259 was selected to study the effects of different culture conditions on biomass and lactic acid production. The inhibitory effect of lactic acid produced by this strain on the growth of a human uropathogenic *Escherichia coli* strain was also determined.

Abbreviation: LDH, lactic acid dehydrogenase.

METHODS

Micro-organisms and culture media. Vaginal lactobacilli strains ($n = 134$) that had been isolated previously from vaginal samples of healthy women from 19 to 45 years old from Tucumán, Argentina (Ocaña *et al.*, 1999a), were studied. The following human uropathogenic micro-organisms were employed: *E. coli*, *Klebsiella* sp., group B *Streptococcus* sp., *Enterococcus faecalis*, *Staphylococcus aureus*, *Neisseria gonorrhoeae*, *Candida* sp. and *Gardnerella* sp. (provided by the Instituto

de Microbiología 'Luis Verna' of the Universidad Nacional de Tucumán) and *Streptococcus agalactiae* ATCC 27956 (CRL 1022) (from the American Type Culture Collection). The strain of *E. coli* that was used for mixed cultures had the following urovirulence characteristics: type P fimbriae (as demonstrated by the haemagglutination test), production of haemolysins and pyelonephritogenic effects, as tested in mice (Silva-Ruiz *et al.*, 2001).

All micro-organisms were stored in milk/yeast extract (130 g non-fat milk, 5 g yeast extract and 10 g glucose l^{-1}) at −20 °C, except for *N. gonorrhoeae* and *G. vaginalis*, which were used as soon as they had been isolated. Stored lactobacilli and pathogens were subcultured three times for 12 h in LAPTg (yeast extract/peptone/tryptone/Tween 80/glucose) broth (Raibaud *et al.*, 1973), prior to screening for production of inhibitory substances.

Before the growth experiments, *L. acidophilus* CRL 1259 was subcultivated in either MRS (De Man–Rogosa–Sharpe; De Man *et al.*, 1960) broth (Biokar Diagnostics) or LAPTg broth. The inoculum was prepared as described previously (Juárez Tomás *et al.*, 2002a).

Screening for production of inhibitory levels of antagonistic substances. The effects of supernatant fluid of 134 strains of vaginal lactobacilli on the growth of uropathogens were studied by employing the plate-diffusion technique (Jack *et al.*, 1995). Briefly, LAPTg agar plates (standardized volume, 15 ml LAPTg broth with 1 % agar) with 10^6–10^7 c.f.u. ml^{-1} of each pathogen were prepared, as described previously (Ocaña *et al.*, 1999b). Standardized aliquots (25 µl) of non-treated and neutralized supernatant of lactobacilli were placed into holes (standardized diameter, 4 mm) in the pathogen-inoculated plates. The plates were incubated for 5 h at room temperature and then for 24 h at 37 °C. A clear inhibition zone of ⩾ 6 mm diameter was defined as a positive result. Control assays with the culture medium (LAPTg broth, pH 4 or 6·5) were also performed.

Growth and lactic acid production by *L. acidophilus* CRL 1259. Combinations of two culture media (LAPTg or MRS broth), three temperatures (30, 37 or 44 °C) and three initial pH values (5·0, 6·5 or 8·0) were evaluated. Growth experiments, including preparation of culture media, pH determination and quantification of c.f.u. ml^{-1}, were performed as described previously (Juárez Tomás *et al.*, 2002b).

Amounts of D- and L-lactic acid produced during growth were analysed enzymically by using a lactic acid dehydrogenase (LDH) commercial test kit (Boehringer Mannheim). The assay was performed on supernatant fluids of lactobacilli cultures that were obtained by centrifugation at 5000 r.p.m. for 10 min.

Estimation of growth curves. Growth parameters, estimated by using the four-parameter Gompertz model, are: log (c.f.u. $ml^{-1})_t$ (cell concentration at time t); log (c.f.u. $ml^{-1})_0$ (cell concentration at time zero); A [increase of biomass between log (c.f.u. $ml^{-1})_0$ and log (c.f.u. $ml^{-1})_{max}$]; µ [maximum specific growth rate (h^{-1})]; and λ [duration time of lag phase (h)] (Zwietering *et al.*, 1990; Juárez Tomás *et al.*, 2002a).

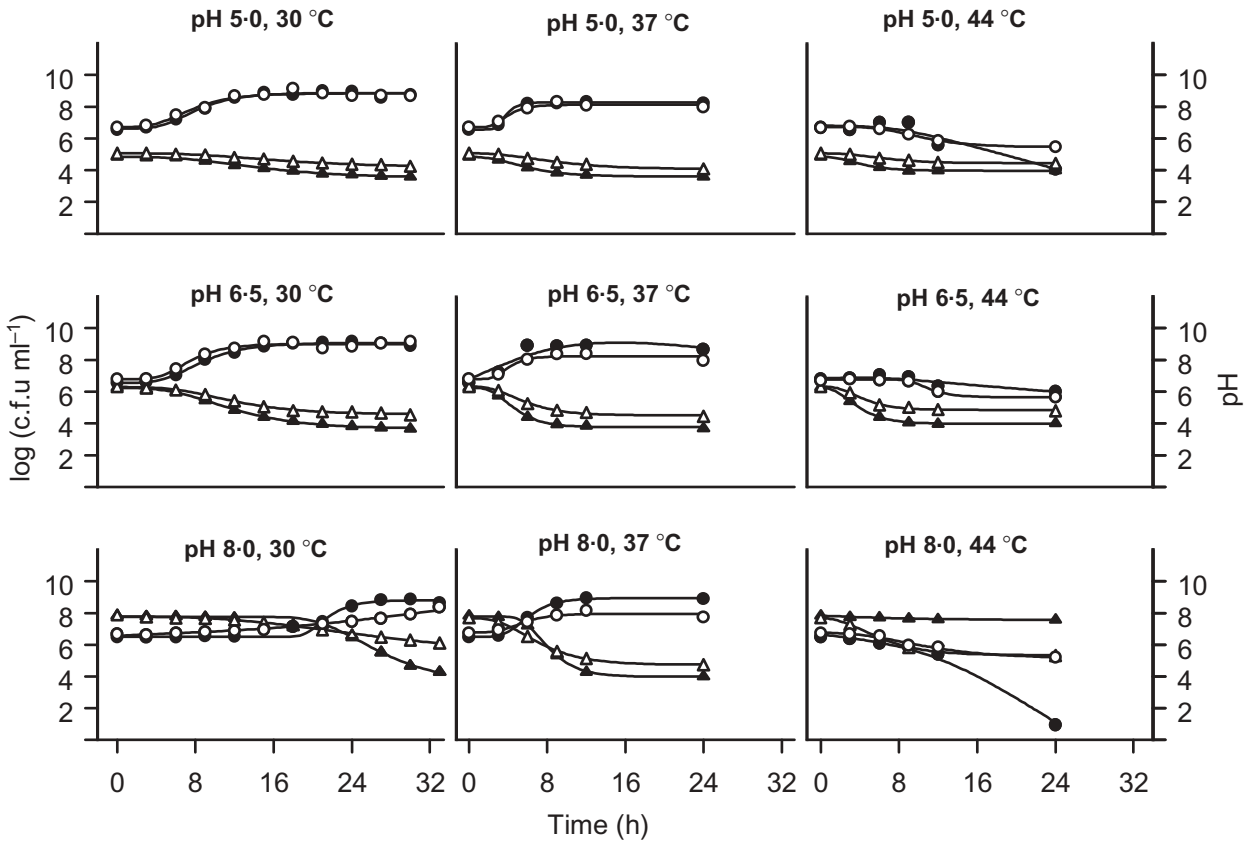

Fig. 1. Kinetics of growth and decrease in pH of *L. acidophilus* CRL 1259 under different culture conditions. Log c.f.u. ml^{-1} in LAPTg broth (●) and MRS broth (○); pH modifications in LAPTg broth (▲) and MRS broth (△).

Standard errors (SE) of the growth parameters were calculated by the bootstrapping method (Efron, 1982; Huet *et al.*, 1996; Juárez Tomás *et al.*, 2002b).

To determine the statistical significance of the effects of each growth medium (LAPTg or MRS broth) on growth parameters, the differences between parameters were included directly in the equation of the model, in order to estimate confidence intervals (data not shown).

To evaluate multivariate effects of different conditions (temperature, initial pH and culture medium) on growth parameters, the non-linear mixed-effects model [as proposed by Lindstrom & Bates (1990)] was applied by using restricted maximum-likelihood.

For analyses and graphical presentations, the statistical programs SAS 8.2, SPSS 10 and S-Plus 2000 were used.

Mixed cultures of *L. acidophilus* CRL 1259 and *E. coli*. Mixed cultures of *L. acidophilus* CRL 1259 and *E. coli* were performed in LAPTg broth at 37 °C. MRS broth was not used, as *E. coli* grew slowly in this medium. Inocula contained 10^5–10^6 c.f.u. ml^{-1} for *E. coli* and 10^6–10^7 c.f.u. ml^{-1} for lactobacilli. Viable cell counts were determined by the plate-dilution method by using selective culture media: MacConkey agar for *E. coli* and lactobacillus selective medium (LBS) for lactobacilli. MacConkey and LBS plates were incubated at 37 °C for 48 h under aerobic and microaerophilic conditions, respectively.

The pH values and levels of D- and L-lactic acids in pure and mixed cultures were determined as described above. All experiments were performed in triplicate. Means of the data are represented in the graphs.

Determination of the MIC of lactic acid. The diffusion method was applied to agar plates that were prepared as described above and contained uropathogenic *E. coli*. Decreasing concentrations of lactic acid were evaluated (111–1·1 mM). The MIC was defined as the lowest amount of lactic acid that produced a clear inhibition zone.

RESULTS

Inhibition of pathogens by lactobacilli supernatants

Among the 134 strains of vaginal lactobacilli isolated previously (Ocaña *et al.*, 1999a), only *Lactobacillus brevis* CRL 1335 and *L. acidophilus* strains CRL 1259, CRL 1307, CRL 1320 and CRL 1324 were able to inhibit the growth of *E. coli*, *Staphylococcus aureus*, *Streptococcus agalactiae*, *Enterococcus faecalis*, *Klebsiella* sp., *N. gonorrhoeae* and *G. vaginalis*. Inhibition haloes were shown to be produced by the low pH of the lactobacilli supernatants, as they disappeared when the supernatants were neutralized. *L. acidophilus* CRL 1259 produced bigger inhibition haloes on the pathogen plates (data not shown).

Lactobacillus salivarius subsp. *salivarius* CRL 1328 was able to inhibit the growth of *E. coli*, *Klebsiella* sp., *G. vaginalis*, *Staphylococcus aureus* and *Streptococcus agalactiae* by the effect of pH, and *N. gonorrhoeae* and *Enterococcus faecalis* by a bacteriocin-like substance that was reported previously (Ocaña *et al.*, 1999d). *Lactobacillus crispatus* CRL 1266 only inhibited the growth of *S. aureus* by the effect of H_2O_2 (a catalase-sensitive metabolite) (Ocaña *et al.*, 1999b).

Table 1. Estimation of growth parameters of *L. acidophilus* CRL 1259 under different growth conditions by application of the Gompertz model

Parameters of the Gompertz model (± SE): log (c.f.u. ml^{-1})$_0$, initial biomass; A, increase between initial and final biomass; μ, maximum specific growth rate; λ, lag phase.

Conditions	log (c.f.u. ml^{-1})$_0$	A	μ (h^{-1})	λ (h)
30 °C				
pH 5·0				
LAPTg	6·62 ± 0·27	2·23 ± 0·28	0·30 ± 0·28	4·23 ± 1·98
MRS	6·72 ± 0·66	2·11 ± 0·72	0·27 ± 0·29	3·55 ± 2·73
pH 6·5				
LAPTg	6·56 ± 0·25	2·51 ± 0·27	0·30 ± 0·09	4·30 ± 1·61
MRS	6·79 ± 0·14	2·21 ± 0·17	0·35 ± 0·28	4·16 ± 1·83
pH 8·0				
LAPTg	6·51 ± 0·02	2·29 ± 0·10	0·48 ± 0·26	19·02 ± 2·66
MRS	6·71 ± 0·06	3·00 ± 0·29	0·08 ± 0·01	13·90 ± 2·09
37 °C				
pH 5·0				
LAPTg	6·56 ± 0·32	1·70 ± 0·32	0·80 ± 0·23	2·64 ± 1·42
MRS	6·70 ± 0·37	1·42 ± 0·39	0·41 ± 0·29	2·15 ± 1·71
pH 6·5				
LAPTg	6·57 ± 0·99	2·28 ± 1·08	1·00 ± 0·07	2·33 ± 1·05
MRS	6·79 ± 0·28	1·44 ± 0·33	0·43 ± 0·27	2·32 ± 1·34
pH 8·0				
LAPTg	6·51 ± 0·34	2·44 ± 0·35	0·49 ± 0·22	3·52 ± 0·98
MRS	6·79 ± 0·31	1·16 ± 0·54	0·23 ± 0·37	2·89 ± 1·66

Optimization of growth conditions of *L. acidophilus* CRL 1259

Fig. 1 shows the growth and pH decrease of *L. acidophilus* CRL 1259 in LAPTg and MRS broth under different combinations of initial pH and temperature. At 44 °C, the viability of the micro-organisms decreased after a short time. In this case, growth-parameter estimation and lactic acid determination were not performed.

Values of the growth parameters obtained varied with the culture conditions tested (Table 1). For all conditions tested, LAPTg broth supported higher growth than MRS broth, but this was statistically significant only at an initial pH of 8·0 and 37 °C. For all growth media and pH values assayed, growth rates were higher at 37 °C. Length of lag phases was inversely related to temperature. When the two types of broth were incubated at the same temperature, lag phases were longer at an initial pH of 8·0.

According to statistical analysis performed with the non-linear mixed-effects model, initial pH of the culture medium and temperature of incubation showed significant effects ($P < 0{\cdot}05$) on all growth parameters tested (increase of biomass, growth rate and lag phase). However, culture medium only affected the final biomass significantly.

Optimal conditions for the growth of *L. acidophilus* were LAPTg broth with an initial pH of 6·5 and at 37 °C. Under these conditions, the highest biomass and growth rates, together with shorter lag phases, were obtained. Similar growth was observed in LAPTg broth at 37 °C and an initial pH of 8·0.

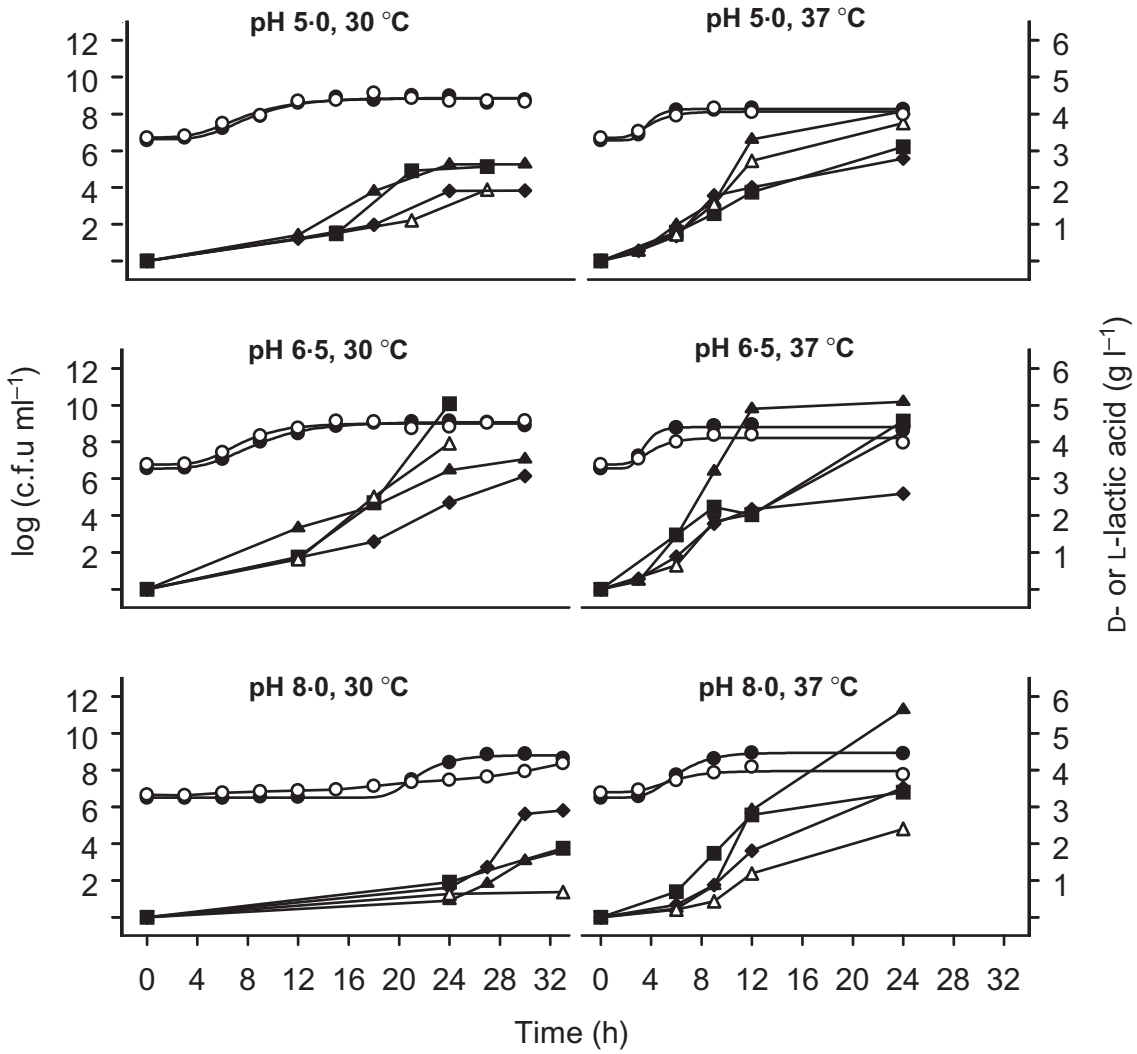

Fig. 2. Kinetics of growth and lactic acid production by *L. acidophilus* CRL 1259 under different culture conditions. Log c.f.u. ml^{-1} in LAPTg broth (●) and MRS broth (○); levels of D-lactic acid in LAPTg broth (▲) and MRS broth (△); levels of L-lactic acid in LAPTg broth (◆) and MRS broth (■).

pH decrease by *L. acidophilus* CRL 1259 under different growth conditions

Decrease in pH and acidification rates were significantly higher in LAPTg broth than in MRS broth, due to the higher ion content and buffering capacity of the latter medium (Fig. 1). The difference between initial and final pH of *L. acidophilus* cultures was related directly to initial pH when LAPTg or MRS broth was incubated at the same temperature. The same behaviour was observed with acidification rates. The largest decrease in pH was obtained in LAPTg broth at an initial pH of 6·5 or 8·0 and at 37 °C. This effect was also observed at 30 °C, but after a longer incubation time.

Lactic acid production by *L. acidophilus* CRL 1259

Relative proportions of D- and L-lactic acid varied according to the growth medium used (Fig. 2). In general, levels of the D-isomer produced in LAPTg (expressed as g l^{-1}; Fig. 2) were higher than those of the L-isomer. An inverse relationship was observed in MRS broth.

In both growth media at different initial pH levels, production of the L- and D-isomers was maximal at 37 °C. When the two types of broth were incubated at the same temperature (except for LAPTg broth at 37 °C), higher amounts of D- and L-lactic acid (expressed as g l^{-1}) were observed at pH 6·5. Maximal concentrations of D-lactic acid were obtained in LAPTg broth at 37 °C and pH 6·5 (5·09 g l^{-1} after 12 h culture) or 8·0 (5·64 g l^{-1} after 24 h). The best conditions for production of L-lactic acid were MRS broth at an initial pH of 6·5 and 30 or 37 °C (5·04 and 4·57 g l^{-1}, respectively, both after 24 h culture).

Levels of D-, L- and total lactic acid produced by 10^7 c.f.u. were higher in MRS broth than in LAPTg broth (Table 2). This indicates that *L. acidophilus* is more active, from a metabolic point of view, in MRS broth.

Mixed cultures of *L. acidophilus* CRL 1259 and *E. coli*

Results from mixed cultures of *L. acidophilus* CRL 1259 and *E. coli* are shown in Fig. 3. When using an *E. coli* inoculum of $1{\cdot}01\times10^6$ c.f.u. ml^{-1}, complete inhibition of pathogen growth was observed after 21 h, whereas when the inoculum of *E. coli* was $2{\cdot}4\times10^5$ c.f.u. ml^{-1}, 100 % inhibition of pathogen growth was observed after 15 h.

Levels of L- and D-lactic acid produced by lactobacilli, either in pure or mixed culture, were two times higher than those produced by pure *E. coli* cultures at both inoculum levels. In mixed cultures, the concentrations were 5·5 g l^{-1} for D-lactic acid and 2·8 g l^{-1} for L-lactic acid.

Table 2. Mean values of maximal lactic acid concentration produced by *L. acidophilus* CRL 1259 under different culture conditions

Conditions	Total lactic acid (g l^{-1})	Lactic acid [mg ml^{-1} (10^7 c.f.u.)$^{-1}$]		
		D-isomer	L-isomer	Total
30 °C				
pH 5·0				
LAPTg	4·54	0·04	0·03	0·07
MRS	4·51	0·03	0·05	0·08
pH 6·5				
LAPTg	6·61	0·04	0·04	0·08
MRS	9·00	0·05	0·07	0·12
pH 8·0				
LAPTg	4·70	0·04	0·07	0·11
MRS	2·56	0·03	0·08	0·11
37 °C				
pH 5·0				
LAPTg	6·85	0·23	0·15	0·38
MRS	6·85	0·39	0·33	0·72
pH 6·5				
LAPTg	7·69	0·09	0·05	0·14
MRS	8·85	0·48	0·51	0·99
pH 8·0				
LAPTg	9·16	0·04	0·02	0·06
MRS	5·80	0·41	0·59	1·00

Maximal amounts of lactic acid were obtained between 24 and 30 h culture at 30 °C or after 24 h for cultures at 37 °C.

Determination of MIC

The MIC of lactic acid for *E. coli* was 55·49 mM (equivalent to 5·0 g l^{-1}). This value was lower than the lactic acid levels produced by *L. acidophilus* CRL 1259 after 9 h in mixed cultures, when pathogen viability decreased.

DISCUSSION

Primary selection of potentially probiotic strains must be performed through the application of '*in vitro*' techniques. Production of antagonistic substances (organic acids, hydrogen peroxide or bacteriocins) against pathogens is a technique that is widely used (McLean & Rosenstein, 2000; Aroutcheva *et al.*, 2001; Strus *et al.*, 2002). Among 134 vaginal *Lactobacillus* strains isolated previously in our laboratory (Ocaña *et al.*, 1999a), only six strains were able to inhibit all the pathogens under study, except for *C. albicans*. Inhibition of pathogenic micro-organisms that cause urogenital infections increases the relevance of these wild strains of *Lactobacillus* for use in probiotic products.

In this study, we employed two culture media that are commonly used for lactobacilli and pH levels other than 4

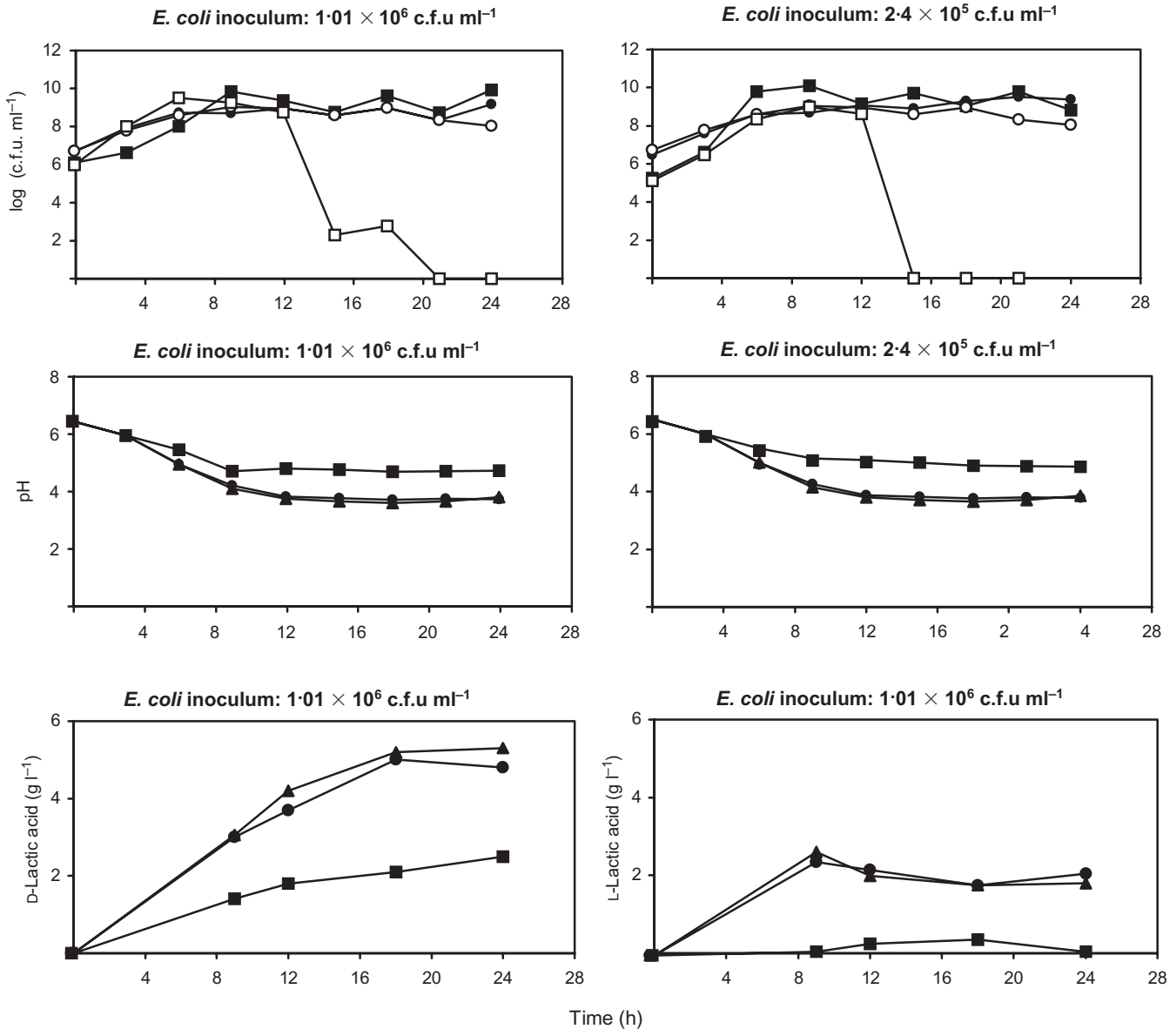

Fig. 3. Pure and mixed cultures of *L. acidophilus* CRL 1259 and *E. coli*. Log c.f.u. ml^{-1}, pH or lactic acid levels of *E. coli* in pure (■) or mixed (□) cultures; log c.f.u. ml^{-1}, pH or lactic acid levels of *L. acidophilus* in pure (●) or mixed (○) cultures; pH or lactic acid levels in mixed cultures (▲).

(the vaginal pH), instead of a chemically defined medium designed to simulate genital secretions (Geshnizgani & Onderdock, 1992). The objective of the present work was not to simulate vaginal conditions, but to assess the most favourable conditions to produce the highest biomass of *L. acidophilus* CRL 1259 in the shortest time and to evaluate factors that affect the production of lactic acid in laboratory assays.

Under conditions of good growth for *L. acidophilus* CRL 1259, the final pH values reached (3·5–4·6) were comparable to those determined in the healthy vagina (Andersch *et al.*, 1986; Tevi-Bénissan *et al.*, 1997). Boskey *et al.* (1999) reported that eight vaginal *Lactobacillus* strains acidified the growth medium to an asymptotic pH of 3·2–4·8. This fact suggests that lactobacilli create an acidic environment that can inhibit the growth of other micro-organisms.

Production of D- and L-lactic acid by *L. acidophilus* CRL 1259 was dependent on the three factors tested (growth medium, pH and temperature). Kylä-Nikkilä *et al.* (2000) reported that the level of production of each isomer only seemed to be dependent to a limited extent on change in expression of the genes responsible for D- and L-LDH. These authors observed different kinetics of production of D- and L-lactic acid by *Lactobacillus helveticus* CNRZ32 and suggested that different intracellular conditions can change either the catalytic activity of enzymes (D- or L-LDH) or their affinity for the substrate (pyruvate).

Optimal pH and temperature for maximum production of lactic acid were the same as those required for growth. Levels of total lactic acid produced by this micro-organism under different culture conditions (2·56–9·16 g l^{-1}) were higher than those found in vaginal secretions of women (0·90–4·00 g l^{-1}) (Boskey *et al.*, 2001).

Mixed cultures showed that *L. acidophilus* CRL 1259 was able to inhibit the growth of *E. coli* at different incubation times, depending on the initial inoculum of pathogen. The final pH reached in mixed cultures was around 4·0. Stamey & Timothy (1975) observed that when the vaginal pH is $< 4{\cdot}5$, colonization of the introitus by *E. coli* is not frequent, whereas the frequency of urinary tract infections is higher when the pH is $> 4{\cdot}5$.

In vitro studies of interactions between organisms are over-simplified, compared with the complexity of human mucosal flora. Although its relevance to the *in vivo* situation is questionable, *in vitro* experimentation provides an approach for determination of the ability of lactobacilli to inhibit the growth of pathogens. In an animal model, *L. fermentum* CRL 1058 contained in agarose beads completely inhibited *E. coli* colonization of the urinary tract of mice (Silva-Ruiz *et al.*, 1993, 1996; Nader de Macías *et al.*, 1996). Reid *et al.* (1985) also reported that vaginal instillation of lactobacilli in mice protected against uropathogenic *E. coli* colonization and later reported similar observations for colonization of the human vagina (Reid *et al.*, 1992).

In summary, the results of this study demonstrate that vaginal *Lactobacillus* strains isolated from Tucumán, Argentina, are able to inhibit the growth of uropathogens by the effect of lactic acid. The results of growth, lactic acid production and mixed cultures with *E. coli* strongly suggest that *L. acidophilus* CRL 1259, alone or combined with other strains of lactobacilli, can be used in probiotic products to prevent infections of the urogenital tract.

ACKNOWLEDGEMENTS

This paper was supported by Carrillo-Oñativia grants (from the Subsecretaría de Ciencia y Tecnología del Ministerio de Salud Pública de la República Argentina) with PID-BID 385 from CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas –Argentina). B. W. is supported by BMBF, Germany, and by SETCIP (bilateral co-operation project ARG 99/025). We thank Elena Bru de Labanda for her help in the experimental design of this study.

REFERENCES

Andersch, B., Forssman, L., Lincoln, K. & Tortensson, P. (1986). Treatment of bacterial vaginosis with an acid cream: a comparison between the effect of lactate-gel and metronidazole. *Gynecol Obstet Invest* **21**, 19–25.

Aroutcheva, A., Gariti, D., Simon, M., Shott, S., Faro, J., Simoes, J. A., Gurguis, A. & Faro, S. (2001). Defense factors of vaginal lactobacilli. *Am J Obstet Gynecol* **185**, 375–379.

Boskey, E. R., Telsch, K. M., Whaley, K. J., Moench, T. R. & Cone, R. A. (1999). Acid production by vaginal flora in vitro is consistent with the rate and extent of vaginal acidification. *Infect Immun* **67**, 5170–5175.

Boskey, E. R., Cone, R. A., Whaley, K. J. & Moench, T. R. (2001). Origins of vaginal acidity: high D/L lactate ratio is consistent with bacteria being the primary source. *Hum Reprod* **16**, 1809–1813.

De Man, J. C., Rogosa, M. & Sharpe, M. E. (1960). A medium for the cultivation of lactobacilli. *J Appl Bacteriol* **23**, 130–135.

Efron, B. (1982). The bootstrap. In *The Jackknife, the Bootstrap, and Other Resampling Plans*, pp. 27–36. Edited by B. Efron. Philadelphia, PA: Society for Industrial and Applied Mathematics.

Eschenbach, D. A. (1993). History and review of bacterial vaginosis. *Am J Obstet Gynecol* **169**, 441–445.

Geshnizgani, A. M. & Onderdock, A. B. (1992). Defined medium simulating genital tract secretions for growth of vaginal microflora. *J Clin Microbiol* **30**, 1323–1326.

Hanna, N. F., Taylor-Robinson, D., Kalodiki-Karamanoli, M., Harris, J. R. & McFadyen, I. R. (1985). The relation between vaginal pH and the microbiological status in vaginitis. *Br J Obstet Gynaecol* **92**, 1267–1271.

Huet, S., Bouvier, A., Gruet, M.-A. & Jolivet, E. (1996). Accuracy of estimators, confidence intervals and tests. In *Statistical Tools for Non-linear Regression*, pp. 29–59. New York: Springer.

Jack, R. W., Tagg, J. R. & Ray, B. (1995). Bacteriocins of Gram-positive bacteria. *Microbiol Rev* **59**, 171–200.

Juárez Tomás, M. S., Bru de Labanda, E., de Ruiz Holgado, A. P. & Nader-Macías, M. E. (2002a). Estimation of vaginal probiotic lactobacilli growth parameters with the application of the Gompertz model. *Can J Microbiol* **48**, 82–92.

Juárez Tomás, M. S., Bru, E., Wiese, B., de Ruiz Holgado, A. A. P. & Nader-Macías, M. E. (2002b). Influence of pH, temperature and culture media on the growth and bacteriocin production by vaginal *Lactobacillus salivarius* CRL 1328. *J Appl Microbiol* **93**, 714–724.

Kotarski, S. F. & Savage, D. C. (1979). Models for study of the specificity by which indigenous lactobacilli adhere to murine gastric epithelia. *Infect Immun* **26**, 966–975.

Kylä-Nikkilä, K., Hujanen, M., Leisola, M. & Palva, A. (2000). Metabolic engineering of *Lactobacillus helveticus* CNRZ32 for production of pure L-(+)-lactic acid. *Appl Environ Microbiol* **66**, 3835–3841.

Lindstrom, M. J. & Bates, D. M. (1990). Nonlinear mixed effects models for repeated measures data. *Biometrics* **46**, 673–687.

McLean, N. W. & Rosenstein, I. J. (2000). Characterisation and selection of a *Lactobacillus* species to re-colonise the vagina of women with recurrent bacterial vaginosis. *J Med Microbiol* **49**, 543–552.

Melis, G. B., Ibba, M. T., Steri, B., Kotsonis, P., Matta, V. & Paoletti, A. M. (2000). Role of pH as a regulator of vaginal physiological environment. *Minerva Ginecol* **52**, 111–121 (in Italian).

Nader de Macías, M. E., de Ruiz, C. S., López de Bocanera, M. E. & Pesce de Ruiz Holgado, A. A. (1996). Preventive and therapeutic effects of lactobacilli on urinary tract infections in mice. *Anaerobe* **2**, 85–93.

Ocaña, V. S., Bru, E., de Ruiz Holgado, A. A. & Nader-Macías, M. E. (1999a). Surface characteristics of lactobacilli isolated from human vagina. *J Gen Appl Microbiol* **45**, 203–212.

Ocaña, V. S., Pesce de Ruiz Holgado, A. A. & Nader-Macías, M. E. (1999b). Selection of vaginal H_2O_2-generating *Lactobacillus* species for probiotic use. *Curr Microbiol* **38**, 279–284.

Ocaña, V. S., de Ruiz Holgado, A. A. & Nader-Macías, M. E. (1999c). Growth inhibition of *Staphylococcus aureus* by H_2O_2-producing *Lactobacillus paracasei* subsp. *paracasei* isolated from the human vagina. *FEMS Immunol Med Microbiol* **23**, 87–92.

Ocaña, V. S., Pesce de Ruiz Holgado, A. A. & Nader-Macías, M. E. (1999d). Characterization of a bacteriocin-like substance produced by a vaginal *Lactobacillus salivarius* strain. *Appl Environ Microbiol* **65**, 5631–5635.

Paavonen, J. (1983). Physiology and ecology of the vagina. *Scand J Infect Dis Suppl* **40**, 31–35.

Passos, F. V., Fleming, H. P., Ollis, D. F., Felder, R. M. & McFeeters, R. F. (1994). Kinetics and modeling of lactic acid production by *Lactobacillus plantarum*. *Appl Environ Microbiol* **60**, 2627–2636.

Raibaud, P., Galpin, J. V., Ducluzeau, R., Mocquot, G. & Oliver, G. (1973). Le genre *Lactobacillus* dans le tube digestif du rat. II. Caractères de souches heterofermentaires isolées de rats ''Holo'' et ''Gnotoxéniques''. *Ann Inst Pasteur Microbiol* **124A**, 2223–2235 (in French).

Redondo-López, V., Cook, R. L. & Sobel, J. D. (1990). Emerging role of lactobacilli in the control and maintenance of the vaginal bacterial microflora. *Rev Infect Dis* **12**, 856–872.

Reid, G., Chan, R. C., Bruce, A. W. & Costerton, J. W. (1985). Prevention of urinary tract infection in rats with an indigenous *Lactobacillus casei* strain. *Infect Immun* **49**, 320–324.

Reid, G., Bruce, A. W. & Taylor, M. (1992). Influence of three-day antimicrobial therapy and *Lactobacillus* vaginal suppositories on recurrence of urinary tract infections. *Clin Ther* **14**, 11–16.

Silva-Ruiz, C., Nader-Macias, M. E., Lopez-Bocanera, M. E. & Pesce-Ruiz Holgado, A. (1993). *Lactobacillus fermentum* administered in suspensions and in agarose beads to mice: a comparative study. *Microbiol Alim Nutr* **11**, 391–397.

Silva-Ruiz, C., Lopez-Bocanera, M. E., Nader-Macias, M. E. & Pesce-Ruiz Holgado, A. (1996). Effect of lactobacilli and antibiotics on *E. coli* urinary infections in mice. *Biol Pharm Bull* **19**, 88–93.

Silva-Ruiz, C., Rey, M. R., de Ruiz Holgado, A. P. & Nader-Macias, M. E. (2001). Experimental administration of estradiol on the colonization of *Lactobacillus fermentum* and *Escherichia coli* in the urogenital tract of mice. *Biol Pharm Bull* **24**, 127–134.

Stamey, T. A. & Kaufman, M. F. (1975). Studies of introital colonization in women with recurrent urinary infections. II. A comparison of growth in normal vaginal fluid of common versus uncommom serogroups of *Escherichia coli*. *J Urol* **114**, 264–267.

Stamey, T. A. & Timothy, M. M. (1975). Studies of introital colonization in women with recurrent urinary infections. I. The role of vaginal pH. *J Urol* **114**, 261–263.

Strus, M., Malinowska, M. & Heczko, P. B. (2002). *In vitro* antagonistic effect of *Lactobacillus* on organisms associated with bacterial vaginosis. *J Reprod Med* **47**, 41–46.

Tevi-Bénissan, C., Bélec, L., Lévy, M., Schneider-Fauveau, V., Si Mohamed, A., Hallouin, M.-C., Matta, M. & Grésenguet, G. (1997). In vivo semen-associated pH neutralization of cervicovaginal secretions. *Clin Diagn Lab Immunol* **4**, 367–374.

Zwietering, M. H., Jongenburger, I., Rombouts, F. M. & Van't Riet, K. (1990). Modeling of the bacterial growth curve. *Appl Environ Microbiol* **56**, 1875–1881.