Vaginal lactobacilli: self- and co-aggregating ability

VIRGINIA S. OCAÑA and MARÍA ELENA NADER-MACÍAS
CERELA (Centro de Referencia para Lactobacilos)-CONICET, Chacabuco 145, 4000 Tucumán, Argentina

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ABSTRACT

Lactic acid bacteria are the dominant bacteria of the vaginal tract in healthy women. *Lactobacillus* species form a barrier population that protects from pathogen colonisation by mechanisms that include adhesion to epithelial surfaces, self-aggregation and co-aggregation. In this study, factors involved in the self-aggregating ability of vaginal lactobacilli and in the co-aggregation of these microorganisms with *Candida* spp. are studied. Both self-aggregation and co-aggregation are monitored quantitatively by the decrease in the absorbance of suspensions of the microorganisms and qualitatively by light microscopy. The self-aggregating ability of four vaginal lactobacilli was shown to be caused by a peptide or protein sensitive to trypsin. However, in self-aggregating *Lactobacillus acidophilus* CRL 1294 the factor was resistant to trypsin and sensitive to pepsin. Among self-aggregating lactobacilli, *L. acidophilus* CRL 1294 and *L. salivarius* CRL 1328 were able to co-aggregate with *Candida* spp. The co-aggregating factor for both strains proved to be peptide of the surface anda peptide on the bacterial surface, while the receptor on the yeast was a carbohydrate. Co-aggregation of both lactobacilli and *Candida* spp. was inhibited by the addition of mannose but was not affected by other carbohydrates. Self and co-aggregation factors were not able to induce aggregation in non-aggregating lactobacilli.

KEY WORDS: Lactobacillus.
Candida.
Aggregation.

Introduction

Lactobacilli are used in the fermented food industry and as probiotics for human and animal nutrition.[1] Lately, however, they have also been suggested as candidate microorganisms to be included in probiotics for vaginal use,[2,3] as application of these microorganisms in the female urogenital tract would contribute to the reestablishment of the normal vaginal flora and the prevention of urogenital infections.[4]

Lactobacilli are thought to exert a protective effect by mechanisms such as production of antimicrobial substances and competitive exclusion.[5] It has been shown that lactic acid bacteria form a barrier population on the vaginal mucosa that protects it from pathogen colonization, and both steric hindrance (specific) and/or competition for receptors (non-specific) are suggested mechanisms for this.[2]

Adhesion to epithelial cells and self- and co-aggregation are phenomena shown to contribute to the formation and stability of biofilms on the mucosa.[6-8] The term self-aggregation is employed for the aggregation of microorganisms of the same strain, while co-aggregation is used for different bacterial lineages or microorganisms.[9] These phenomena have been studied widely for oral microorganisms, and their role in dental plaque formation is well established.[10,11] Self- and co-aggregation are also involved in the microbial colonisation of the gastro-intestinal[12,13] and urogenital tracts,[14] but is not known if these phenomena and the persistence of lactobacilli in the intestinal or vaginal tract are related.

It has also been observed that co-aggregation of lactobacilli (either indigenous microflora or exogenously applied into the vagina) and *Escherichia coli*[15] or a *Candida* sp.[14] constitute a defence mechanism against urogenital tract infections caused by other pathogens.

In order to study the self- and co-aggregating properties of lactobacilli isolated from women in Tucuman, Argentina, previously selected self-aggregating lactobacilli[16] are screened for their ability to co-aggregate with a *Candida* sp. The nature, localisation and specificity of self-aggregating and co-aggregating factors are also determined in order to further understand these phenomena.

Correspondence to: Dr María Elena Nader-Macías.
Email: mmacias@cerela.org.ar

Materials and methods

Microorganisms and culture conditions

Five self-aggregating lactobacilli were employed for this study and included *Lactobacillus acidophilus* CRL (Centro de Referencia para Lactobacilos Collection Strain) 1294, *L. salivarius* subsp. *salivarius* CRL 1328, *L. delbrueckii* subsp. *delbrueckii* CRL 1317, *L. delbrueckii* subsp. *delbrueckii* CRL 1313 and *L. gasseri* CRL 1372. All had been selected previously from 134 lactobacilli isolated from the vaginas of healthy women in Tucuman, Argentina, for their ability to aggregate in the presence and absence of ammonium sulphate, using a salt aggregation test.[16]

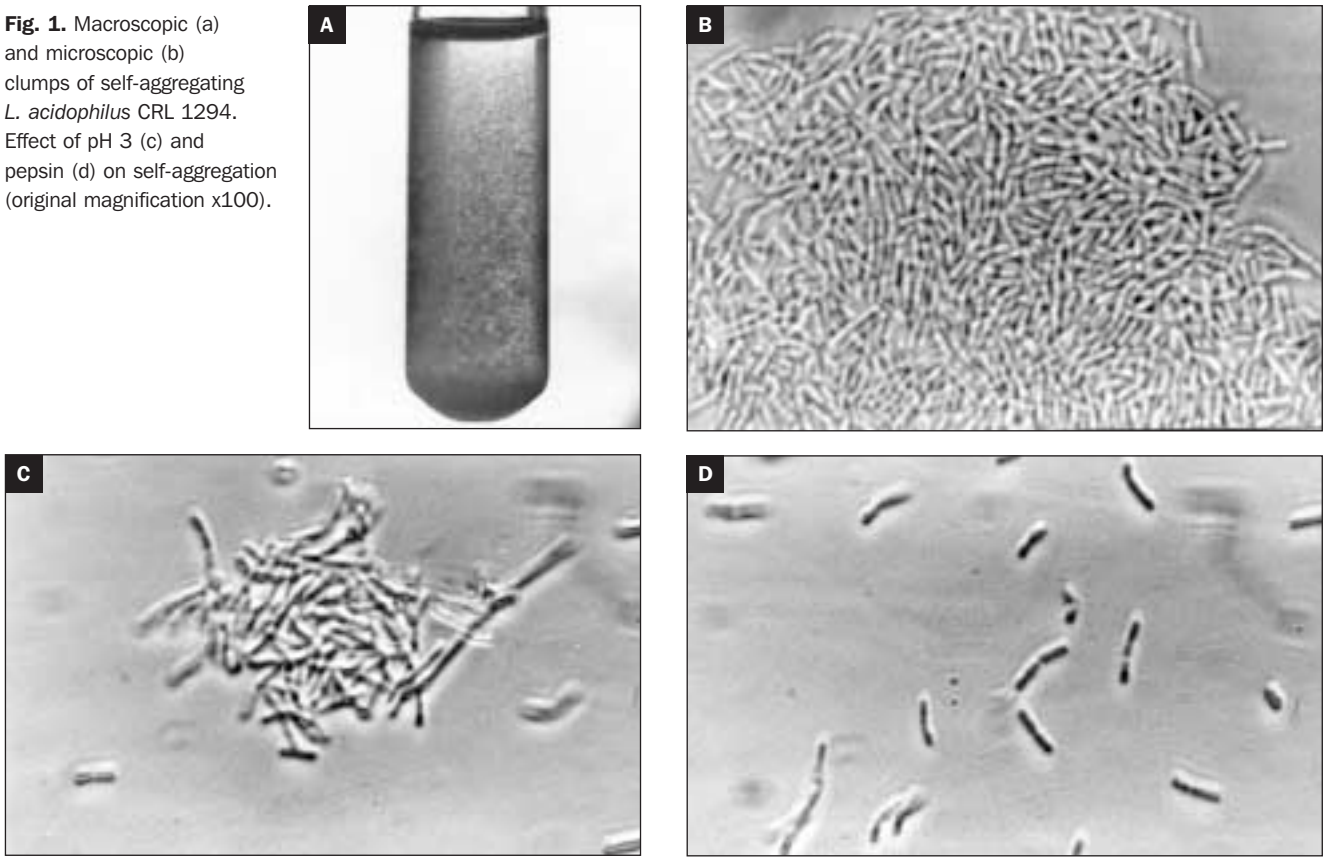

Fig. 1. Macroscopic (a) and microscopic (b) clumps of self-aggregating *L. acidophilus* CRL 1294. Effect of pH 3 (c) and pepsin (d) on self-aggregation (original magnification x100).

Table 1. Self-aggregation (expressed as percentages) of vaginal lactobacilli in PBS and in distilled water

		Lactobacillus strains				
Time	Media	CRL 1294	CRL 1328	CRL 1317	CRL 1313	CRL1372
1 h.	PBS	77	8	8	17	18
4 h.	PBS	80	25	35	32	35
1 h.	Dist. water	22	13	0	0 4	
4 h.	Dist. water	43	32	0	0	11

Table 2. Effect of treatment with enzymes and sodium m-periodate on self-aggregation of vaginal lactobacilli

Strain	Trypsin*	Pepsin*	Periodate*	Lipase*
L. acidophilus CRL 1294	–	+	–	–
L. salivarius CRL 1328	+	Nd	–	–
L. delbrueckii CRL 1317	+	Nd	–	–
L. delbrueckii CRL 1313	+	Nd	–	–
L. gasseri CRL1372	+	Nd	–	–

Aggregation sensitive (+) and resistant (-). Nd: not determined.
(*) Concentrations are indicated in Materials and methods section.

Candida sp. isolated from the vagina of a woman with candidiasis was also employed to study the co-aggregation ability of *Lactobacillus* strains with the yeast. Nine non-aggregating vaginal lactobacilli and non-aggregating *L. salivarius* S253 from human saliva were also employed to test the diffusible nature of the aggregating factors.

All the microorganisms were stored in milk-yeast extract at -20°C and subcultured three times in LAPTg[17] (1.5% peptone, 1% tryptone, 1% yeast extract, 1% glucose, 0.1% Tween 80 [pH 6.5]) prior to the aggregation studies.

Self-aggregation test

The aggregation phenomenon was characterised by the formation of clumps that were able to gravitate and leave a clear supernatant fluid.[7] Self-aggregation was monitored using a method previously described.[18] Briefly, cultures of self-aggregating lactobacilli grown for 12 h in LAPTg broth were centrifuged 2000 rpm for 15 min, washed with phosphate-buffered saline (PBS, pH 6.5) containing NaCl

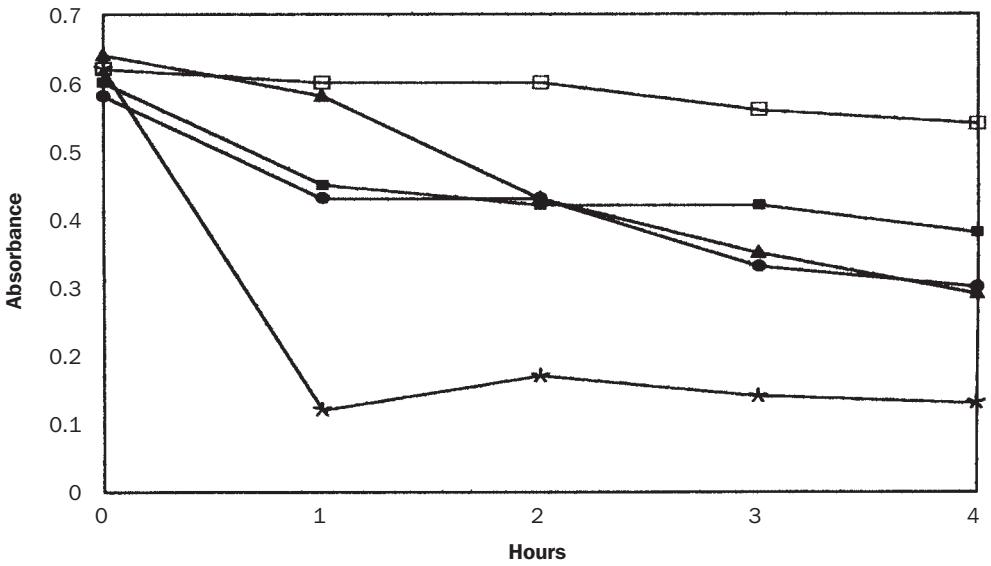

Fig. 2. Self-aggregation of vaginal *Lactobacillus* strains monitored spectrophotometrically (A_{600}). Aggregation of *L. salivarius* subsp. *salivarius* CRL 1328 (□), L. acidophilus CRL 1294 (*), *L. delbrueckii* subsp. *delbrueckii* CRL 1317 (▲), *L. delbrueckii* subsp. *delbrueckii* CRL 1313 (●) and *L. gasseri* CRL 1372 (■).

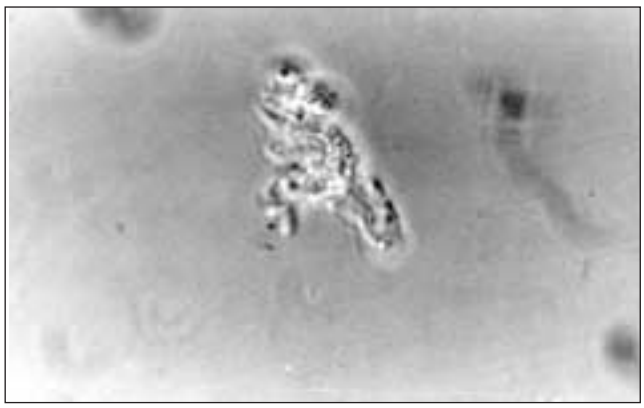

Fig. 3. Microscopic observation of self-aggregating *L. salivarius* CRL 1328 (original magnification x400).

Table 3. Effect of different treatments of Candida sp. on the co-aggregation of the yeast and vaginal lactobacilli

	Treatment				
Strain	Pepsin	Trypsin	Lipase	Periodate	pH 3
L. acidophilus CRL1294	+	+	+	-	+
L. salivarius CRL 1328	+	+	+	-	+

Co-aggregation resistant (+) and sensitive (-)

(8 g/L), KH_2PO_4 (0.34 g/L) and K_2HPO_4 (1.21 g/L) and resuspended in the same buffer to give an absorbance (A) of 0.6 ± 0.05 at 600 nm (A_{600}). Aggregation was monitored spectrophotometrically over 4 h (leaving the suspensions to sediment during the spectrophotometric determinations). Degree of aggregation was express as follows:

$$\% \text{ aggregation: } \frac{(1- A_{final})}{A_{initial}} \times 100$$

Aggregation was confirmed by microscopic observation using a Leitz microscope at x100 magnification.

Chemical composition or nature of the self-aggregation factors

Chemical composition of the self-aggregation factors was determined by the treatment of the microorganism suspensions (A_{600}: 0.8) with proteases (trypsin; 1 mg/mL in PBS [pH 6.8], and pepsin; 1 mg/mL in phosphate/citrate buffer [pH 3]), lipase (10 mg/mL in Tris buffer [pH 8]) and sodium m-periodate (10 mmol/L in acetate buffer [pH 4.5]) for 1 h at room temperature. Treated microorganisms were separated by centrifugation 2000 rpm for 15 min and washed twice with PBS. Self-aggregation was studied as described previously and the appropriate controls were employed for each experiment.

The presence of self-aggregation factors in the supernatant from aggregating lactobacilli was studied by resuspending non-aggregating lactobacilli in the filter-sterilised supernatant from aggregating microorganisms. Subsequent aggregation of non-aggregating lactobacilli would suggest the presence of a diffusible compound in the supernatant. For this experiment, non-aggregating lactobacilli were centrifuged, washed with PBS and resuspended in the filter-sterilised supernatant from self-aggregating *L. acidophilus* CRL 1294 and *L. salivarius* subsp. *salivarius* CRL 1328. Aggregation was evaluated as described previously.

The effect of the ions present in PBS was evaluated by resuspending the cells in distilled water comparing the results obtained.

Co-aggregation among self-aggregating lactobacilli

Co-aggregation of *Lactobacillus* strains and a *Candida* sp. was studied as previously described.[19] Briefly, cultures of self-aggregating lactobacilli grown in LAPTg for 12 h and a *Candida* sp. were centrifuged and washed in PBS (x2).

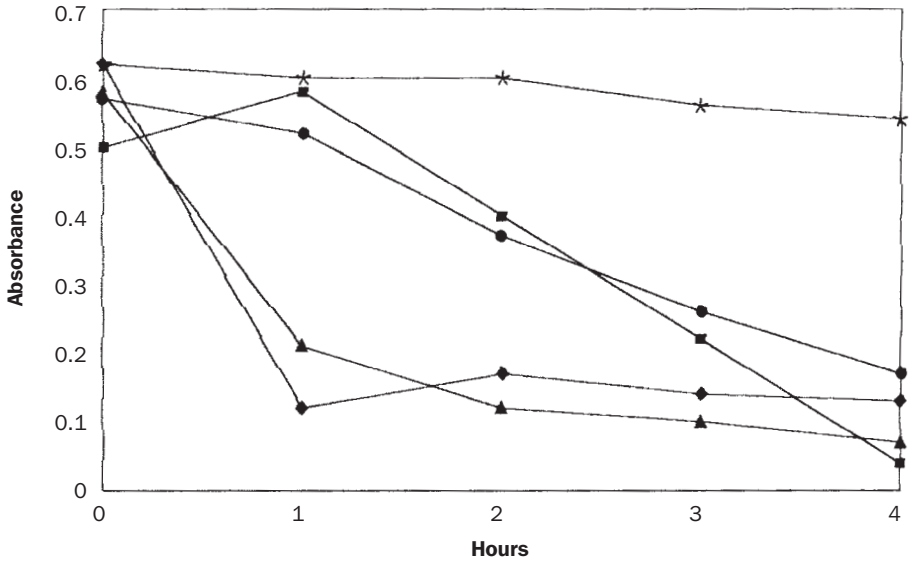

Fig. 4. Co-aggregation of *L. salivarius* CRL 1328 and *L. acidophilus* CRL 1294 monitored spectrophotometrically (A_{600}). Self-aggregation of *Candida* sp. (●), *L. acidophilus* CRL 1294 (◊) and *L. salivarius* CRL 1328 (*). Co-aggregation of *Candida* sp. with *L. acidophilus* CRL 1294(■) and with *L. salivarius* CRL 1328 (▲).

Cells were resuspended in PBS and the A600 adjusted to 0.6 ± 0.02. Bacterial and yeast suspensions (1.5 mL of each) were mixed and co-aggregation was studied as described for self-aggregation. Controls containing single suspensions of microorganism were employed.

Chemical nature of bacterial and yeast factors involved in co-aggregation

The chemical nature of the bacterial factors involved in the co-aggregation phenomenon was determined by treating suspensions of lactobacilli (A_{600}: 0.8) with trypsin, pepsin and sodium m-periodate for 1 h at room temperature. Treated bacteria were washed with PBS prior to adjusting the A_{600} to 0.6 ± 0.2 in the same buffer and then mixed with a non-treated *Candida* sp. Co-aggregation was studied as described previously.

The chemical nature of yeast receptors was studied by treating the *Candida* sp. with proteases, lipase and sodium m-periodate and evaluating co-aggregation as described before.

The effect of carbohydrates on the co-aggregation was determined by adding monosaccharides and disaccharides to the mixture of lactobacilli and Candida sp. mixture. The carbohydrates tested were galactose, mannose, fructose, saccharose, maltose and lactose; all at final concentrations of 0.12 and 0.24 mol/L.

Results

Self-aggregation of lactobacilli

Different self-aggregation percentages were obtained for the vaginal *Lactobacillus* strains studied (Table 1). *L. acidophilus* CRL 1294 formed clumps that sedimented in a short time, leaving the supernatant clear after 1 h (Figure 1a) and an aggregation level of 77%, while other microorganisms showed aggregation percentages between 8% and 18% (Table 1). The decrease in A_{600} for all the lactobacilli studied is shown in Figure 2. *L. salivarius* CRL 1328 did not showed a significant decrease in A_{600}; however, clumps were observed by light microscopy (Figure 3). The ability of *L. acidophilus* CRL 1294 to form clumps was confirmed by microscopy (Figure 1b), and with this strain aggregation was observed in all microscope field.

Chemical nature of self-aggregation factors

The effect of proteases, lipase and sodium m-periodate on self-aggregation of different *Lactobacillus* strains is shown in Table 2. The chemical nature of the factor involved in aggregation proved to be a peptide or protein sensitive to trypsin for *L. salivarius* subsp. *salivarius* CRL 1328, *L. delbrueckii* subsp. *delbrueckii* CRL 1317, *L. delbrueckii* subsp. *delbrueckii* CRL 1313 and L. gasseri CRL 1372; and resistant to trypsin but sensitive to pepsin for *L. acidophilus* CRL 1294. Lipase and sodium m-periodate did not affect self-aggregation of any of the tested lactobacilli.

The effect of buffer (pH 3) and pepsin on self-aggregation of *L. acidophilus* CRL 1294 is shown in Figures 1c and 1d. The buffer (employed to study the effect of pepsin) decreased the size of macroscopic aggregates.

Distilled water instead of PBS demonstrated that the presence of ions affected aggregation. In all but one of the vaginal lactobacilli strains tested, the absence of Na^+, K^+, Cl^+ and $P0_4^{2-}$ decreased aggregation. The exception was *L. salivarius*, which showed a higher rate of sedimentation (Table 1). Filter-sterilised supernatants from both aggregating lactobacilli did not induced aggregation in nine non-aggregating lactobacilli (data not shown).

Co-aggegation of lactobacilli and Candida sp.

Among five strains of self-aggregating vaginal lactobacilli, only *L. acidophilus* CRL 1294 and *L. salivarius* CRL 1328 were able to co-aggregate with the *Candida* sp. *L. acidophilus* CRL 1294 co-aggregated with the *Candida* sp. as soon as both microorganisms were brought into contact. Lactobacilli produce an increase in the rate of sedimentation of the yeast (Figure 4).

L. salivarius subsp. *salivarius* CRL 1328 showed a different pattern of sedimentation, as the decrease in A_{600} of the pure bacterial suspension was not significant, but it increased after being mixed with the yeast. Microscopic co-aggregation of both lactobaciili strains and the *Candida* sp. is shown in Figure 5. Filter-sterilised supernatants of both lactobacilli did not induced aggregation of the *Candida* sp. (data not shown).

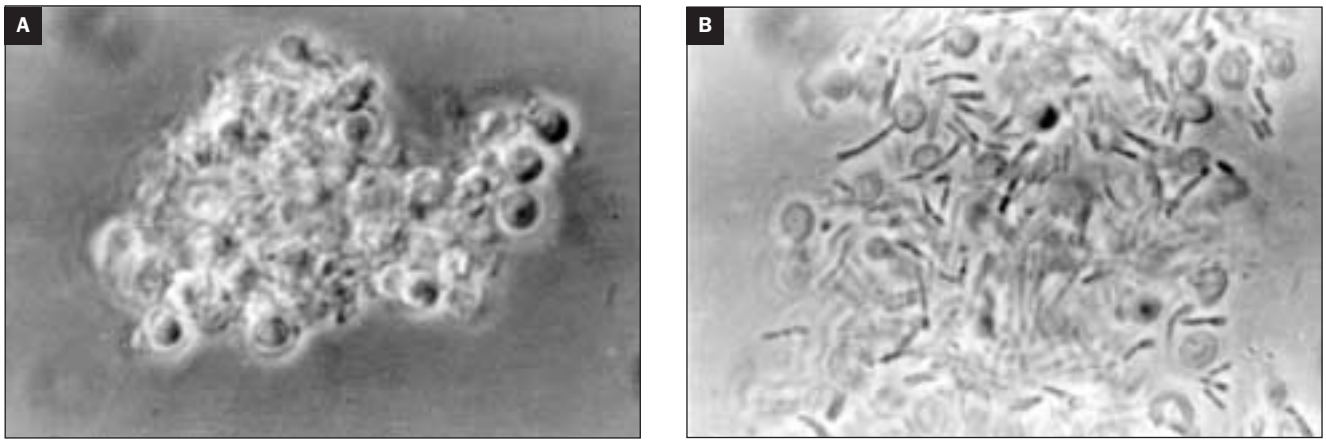

Fig. 5. Microscopic co-aggregation of *L. salivarius* CRL 1328 (A) and *L. acidophilus* CRL 1294 (B) with *Candida* sp. (original magnification x400).

Chemical nature of bacterial and yeast co-aggregating factors
Trypsin and pepsin treatments inhibited the co-aggregation of *L. salivarius* subsp. *salivarius* CRL 1328 but the co-aggregation properties of *L. acidophilus* CRL 1294 were affected only by pepsin. Bacterial co-aggregation factors were not affected by lipase and sodium m-periodate (data not shown). The effect of buffer (pH 3) and pepsin treatment on *L. acidophilus* CRL 1294 and the *Candida* sp. is shown in Figure 6.

Treatment of the *Candida* sp. with sodium *m*-periodate affected the co-aggregation properties of the yeast, but the proteases and lipase did not (Table 3). Addition of mannose (at concentrations of 0.12 mol/L and 0.24 mol/L) inhibited the co-aggregation phenomenon, indicating that a lectin-like adhesin could be involved.

Addition of galactose, fructose, saccharose, maltose and lactose did not inhibit co-aggregation (data not shown). In Figure 7, the synergic effect of pepsin treatment on the *L. acidophilus* factor and the addition of mannose can be appreciated.

Discussion

Protection of the vaginal ecosystem by lactobacilli may be accomplished by different mechanisms that include adherence to the mucosa, aggregation and co-aggregation that form a barrier that prevents colonisation by pathogenic microorganisms. The prevention of adhesion or colonisation of microorganisms at mucosal surfaces has been achieved using a number of approaches, as summarised by Ma and Kelly.[19]

Even though some workers considered adhesion to be a phenomenon implicating surface components other than those of aggregation or co-aggregation, other researchers believed the association between the ability of bacteria to adhere to epithelial cells is due to aggregation activity and bacterial surface hydrophobicity,[13] as in the case of lactobacilli and *E. coli*. Kmet *et al*.[20] examined the expression of auto-aggregation and cell-surface hydrophobicity – characteristics related only in 12 homofermentative strains – in 60 vaginal isolates. Co-aggregation with *E. coli* was positive in only three of the lactobacilli strains.

The relationship of aggregation and colonisation has also been described previously by Cesena *et al*.[21] for self-aggregating *L. crispatus* in *in vitro* and *in vivo* assays. They demonstrated that the wild-type aggregating *L. crispatus* adhered better to Caco-2 cells and mucus than did the non-aggregating mutant, and that the wild-type was recovered more frequently from humans previously fed with the wild-type than those fed with the mutant. Vandevoorde[18] also observed greater colonisation ability in the chicken gut by self-aggregating lactobacilli. However, further study is needed to understand the exact nature of the factors involved in the adhesion, colonisation and aggregation phenomena.

The mechanisms of induction and the identification of the components that mediate co-aggregation could mediate protection of the mucosa through the formation of protecting bacterial biofilms that impede the access of undesirable microorganisms. Characterisation of self- and co-aggregation properties will contribute to the knowledge of the role of these phenomena in biofilm formation and the protective effect of lactobacilli on the mucosa.[22]

In the present study, self- and co-aggregation of vaginal lactobacilli were studied quantitatively by the decrease in absorbance at 600 nm of both pure or mixed microorganism suspensions over four hours. Handley *et al*.[23] and Boris *et al*.[7] observed that aggregating bacteria were able to sediment in one hour. According to our results, not all aggregating bacteria sediment in such a short period of time; however, self-aggregating *L. salivarius* CRL 1328 did not show a significant decrease in A_{600} after the four-hour period, but macroscopic and microscopic observations confirmed the presence of aggregates.

Microbial colonisation of human or animal tissues depends on multiple factors including growth rate, adhesion ability, resistance to the immune system and production of antimicrobial substances.[22] It has also been observed that self- and co-aggregation of bacteria are important characteristics in the maintenance of stable populations on tissue surfaces.[7] These properties are associated with a high frequency of conjugation among Gram-positive bacteria[24] and in *Enterococcus faecium*,[25] *Bacillus thuringiensis*[26] and *Lactococcus lactis*.[27]

In lactobacilli, a close relationship between genetic exchange and aggregation has been observed. Ross[28] reported that the aggregating factor of *L. reuteri* shows high

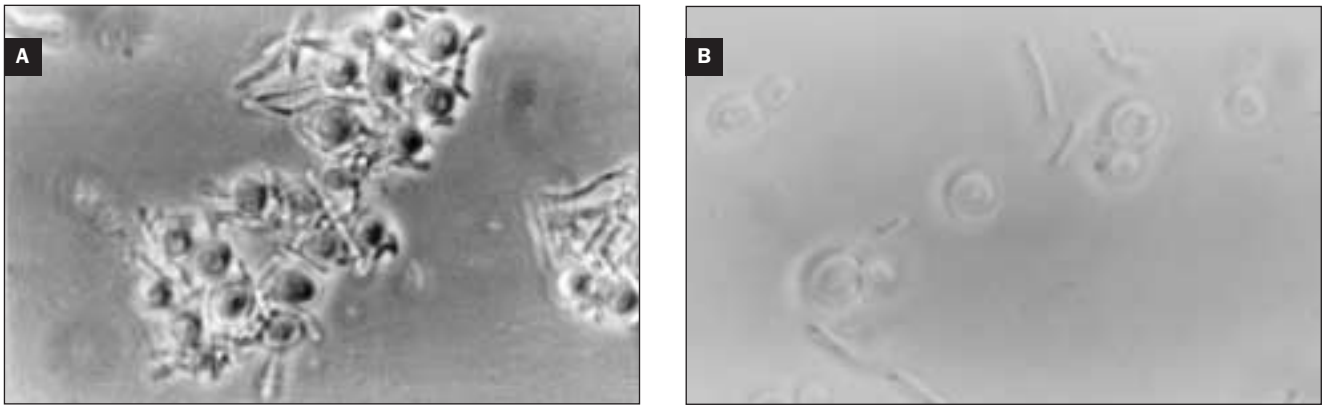

Fig. 6. Effect of pH 3 (A) and pepsin (B) on *L. acidophilus* CRL 1294 and *Candida* sp. co-aggregation (original magnification x400).

homology with the DNA-dependent RNA helicases, indicating that this factor is involved in gene transference. For *L. plantarum*, a soluble protein was shown to promote conjugation.[24]

The surface characteristics of lactobacilli have been demonstrated in a wide range of microorganisms isolated from different sources. Although the interaction of pathogenic microorganisms with other microorganisms or with host cells is generally related to defined structures present in the external membranes or surfaces, those associated with members of the normal flora are more variable. It has been suggested that lipoteichoic acids, protein and carbohydrates on the bacterial surface,[10] soluble proteins[24] or pheromones[29] are involved in the aggregation ability of bacteria.

Self-aggregation of the five vaginal lactobacilli strains studied here was shown to be related to a protein or peptide present on the bacterial cell surface. In four out of the five tested strains, the peptide was sensitive to trypsin; however, with *L. acidophilus* CRL 1294 the factor was sensitive to pepsin but resistant to trypsin.

In *E. faecium*, Ehrenfeld *et al*.[30] found that self-aggregation was related to components of the bacterial surface characterised as teichoic acids. Boris *et al*.[7] demonstrated that the self-aggregation factor of a vaginal *L. gasseri* was a protein while in *L. jensenii* and *L. acidophilus* it was a lipoprotein. The factor associated with *L. gasseri* has been found in the filter-sterilised supernatant obtained from this microorganism and this induced aggregation of non-aggregating lactobacilli.[7]

In the present study, none of the filter-sterilised supernatant fluids induced aggregation of non-aggregating lactobacilli, indicating that aggregating factors were not present or that they were strain specific. It was also found that the ions present in PBS affected aggregate formation and sedimentation rate, with absence of Na^+, K^+, PO_4^{3-} and Cl^- decreasing the self-aggregation properties of all the lactobacilli studied except *L. salivarius* CRL 1328. The role of the ions in the aggregation phenomenon is unclear, however, in oral biofilms the proximity to neighbouring streptococci was dependent on calcium bridging.[31]

Vulvovaginal candidiasis is a widespread and common disease affecting about a third of all women at least once in their lifetime, and approximately 5% experience recurrent disease.[32,33] Clinical and immunological observations have not provided firm evidence for the presence of protective antibodies in the vagina after resolution of infection. However, although raised anti-candida antibody titres were detected in some patients suffering from recurrent vaginitis, all anti-candida antibodies are protective.[32]

Fidel *et al*.[33] have suggested that local host-defence mechanisms are more important than systemic ones, and De Bernardis *et al*.[34] have studied a rat model of a candidal vaginal infection and reported a protective effect for anti-mannan and anti-aspartyl proteinase antibodies present in the vaginal fluid following a primary infection. They also discussed the T-cell dependence of this protection.

Co-aggregation is a highly specific property of some genetically different bacteria[9]. It was first observed in streptococci and actinomyces of the oral mucosa[35] and in microorganisms belonging to the Genus Fusobacterium, Veillonella and Bacteroides.[36,37] It has also been suggested that the interaction of *Streptococcus sanguis* and *Prevotella loescheii* is mediated by lectins present on the surface of *S. sanguis*, and by other adhesions in *S. gordonii*.[7]

In dental plaque, the ability of microorganisms to co-aggregate provides an advantage over non-co-aggregating strains, which are easily removed by saliva. There is also some evidence to suggest a role for co-aggregation in the gastrointestinal and urogenital tract. Wadstrom *et al*.[38] have suggested that co-aggregation of lactobacilli in the gastrointestinal tract of pigs favours colonisation and genetic transference. Elsewhere, the competitive exclusion of urogenital tract pathogens has been related to the ability of lactobacilli to interact closely with pathogens.[15] This indicates that co-aggregation is not associated with pathology, as in the bacterial plaque, but contributes to the maintenance of the normal flora.[22]

Experimental models used by Van der Mei *et al*[39] demonstrated the influence of probiotic bacteria on the prevalence of yeast in oropharyngeal biofilms on silicone rubber voice prostheses. Different probiotic bacteria led to a reduction in the number of yeast cells in the biofilm, indicating that prevalence in this experimental model might be controlled by consumption of probiotic bacteria.

Characteristics of self-aggregation and co-aggregation in the lactic acid bacteria studied here are coded by chromosomal genes, as there are no plasmids or bacteriophages present in the five strains studied. These are strain-specific characteristic because they appeared only in some of the isolated strains, and were not related to a specific species or metabolic group.

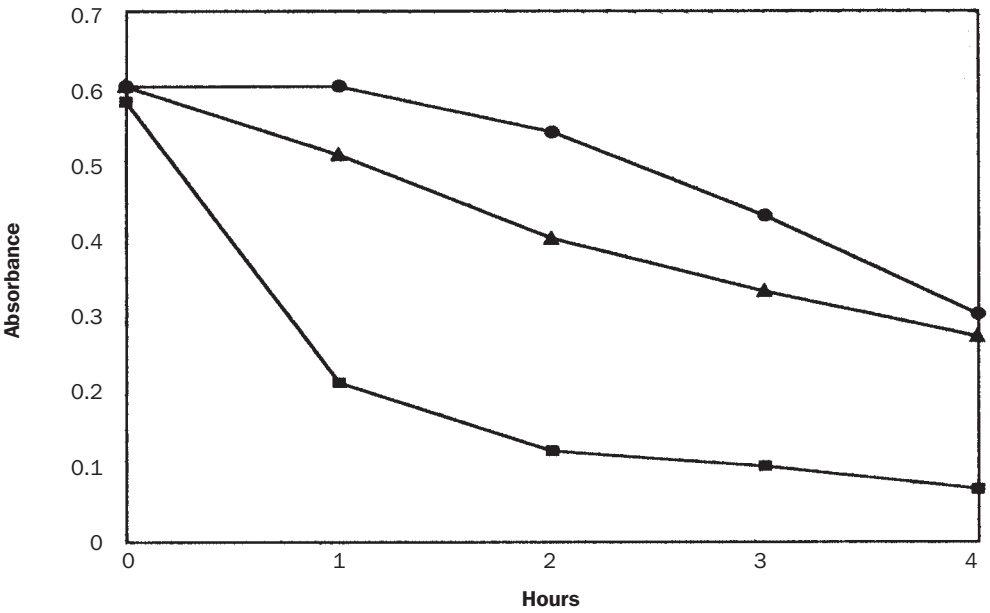

Fig. 7. Synergic effect of pepsin treatment on *L. acidophilus* CRL 1294 factor and the addition of mannose on the co-aggregation of the lactobacilli and *Candida* sp. Co-aggregation of *Candida* sp. and *L. acidophilus* CRl 1294 (■), *Candida* sp. and *L. acidophilus* CRL 1294 in presence of mannose (▲), and *Candida* sp. and pepsin-treated *L. acidophilus* CRL 1294 in presence of mannose (●).

Lack of evidence about the role of co-aggregation of lactobacilli and Candida in the colonisation of the urogenital tract by the yeast should encourage further research to determine whether co-aggregation is a desirable or non-desirable characteristic in selecting probiotic strains, as some of the strains tested here presented other probiotic characteristics.[40,41]

Currently, the effect of co-aggregating lactobacilli on candidal colonisation is being studied in a murine model in our laboratory. □

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