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Factors affecting the coaggregation ability of vaginal Lactobacilli with *Candida* spp.

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Abstract - In this study, the capacity of 30 strains of lactobacilli to coaggregate with *Candida albicans* ATCC 10239, *C. albicans* AJD 180 and *Candida krusei* ATCC 6258 were studied *in vitro*. A marked coaggregation with *C. albicans* was observed for two strains of *Lactobacillus crispatus*, a strain of *Lactobacillus cellobiosus* and a strain of *Lactobacillus salivarius*. Coaggregation occurred at a pH range from 3 to 7, some strains showing optimal binding at high and other strains at low pH. Treatment of lactobacilli at 70 or 85 °C for 20 min or treatment of the bacteria with pepsin abolished their capacity of coaggregation. The results may be of importance when trying to establish probiotics for vaginal use.

Key words: coaggregation; *Lactobacillus* spp.; *Candida* sp.; vagina; probiotic.

INTRODUCTION

Lactic acid bacteria (LAB) grow in a variety of habitats, such as the mucosa and intestines of humans and animals. Also, lactobacilli are used in the fermented food industry and as probiotics for human and animal nutrition. Lately, they have also been suggested as candidate microorganisms to be included in probiotics for vaginal use, as application of these microorganisms in the female urogenital tract would contribute to the reestablishment of the normal vaginal flora and prevention of urogenital infections (Ocaňa and Nader-Macías, 2002). Lactobacilli are dominant in this habitat, at 10^7 to 10^8 CFU/g of vaginal fluid in healthy premenopausal women (Sobel, 1996). Lactobacilli are believed to interfere with pathogens by various mechanisms. The first is competitive exclusion of genitourinary epithelium (Chan *et al*., 1995). Second, lactobacilli coaggregate with some uropathogenic bacteria, a process that when linked to the production of antimicrobial compounds, such as lactic acid, hydrogen peroxide, bacteriocin-like substances and possibly biosurfactants, would result in inhibition of the growth of pathogen (Boris *et al*., 1998). Their ability to form multicellular aggregates has been shown to play an important role in colonization of the oral cavity and the urogenital tract (Reid *et al*. 1990). Autoaggregation and coaggregation are involved in the microbial colonisation of the gastrointestinal and urogenital tracts, but it is not known if these phenomena and the persistence of lactobacilli in the intestinal or vaginal tract are related (Ocaňa and Nader-Macías, 2002).

Studies on the mechanism of autoaggregation in lactobacilli showed that proteins present in the culture supernatant and proteins or lipoproteins located on the cell surface are involved in the cell aggregation. Furthermore, it was observed that spent culture supernatants of autoaggregating lactobacilli mediate not only the aggregation of cells of the producer strain but also aggregation of other lactic acid bacteria and even *Escherichia coli* (Schachtsiek *et al*., 2004).

Mucosal candidiasis is a significant problem in both immunocompetent and immunocompromised individuals. Most episodes of oropharyngeal and vulvovaginal candidasis, the most common forms of mucosal candidiasis, are caused by *Candida albicans*, a commensal dimorphic fungal organism of the gastrointestinal and female reproductive tracts (Steele *et al*., 2002). It has been observed that coaggregation of lactobacilli (either indigenous microflora or exogenously applied into the vagina) and *E. coli* or a *Candida* spp. constitute a defence mechanism against urogenital tract infections caused by other pathogens (Kmet and Lucchini, 1997; Reid *et al*., 1988).

The purpose of this study was to determine coaggregation ability of vaginal lactobacilli with *Candida* spp. strains. The effect of pH, sonication, heat, some enzymes, sodium periodate, conditions on coaggregation ability was also investigated. These evaluations may be performed as an initial step in establishing rational criteria for screening and selecting microorganisms with human probiotic properties.

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MATERIALS AND METHODS

Bacterial strains and culture conditions. *Lactobacillus* spp. were isolated from the lateral vaginal wall of 19 patients at the University of Gazi, Faculty of Medicine, Department of Gynecology. The isolates were classified by their morphologic and cultural properties, catalase test (negative), and the API 50 CHL kit analyzed API LAB plus software version 4.0 database (Bio-Merieux, France) (Kilic *et al*., 2005; Aslim and Kilic, 2006). All the *Lactobacillus* spp. strains were classified by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of whole cell proteins (data not shown). In addition, *Lactobacillus* spp. strains were characterized by Gram staining, growth at various temperatures (15, 45, and 50 °C) and tolerance of different salt levels (2, 4, and 6.5% NaCl). *Lactobacillus* spp. strains were grown in de Man, Rogosa and Sharpe medium (MRS, Oxoid) for 16 h (exponential growth phase) at 37 °C. *Candida albicans* ATCC 10239, *Candida krusei* ATCC 6258 (American Type Culture Collection, Rockville, MD, USA), *C. albicans* AJD 180 were used as the test bacteria. *Candida* spp. strains were maintained on YEPD agar and cultured in YEPD broth at 37 °C for 16 h before use. All tests were carried out in three independent assays.

Aggregation assays. Autoaggregation and coaggregation experiments of *Lactobacillus* and *Candida* spp. strains were performed, with some modification, as described by Jabra-Rizk *et al*. (1999).

The degree of autoaggregation and coaggregation was recorded macroscopic on a scale of 0 to 4+ as follows: a score of 0 for no visible aggregates in the cell suspension, 1+ for small uniform coaggregates in the suspension, 2+ for coaggregates that are easily seen but no immediate settling of coaggregates, 3+ for large coaggregates which settle rapidly and leave some turbidity in the supernatant fluid, and 4+ for large coaggregates which settle immediately and leave clear supernatant fluid.

Also, all of the suspensions were observed by inversion light microscopy and scored for aggregation (1+, partial coaggregation; 2+, moderate coaggregation; 3+, good coaggregation; 4+, high coaggregation).

Cell surface hydrophobicity of strains (A1, I1, I2, I3, O3 and S1) was determined by the bacterial adherence to xylene and toluene hydrocarbons (Sweet *et al*., 1987).

Treatment of bacteria. Cultures were harvested by centrifugation at 5000 *x g* for 15 min, washed twice with distilled water and re-suspended in the appropriate buffer (PBS, citrate/phosphate buffer, acetate buffers used for following tests, respectively) with turbidity adjusted according to McFarland no 4. Cell suspensions were subjected to heat, lipase, pepsin and sodium periodate. The heat treatment was assayed at both 20 min at 85 °C and 30 min at 70 °C. Unheated [Room Temperature (RT), ≈22-24 °C] cell suspensions were also used. Bacterial cells were examined for coaggregation at different pH values ranging from 3 to 7. Washed bacterial cells were also subjected to a sonication treatment Vibra Cell Model, sonic materials inc. at 50 MHz for 12 min. The sonicated cells and supernatant fluid were respectively examined for coaggregation ability (Vandevoorde *et al*., 1992).

RESULTS AND DISCUSSION

There has been an increasing recognition of the role of lactobacilli in the maintenance of homeostasis within dynamic ecosystems such as the vagina, and in prevention of colonization and infection caused by pathogenic organisms (McGroarty, 1993). Reid *et al*. (1988) showed that certain *Lactobacillus* strains undergo coaggregation with uropathogens and suggested that this phenomenon is an important factor in the establishment and maintenance of a healthy urogenital flora. For this reason, coaggregation ability is considered an important parameter in the selection of probiotic strains for vaginal use. Recently, a few reports on the coaggregation abilities of vaginal lactobacilli have also been published (Ocaňa and Nader-Macías, 2002; Reid and Bruce, 2003). However, probiotic importance of coaggregation in vaginal lactobacilli has never been explained.

In this study, thirty *Lactobacillus* spp. strains were selected for assay of coaggregation with *C. albicans* ATCC 10239, *C. albicans* AJD 180 and *C. krusei* ATCC 6258. Some probiotic properties of these strains were determined in our previous works (Kilic *et al*., 2005; Aslim and Kilic, 2006).

Yeast vaginits is estimated to affect around 1:5 black American women and close to 1:10 white women during any given two month time frame, 1:12 reporting four or more episodes per year. While *C. albicans* is the major cause of infections (around 85%), other yeasts such as *Candida glabrata*, *C. krusei*, and *Candida tropicalis* are also involved (Reid and Bruce, 2003). In the present study, all thirty strains of lactobacilli showed coaggregation with two or more yeast strains (Table 1). Microscopic and macroscopic coaggregation of both *Lactobacillus crispatus* G10 and *C. albicans* ATCC 10239 is shown in Fig. 1 and Fig. 2B, respectively. Two strains, *Lactobacillus cellobiosus* I3 and *Lactobacillus salivarius* I1, in particular showed the greatest coaggregation against *C. albicans* ATCC 10239 and *C. albicans* AJD 180. In general, scores of coaggregation were higher with the two strains of *C. albicans* than with *C. krusei*. Among the different strains of *Lactobacillus* spp., the interaction with yeasts varied from partial (1+) to high coaggregation capacity (4+). Similar results were found by Mastromarino *et al*. (2002). They found that different *Lactobacillus gasseri* strains showed different degrees of coaggregation activity with *C. albicans* and *Gardnerella vaginalis*. An association was observed between autoaggregation and coaggregation activities. *L. cellobiosus* I3 and *L. salivarius* I1 strains that exhibited greatest coaggregation activity also showed highest autoaggregation activity. Partial coaggregation (1+) occurred with all of *Candida* spp. strains and *L. acidophilus* S1, S2, *L. gasseri* L1, *L. crispatus* O3, *Lactobacillus jensenii* R11 strains, and also these strains exhibited partial autoaggregation activity. This kind of coggregation is demonstrated in Fig. 2A for *Lactobacillus paracasei* A2 strain.

Hydrophobicity abilities of *Lactobacillus* sp. strains ranged between 31 to 86% (toluene) and 33 to 85% (xylene), respectively (data not shown). The *L. cellobiosus* I3 strain showed greater autoaggregation (4+) and hydrophobicity (85-86%) than the other strains. Kmet and Lucchini (1995) reported an association between autoaggregation activity and bacterial surface hydrophobicity in lactobacilli. In this research, bacterial cell surface hydrophobicity was also similarly related to autoaggregation activity.

The mechanism of lactobacilli coaggregation with uropathogens has yet to be fully elucidated, although a number of properties were ascertained. Coaggregation was optimal at physiological pH, as found previously for *Fusobacterium nucleatum* and streptococci (Kelstrup and Funder-Nielsen, 1974). In the

TABLE 1 - Coaggregation ability of *Lactobacillus* spp. strains with different *Candida* spp. strains

Lactobacillus strains	Autoaggregation for control*	Coaggregation with *Candida* spp.		
		C. albicans ATCC 10239	*C. albicans* AJD 180	*C. krusei* ATCC 6258
L. acidophilus S1	1+	1+	1+	1+
L. acidophilus S2	1+	1+	1+	1+
L. acidophilus G6	2+	1+	1+	2+
L. acidophilus R13	2+	2+	1+	2+
L. acidophilus G11	2+	2+	3+	1+
L. acidophilus R9	3+	1+	2+	1+
L. acidophilus G8	2+	1+	1+	1+
L. gasseri I2	1+	1+	2+	3+
L. gasseri R2	3+	1+	2+	1+
L. gasseri R3	3+	1+	2+	2+
L. gasseri R5	1+	2+	1+	1+
L. gasseri L1	1+	1+	1+	1+
L. crispatus G9	1+	3+	2+	1+
L. crispatus G10	4+	4+	2+	1+
L. crispatus O3	1+	1+	1+	1+
L. plantarum H17	1+	2+	1+	1+
L. plantarum T1	3+	2+	2+	1+
L. plantarum I4	3+	1+	1+	3+
L. delbrueckii G13	3+	1+	2+	2+
L. delbrueckii H 9	3+	2+	1+	1+
L. delbrueckii H10	2+	2+	1+	1+
L. jensenii R11	1+	1+	1+	1+
L. jensenii A1	1+	1+	1+	2+
L. curvatus L3	3+	2+	2+	2+
L. curvatus H6	1+	1+	1+	1+
L. cellobiosus I3	4+	4+	4+	1+
L. cellobiosus L2	4+	2+	1+	1+
L. vaginalis H8	2+	1+	1+	1+
L. salivarius I1	4+	4+	3+	1+
L. paracasei A2	2+	1+	1+	1+

* The score is (1+) partial autoaggregation or coaggregation, (2+) moderate autoaggregation or coaggregation, (3+) good autoaggregation or coaggregation, (4+) high autoaggregation or coaggregation.

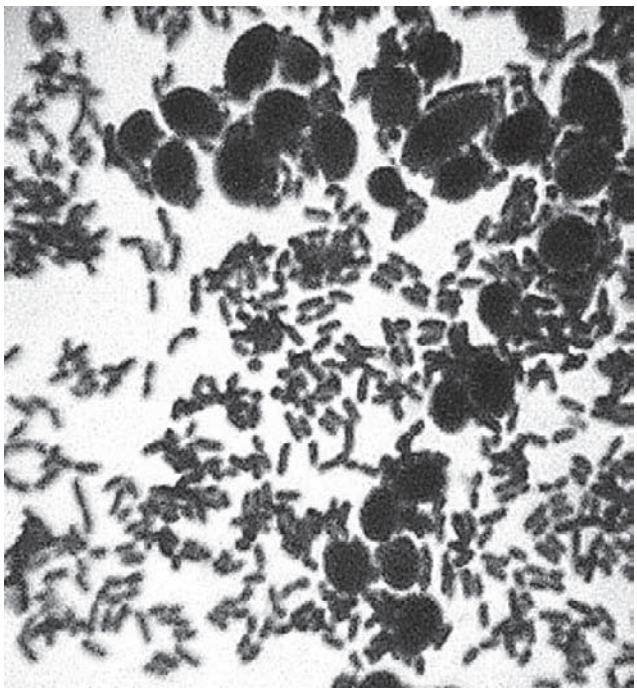

FIG. 1 - High (4+) coaggregation of *Lactobacillus crispatus* G10 with *Candida albicans* ATCC 10239.

FIG. 2 - A: partial (1+) coaggregation of *Lactobacillus paracasei* A2 with *Candida albicans* ATCC 10239. B: high (4+) coaggregation of *Lactobacillus crispatus* G10 with *C. albicans* ATCC 10239.

TABLE 2 - Effect of pH on coaggregation ability of *Lactobacillus* spp. strains with *Candida* spp.

Lactobacillus strains	*Candida albicans* AJD 180			*Candida albicans* ATCC 10239			*Candida krusei* ATCC 6258		
	pH			pH			pH		
	3	5	7	3	5	7	3	5	7
L. jensenii A1	4+	3+	2+	3+	2+	1+	3+	3+	2+
L. acidophilus S1	3+	3+	2+	4+	3+	2+	3+	2+	2+
L. salivarius I1	2+	2+	3+	2+	2+	3+	0	0	1+
L. gasseri I2	4+	3+	2+	3+	3+	2+	1+	1+	2+
L. cellobiosus I3	1+	2+	3+	2+	2+	3+	3+	3+	2+
L. crispatus O3	3+	2+	2+	2+	2+	1+	3+	3+	2+

The score is (0) no coaggregation, (1+) partial coaggregation, (2+) moderate coaggregation, (3+) good coaggregation, (4+) high coaggregation.

TABLE 3. Effect of heat on coaggregation ability of *Lactobacillus* spp. strains with *Candida* spp.

Lactobacillus strains	*Candida albicans* AJD 180			*Candida albicans* ATCC 10239			*Candida krusei* ATCC 6258		
	R*	70 °C	85 °C	R*	70 °C	85 °C	R*	70 °C	85 °C
L. jensenii A1	2+	1+	0	2+	1+	0	2+	1+	0
L. acidophilus S1	1+	0	0	1+	1+	0	1+	1+	0
L. salivarius I1	3+	1+	0	4+	1+	0	1+	1+	0
L. gasseri I2	2+	1+	0	2+	1+	0	2+	1+	0
L. cellobiosus I3	3+	1+	0	4+	1+	0	1+	1+	0
L. crispatus O3	1+	0	0	2+	1+	0	2+	1+	0

* R: Room temperature.
The score is (0) no coaggregation, (1+) partial coaggregation, (2+) moderate coaggregation, (3+) good coaggregation, (4+) high coaggregation.

present study, while I1 and I3 strains showed high coaggregation activity with AJD 180 and ATCC 10239 at pH 7; A1, O3 and S1 strains exhibited coaggregation with three yeast strains low pH (Table 2). In a previous study, coaggregation was found to require an optimum of 3-4 h incubation at 37 °C and to occur at room temperature (Reid *et al*., 1988). In the present study, optimum coaggregation occurred at room temperature and heat treatment of lactobacillus reduced the coaggregation scores (Table 3). Grimaudo and Nesbitt (1997) showed that exposure of all coaggregating strains of *Fusobacterium* to heat was sufficient to completely inhibit coaggregation with all tested *Candida* species. There is some evidence to suggest that heat-sensitive surface components on the lactobacilli and uropathogens are also involved in certain coaggregation reactions.

The surface characteristics of lactobacilli strains have been demonstrated in a wide range of microorganisms which isolated from different sources. It has been suggested that lipoteichoic acids, protein and carbohydrates on the bacterial surface, soluble proteins or pheromones are involved in the aggregation ability of bacteria (Ocaňa and Nader-Macías, 2002). In this present study, coaggregation properties of *L. salivarus* I1 and *L. cellobiosus* I3 strains were affected only by pepsin. Bacterial coaggregation factors were not affected by lipase and sodium meta periodate (Table 4). Grimaudo and Nesbitt (1997) found that periodate oxidation of the *Fusobacterum* spp. did not prevent coaggregation. For this reason, it may be suggested that a proteinaceous surface component mediates the coaggregation of *L. salivarus* I1 and *L. cellobiosus* I3 with *Candida* spp. strains. On the other hand, it appears that structures mediating coaggregation could be released from the cell wall by sonication. The sonicated cells of *L. salivarius* I1 and *L. cellobiosus* I3 strains showed lower coaggregation than the control, the supernatant fluid of these sonicated cells showed similar coaggregation ability to the control (Table 4).

In conclusion, there is little evidence that probiotics can effectively cure a symptomatic yeast vaginitis. However, the lactobacilli used in this study may protect the vaginal epithelium through the coaggregation mechanism. Consequently, they may be excellent candidates for eventual use as probiotic agents. Studies to further evaluate their feasibility as such are under way.

TABLE 4 - Effect of treatment with some enzymes, sonication (pellet and supernatant) and sodium periodate (SMP) on coaggregation ability of *Lactobacillus salivarus* I1 and *Lactobacillus cellobiosus* I3 strains with *Candida albicans* ATCC 10239

Lactobacillus strains	Coaggregation after treatment with sonication			Coaggregation after treatment with enzymes and SMP			
	Control*	Pellet	Supernatant	Control*	Pepsin	Lipase	SMP
L. salivarus I1	4+	2+	4+	4+	2+	3+	3+
L. cellobiosus I3	4+	2+	3+	4+	2+	3+	3+

*: Strains without treatment with sonication or enzymes.
The score is (1+) partial coaggregation, (2+) moderate coaggregation, (3+) good coaggregation, (4+) high coaggregation.

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