

SOME FACTORS AFFECTING THE AUTOAGGREGATION ABILITY OF VAGINAL LACTOBACILLI ISOLATED FROM TURKISH WOMEN

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Abstract — In this work, autoaggregation and factors involved in the autoaggregation ability of vaginal lactobacilli were studied. The autoaggregation ability of 28 lactobacilli strains was positive. The effects of various factors on autoaggregation were also evaluated. *Lactobacillus jensenii* A1, *L. salivarius* I1, and *L. cellobiosus* I3 showed higher autoaggregation in acidic conditions and lower autoaggregation in hot (70 and 85°C) conditions. The *L. salivarius* I1 strain, which exhibited high autoaggregation activity, also showed good autoaggregation in pepsin, lipase, and sodium periodate, as well as under conditions of sonication and heat. The results of this study suggest that lactobacilli showing high autoaggregation may constitute an important host defense mechanism against infections as a probiotic.

Key words: Microbiology, autoaggregation, *Lactobacillus* spp., vagina, probiotic

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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 reestablishment of the normal vaginal flora and prevention of urogenital infections (Ocaña and Nader-Macías, 2002). In healthy premenopausal women, the vaginal bacterial microbiota is dominated by the species *Lactobacillus acidophilus*, *L. crispatus*, *L. gasseri*, *L. plantarum*, *L. casei*, and *L. jensenii* species, as well as *L. vaginalis* and *L. salivarius* (Sobel, 1996). Multicellular aggregates of lactobacilli have been shown to play an important role in colonization of the oral cavity and the urogenital tract (Reid et al., 1990). The aggregation ability comprises autoaggregation, characterized by clumping of cells of the same strain, and coaggregation, in which genetically distinct cells are involved

(Kolenbrander, 1988). Autoaggregation and coaggregation are involved in the microbial colonization 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 (Ocaña and Nader-Macías, 2002).

Cell aggregation seems to involve the interaction of cell surface components such as lipoteichoic acid, proteins, and carbohydrates, as well as soluble proteins (Clewell and Weaver, 1989; Reniero et al., 1991). 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 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).

The objective of this study was to investigate the effects of pH, sonication, heat, some enzymes, sodium periodate, and aerobic and anaerobic conditions

on the autoaggregation ability of vaginal *Lactobacillus* spp. strains. In this study, the occurrence of autoaggregation and the nature of the surface components involved were investigated. Moreover, the ecological and probiotic significance of autoaggregation was also considered.

MATERIALS AND METHODS

Bacterial strains

Lactobacillus spp. were isolated from the lateral vaginal wall of 19 women. The isolates were classified according to their morphological and cultural properties, catalase test result (negative), and results obtained using the API 50 CHL kit and API LAB plus software, version 4.0 (Bio-Merieux, France) (Kilic et al., 2005; Aslim and Kilic, 2006). All the *Lactobacillus* spp. strains were classified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of whole cell proteins. In addition, *Lactobacillus* spp. strains were characterized by their gram staining, growth at various temperatures (15, 45, and 50°C), and tolerance of different salt levels (2, 4, and 6.5% NaCl). *Lactobacillus* strains were grown in de Man, Rogosa, and Sharpe medium (MRS; Oxoid) for 18-20 h (exponential growth phase) at 37°C. The isolates were stored at -80°C in MRS broth containing 30% glycerol (Merck) and subcultured twice before use.

Spectrophotometric autoaggregation assay

Aggregation experiments were performed as described by Vandervoorde et al. (1992) with some modification. *Lactobacillus* spp. strains were grown in aerobic and anaerobic conditions for 16 h at 37°C. Activated cultures were harvested by centrifugation at 10,000 x g for 15 min, washed twice in phosphate-buffered saline (PBS) containing (g/l): NaCl, 8; KH₂PO₄, 0.34; and K₂HPO₄, 1.21 (pH 6.0) and resuspended in PBS to give a final optical density of 0.60±0.02 at 600 nm as measured by a spectrophotometer (Hitachi, T1800). For aerobic and anaerobic autoaggregation experiments, samples consisted of 2 ml of each strain with OD (optical density) measured at 600 nm after 4 h of incubation at 37°C. The percent autoaggregation was expressed as follows:

$$\% \text{ Autoaggregation} = [(OD_1 - OD_2)/(OD_1) \times 100] \quad (1)$$

OD₁: first optical density, OD₂: optical density after 4 h.

Also, all of the suspensions were observed by inversion light microscopy.

Coaggregation assays

Coaggregation experiments were performed between *Lactobacillus* spp. strains according to the method of Vandervoorde et al. (1992). The percent coaggregation was expressed as follows:

$$\% \text{ Coaggregation} = [(OD_1 + OD_2) - 2(OD_3)/(OD_1 + OD_2) \times 100] \quad (2)$$

OD₁ = optical density of strain 1 (*Lactobacillus* spp.); OD₂ = optical density of strain 2 (other *Lactobacillus* spp.); OD₃ = optical density of strain 1 and strain 2.

Treatment of bacteria

Cultures were harvested by centrifugation at 10,000 x g for 15 min, washed twice with distilled water, and resuspended in the appropriate buffer. Cell suspensions were subjected to heat, lipase, pepsin, and sodium periodate. Heat treatment involved both 20 min at 85°C and 30 min at 70°C, and unheated [room temperature; (RT)] cell suspensions were also used. Lipase treatment (0.5 mg/ml, Fluka, Lipase from hog pancreas) was performed in PBS, pH 7.5. The effect of pepsin (0.5 mg/ml, Sigma, pepsin from porcine stomach mucosa) was determined in 0.1 mol/l citrate/phosphate buffer (pH 2.8) and sodium periodate (10 mg/ml) in 0.1 mol/l acetate buffer (pH 4.5). Bacterial cells were examined for autoaggregation at different pH values ranging from 3 to 9. Subsequently, bacterial cell resuspensions were centrifuged and washed twice with PBS prior to the autoaggregation assay. Washed bacterial cells were also subjected to a sonication treatment (12 min). The sonicated cells and supernatant fluid were respectively examined for autoaggregation ability. All tests were performed in three independent assays (Vandervoorde et al., 1992).

Statistical analysis

All experiments were done in three independent as-

says and mean values are presented. Statistical analysis of the data was performed using SPSS 13.0 bivariate correlation analysis. The Pearson rank order correlation test was used between aerobic and anaerobic autoaggregation. The Pearson rank order coefficient was determined for comparison of autoaggregation between heat treatments (at room temperature, 70, and 85°C).

RESULTS

The results of testing aerobic and anaerobic autoaggregation of the 28 vaginal lactobacilli strains are presented in Table 1. Of the tested 28 *Lactobacillus* spp. strains, the *L. salivarius* I1 strain exhibited the highest autoaggregation (97%), while the *L. crispatus*

tus O3 strain showed lower autoaggregation (21%), under aerobic conditions. *Lactobacillus acidophilus* S1 showed remarkable high autoaggregation under anaerobic conditions (92%). On the other hand, *L. cellobiosus* I3, *L. acidophilus* G6, and *L. acidophilus* R9 strains showed strong autoaggregation under both aerobic and anaerobic conditions. In contrast, *L. crispatus* O3, G9, *L. curvatus* H6, *L. gasseri* R5, L1, *L. plantarum* H17, *L. jensenii* A1, and *L. delbrueckii* H10 exhibited weak autoaggregation ability in aerobic and anaerobic conditions. Different autoaggregation percentages were observed between species and strains. The effects of aerobic and anaerobic conditions on autoaggregation ability of all strains were significant ($P < 0.01$).

Table 1. Autoaggregation ability (%) of *Lactobacillus* spp. strains under aerobic and anaerobic conditions.

Strains	Aerobic (%) Autoaggregation	Anerobic (%) Autoaggregation
<i>L. acidophilus</i> S1	56	92
<i>L. acidophilus</i> S2	46	59
<i>L. acidophilus</i> G6	73	88
<i>L. acidophilus</i> G8	59	38
<i>L. acidophilus</i> G11	45	44
<i>L. acidophilus</i> R9	85	90
<i>L. acidophilus</i> R13	60	74
<i>L. plantarum</i> H17	29	44
<i>L. plantarum</i> T1	70	76
<i>L. gasseri</i> L1	38	30
<i>L. gasseri</i> I2	47	87
<i>L. gasseri</i> R3	63	71
<i>L. gasseri</i> R5	29	48
<i>L. gasseri</i> R2	70	67
<i>L. crispatus</i> O3	21	19
<i>L. crispatus</i> G9	41	37
<i>L. crispatus</i> G10	60	65
<i>L. delbrueckii</i> H10	41	49
<i>L. delbrueckii</i> G13	69	77
<i>L. jensenii</i> A1	49	41
<i>L. jensenii</i> R11	39	62
<i>L. curvatus</i> L3	63	59
<i>L. curvatus</i> H6	39	32
<i>L. cellobiosus</i> I3	81	90
<i>L. cellobiosus</i> L2	80	62
<i>L. salivarius</i> I1	97	68
<i>L. vaginalis</i> H8	69	53
<i>L. paracasei</i> A2	73	56

Six strains were selected from 28 isolates on the basis of their low (O3), moderate (A1), and high (S1, I1, I2, I3) autoaggregation ability as indicated by appropriate tests. Different coaggregation abilities were determined among *Lactobacillus* spp. strains (Table 2). The highest coaggregation ability (94%) was observed between *L. cellobiosus* I3 and *L. gasseri* I2.

To study the influence of environmental conditions, bacterial cells were examined for autoaggregation at different pH values. In Table 3, the autoaggregation of A1, I1, I2, I3, O3, and S1 strains was measured at pH values ranging from 3 to 9. Autoaggregation of the *L. salivarius* I1 strain was optimal (98%) at pH 3 and decreased to 74% in a buffer of pH 9. The *L. jensenii* A1 and *L. cellobiosus* I3 strains showed high autoaggregation at low pH values. On the other hand, the *L. cellobiosus* I3, *L. jensenii* A1, and *L. acidophilus* S1 strains exhibited good autoaggregation at high pH values of 7 and 9. These results indicate that decreasing the pH value provoked an increase of autoaggregation activity.

The autoaggregation ability of *Lactobacillus* spp. strains was evaluated in high-temperature experiments at both 70 and 85°C (Table 3). The autoaggregation ability of *L. salivarius* I1 was determined as 87% at room temperature. After heating at 70 and 85°C, the autoaggregation ability of *L. salivarius* I1 decreased to 68 and 53%, respectively. While this strain showed high autoaggregation activity at pH 3, it was sensitive to heat (70 and 85°C). The autoaggre-

Table 2. Percentage of coaggregation between paired strains of vaginal lactobacilli.

Strains	A1	I1	I2	I3	O3	S1
A1	-	66	76	89	60	75
I1	-	-	76	87	89	76
I2	-	-	-	94	74	86
I3	-	-	-	-	79	71
O3	-	-	-	-	-	88
S1	-	-	-	-	-	-

Table 3. Effect of pH and heat on autoaggregation ability (%) of *Lactobacillus* spp. strains. RT¹: Room temperature

Strains	pH				Temperature		
	3	5	7	9	RT ¹	70°C	85°C
<i>L. jensenii</i> A1	94	89	67	51	67	53	23
<i>L. acidophilus</i> S1	84	72	60	55	66	53	22
<i>L. salivarius</i> I1	98	90	88	74	87	68	53
<i>L. gasseri</i> I2	84	75	51	45	68	46	28
<i>L. cellobiosus</i> I3	91	87	80	68	84	55	12
<i>L. crispatus</i> O3	76	65	30	25	46	25	18

Table 4. Effect of treatment with some enzymes, sonication (pellet and supernatant), and sodium periodate (SMP) on autoaggregation ability (%) of *L. salivarius* I1, *L. cellobiosus* I3, and *L. acidophilus* R9 and S1.

Strains	Treatment with sonication			Treatment with enzymes and SMP			
	Control	Pellet	Supernatant	Control	Pepsin	Lipase	SMP
<i>L. salivarius</i> I1	88	20	90	93	52	33	78
<i>L. cellobiosus</i> I3	77	14	76	89	54	31	74
<i>L. acidophilus</i> R9	79	23	77	84	49	22	68
<i>L. acidophilus</i> S1	65	22	70	72	45	53	61

gation abilities of other strains were sensitive to heat. A correlation was observed between autoaggregation and heat. It was determined to be significant between room temperature and 70°C ($P < 0.01$), but the correlation between 70 and 85°C was not statistically significant ($P = 0.579$).

The *L. salivarius* I1, *L. cellobiosus* I3, *L. acidophilus* R9, and *L. acidophilus* S1 strains were selected on the basis of their high autoaggregation ability in aerobic or anaerobic conditions for sonication, enzyme, and sodium meta-periodate treatments. The I1, I3, R9, and S1 strains were sonicated for 12 min in an attempt to remove surface structures and examine the effect of sonication on autoaggregation (Table 4). Besides sonicated cells, the supernatant fluid of the

treated samples was also tested for its ability to prevent autoaggregation by blockage of bacterial receptors. Sonicated cells of the I1, I3, R9, and S1 strains exhibited lower autoaggregation than the control, but the supernatant fluid of these sonicated cells showed autoaggregation ability similar to that in the control. The effects of lipase, pepsin, and sodium periodate on autoaggregation ability of the I1, I3, R9, and S1 strains was also determined (Table 4). These experiments showed that autoaggregation activity of the I1, I3, R9, and S1 strains decreased after sodium periodate and enzyme treatments. Autoaggregation properties of these strains were also affected by pepsin and lipase. Bacterial autoaggregation factors were unaffected only by sodium periodate.

DISCUSSION

In recent years, there has been an increasing recognition of the role of lactobacilli in maintenance of homeostasis within dynamic ecosystems such as the vagina and in prevention of colonization and infection caused by pathogenic organisms. Lactobacilli are believed to interfere with pathogens by various mechanisms. The first is competitive exclusion of genitourinary epithelium (Chan et al., 1985). Secondly, lactobacilli coaggregate with some uropathogenic bacteria, a process that when linked to the production of antimicrobial compounds (lactic acid, hydrogen peroxide, bacteriocin-like substances, and possibly biosurfactants) would result in inhibition of pathogen growth (Boris et al., 1998). The concept of aggregation ability includes autoaggregation, characterized by clumping of cells of the same strain, and coaggregation, in which genetically distinct cells are involved. Both types of aggregation have been described previously for lactobacilli, including *L. crispatus*, *L. gasseri*, and *L. reuteri* (Schachtsiek et al., 2004). Bujnakova and Kmet (2002) determined that only autoaggregating strains show coaggregation ability with different *Escherichia coli* strains. Autoaggregation of probiotic strains appears to be necessary for adhesion to vaginal epithelial cells, with coaggregation resulting in a barrier that prevents colonization by pathogenic microorganisms (Reid et al., 1990; Boris et al., 1997; Del Re et al., 2000). Del Re et al. (2000) showed that the autoaggregation ability in *Bifidobacterium longum* strains ranged from ≥ 89 to $\leq 10\%$ after 2 h of incubation at 37°C. When we compared our results with these findings, autoaggregation phenotypes were identified and defined as follows. Strongly autoaggregating strains (I1, I3, I2, R9, and S1) showed a high autoaggregation percentage ($\geq 85\%$), aggregated immediately, and formed a precipitate resulting in a clear solution. In contrast, strains O3 and A1 showed autoaggregation percentages of 19 and 41%, respectively, and their suspension showed both a precipitate and constant turbidity, results that were verified by light microscopy.

Intestinal and vaginal lactobacilli have been found to coaggregate with each other (Vandevoorde et al., 1992) or with *E. coli* (Reid et al., 1988). The

prevalence of coaggregation among lactobacilli from the vagina gives support to the idea that intrageneric interactions might be of considerable ecological significance. In this study, the highest coaggregation ability was observed between *L. cellobiosus* I3 and *L. gasseri* I2. Coaggregation was optimal at physiological pH, as was found by previous authors for *Fusobacterium nucleatum* and streptococci (Kelstrup and Funder-Nielsen, 1974). But they made no mention of the pH value optimal for autoaggregation. In the present study, the I1 and I3 strains showed high autoaggregation at pH 3, 5, 7, and 9; while the A1, I2, and S1 strains exhibited autoaggregation at low pH values. On the other hand, coaggregation reactions required an optimum of 3-4 h of incubation at 37°C and occurred at room temperature (Reid et al., 1988). In the present study, optimum autoaggregation occurred at room temperature, and heat treatment of lactobacilli reduced autoaggregation scores. There is some evidence to suggest that heat-sensitive surface components on lactobacilli and uropathogens are also involved in certain aggregation reactions (Jabra-Rizk et al., 1999).

The results indicated that autoaggregation ability is dependent on environmental factors (such as pH and heat conditions). Moreover, the cell surface properties of bacteria are thought to play an important role in autoaggregation. It has been suggested that lipoteichoic acids, proteins, and carbohydrates on the bacterial surface, soluble proteins, or pheromones are involved in the aggregation ability of bacteria (Ocaña and Nader-Macías, 2002). In the present study, autoaggregation properties of the *L. salivarius* I1, *L. cellobiosus* I3, *L. acidophilus* R9, and *L. acidophilus* S1 strains were affected by pepsin and lipase. Bacterial autoaggregation factors were not affected by sodium periodate. For this reason, it can be suggested that a proteinaceous surface component mediates the autoaggregation of I1, I3, R9, and S1. On the other hand, it appears that structures mediating autoaggregation can be released from the cell wall by sonication.

In conclusion, the lactobacilli used in this study may protect the vaginal epithelium through a barrier created by autoaggregation. Consequently, they may be excellent candidates for eventual use as a probiot-

ic. Studies to further evaluate their feasibility as such are under way.

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