Aggregation and co-aggregation abilities of potentially probiotic amylolytic lactic acid bacteria strains. By G. Blagoeva[1], V. Gotcheva[1] and A. Angelov[1], [1]*Department of Biotechnology, University of Food Technologies, Plovdiv 4002, Bulgaria.*

INTRODUCTION

The unique natural and climatic conditions in Bulgaria have contributed to the spontaneous evolution of lactic acid bacteria (LAB) strains known to have beneficial effect on the healthy composition and function of the gut microbiome[1,2]. LAB with amylolytic properties represent a rare group of LAB which can assimilate starch in a single step process and could be successfully applied as starter cultures for the production of alternative cereal-based functional foods[3]. However, functional properties of amylolytic lactic acid bacteria (aLAB) are not systematically investigated and probiotic aLAB strains are not reported yet. Therefore, screening and selection of aLAB strains with probiotic functional properties is receiving much attention over the past two decades.

An important parameter for the selection of probiotic probiotic bacteria is adhesion to the host gut, which would ensure bacteria-host interaction to confer health benefits[4]**.** Adhesion of probiotic bacteria to intestinal epithelial cells is an important prerequisite for colonization of probiotic strains in the gastrointestinal tract, preventing their immediate elimination by peristalsis and providing a competitive advantage in this ecosystem[5,6]. Autoaggregation of probiotic strains appears to be necessary for adhesion to intestinal epithelial cells, and co-aggregation abilities may form a barrier that prevents colonization by pathogenic microorganisms[7].

The aim of this study was to assess *in vitro* the aggregation and coaggregation abilities of aLAB strains isolated from Bulgarian cereal-based products for further development of amylolytic probiotic starter cultures for food and biotechnology applications.

MATERIALS AND METHODS

1. Bacterial strains and growth conditions

35 aLAB strains previously isolated from Bulgarian cereal-based fermented products were tested in the present study. Co-aggregation abilities of all isolates were tested with *Lactobacillus bulgaricus* LBB and with two pathogenic strains - *Salmonella sp.* NBIMCC 1425 and *Listeria monocytogenes* NBIMCC 8669 (Bulgarian National Bank of Industrial Microorganisms and Cell Cultures). All LAB species were grown in de Man Rogosa Sharpe (MRS) broth (Oxoid, UK) at 37^{o}C for 24 h and were stored at -20^{o}C in the same culture broth with 25% glycerol. *Salmonella sp.* NBIMCC 1425 and *Listeria monocytogenes* NBIMCC 8669 were cultivated in TSB broth (Merck) at 37^{o}C for 48 h and maintained on Tryptic soy agar (Merck) at 4^{o}C. Before analyses, strains were sub-cultured in MRS or in TSB broth, respectively.

2. Autoaggregation assay

Autoaggregation assays were performed according to the method of Kos *et al*. (2003). Bacteria were grown for 18 h at 37^{o}C in MRS broth. The cells were harvested by centrifugation at 5000 g for 15 min, washed twice and resuspended in their culture

supernatant fluid or in phosphate buffered saline (PBS, pH 7.2) to give viable counts of approximately 10^8 CFU/ml. Cell suspensions (4 ml) were mixed by vortexing for 10 s and autoaggregation was determined during 5 h of incubation at room temperature. At 1 h intervals, 0.1 ml of the upper suspension was transferred to another tube with 3.9 ml of PBS and the absorbance (A_t) at 600 nm was measured in a UV-VIS 1800 Spectrophotometer (Shimadzu, Japan). The autoaggregation percentage was expressed as a function of time until it was constant, using the formula $1-(A_t/A_0)\times 100$, where A_t represents the absorbance at any time (1, 2, 3, 4 or 5 h), and A_0 the absorbance at time t=0 h.

3. Co-aggregation ability of the aLAB strains

Co-aggregation assays were performed according to Del Re *et al*. (2000). Cell suspensions were prepared as described above for autoaggregation assay. Equal volumes (2 ml) of broth cultures of the tested aLAB strains and each pathogen strain were mixed by vortexing for 10 sec. Control tubes were set up at the same time, containing 4 ml of each bacterial suspension independently. Absorbance of the suspensions was measured at 600 nm after mixing and incubation at room temperature for 5 hours. Samples were taken in the same way as in the autoaggregation assay. The percentage of co-aggregation was calculated using the equation of Handley *et al*. (1987):

$$\text{Coaggregation, \%} = \frac{((Ax + Ay)/2 - A(x + y)}{Ax + Ay/2} * 100,$$

where *x* and *y* represent each of the two strains in the control tubes and in the (*x* + *y*) mixture.

RESULTS AND DISCUSSION

1. Autoaggregation ability of the isolated aLAB strains

Assessement of the autoaggregation ability of LAB is an important step in the selection of new probiotic strains[6]. Aggregation ability of bacteria is often related to cell adherence to epithelial cells and mucosal surfaces and is a prerequisite for colonization of probiotic strains in the gastrointestinal tract. In the present study, 35 aLAB strains were tested for their autoaggregation ability.

To avoid the possibility of removing extracellular components, which may have been related to autoaggregation, broth-grown cells were additionally examined for autoaggregation ability resuspended in their own culture supernatant fluid. Results for autoaggregation properties of aLAB strains in MRS broth and in PBS buffer are summarized in Table 1. The sedimentation rate of aLAB species isolated from Bulgarian cereal-based products was measured over a period of 5 h.

Table 1. Autoaggregation ability of aLAB strains

Species	A, % - 1 h		A, % - 2 h		A, % - 3 h		A, % - 4 h		A, % - 5 h	
	MRS	**PBS**	**MRS**	**PBS**	**MRS**	**PBS**	**MRS**	**PBS**	**MRS**	**PBS**
S. bovis	4,72	7,55	5,38	7,94	7,41	20,21	14,67	22,06	18,49	23,66
E. faecium	12,36	35,12	37,27	35,41	39,95	39,89	44,32	40,17	46,66	41,86
Lb. fermentum	9,04	11,91	5,10	10,78	4,51	12,80	7,47	13,65	9,50	16,52
Lb. fermentum	2,85	4,73	7,08	17,97	14,85	26,17	17,25	27,36	20,19	35,49
Lb. fermentum	9,34	14,79	15,96	35,15	49,45	51,09	46,31	51,51	46,42	55,12
E. faecium	5,74	8,87	17,08	19,44	26,77	19,12	34,17	26,72	35,92	32,05

Lb. plantarum	2,05	16,83	10,89	23,59	16,96	26,39	18,10	30,27	19,01	34,78
W. paramesenteroides	5,11	9,67	10,17	10,99	14,90	14,15	11,04	21,02	25,66	38,03
Lb. rhamnosus	2,25	6,49	5,27	5,49	10,00	10,02	16,32	23,87	24,63	32,52
Lb. pentosus	28,64	16,98	31,11	18,96	36,14	17,43	36,96	20,09	38,54	23,63
P. acidilactici	4,32	8,28	5,33	3,98	8,18	8,27	13,90	16,73	17,17	14,90
Lb. fermentum	2,78	3,16	4,54	4,42	9,68	11,85	16,53	35,91	22,25	33,43
P. acidilactici	1,33	1,16	1,40	2,13	4,02	8,62	9,87	12,68	24,59	26,39
Lb. plantarum	1,92	5,42	5,26	7,08	8,73	10,42	14,13	15,41	25,39	32,84
Lb. plantarum	4,97	5,46	6,61	4,22	11,37	10,48	15,06	19,32	20,66	27,81
P. acidilactici	3,58	2,21	3,77	6,14	7,33	9,68	10,84	16,56	27,37	31,03
P. acidilactici	4,58	3,53	6,29	5,03	8,76	7,65	17,23	15,74	19,73	20,92
Lb. plantarum	1,12	5,22	5,51	7,55	8,31	11,13	14,38	14,96	15,72	18,35
Lb. plantarum	1,81	7,01	2,57	12,60	6,52	14,01	5,47	19,89	7,86	22,17
Lb. paralimentarius	3,30	3,84	6,13	10,64	12,44	12,58	19,03	21,33	29,95	33,21
Lb. fermentum	6,62	5,22	18,17	18,10	27,29	30,41	39,23	40,66	41,69	46,56
P. acidilactici	5,54	2,90	14,23	21,94	16,85	29,29	20,56	35,24	29,91	40,50
Lb. paracasei	6,11	7,61	12,40	8,58	18,77	16,40	25,00	23,48	28,40	29,69
Lb. plantarum	2,35	10,32	3,53	8,50	5,71	8,50	7,37	13,72	11,01	16,35
P. pentosaceus	2,60	5,51	3,52	6,76	4,35	6,93	7,73	9,24	13,23	9,95
Lb. plantarum	8,84	5,82	9,13	3,91	19,15	11,46	23,92	17,90	23,83	20,58
P. acidilactici	8,04	2,05	15,28	5,36	13,81	7,78	19,25	18,53	27,48	25,40
Lb. casei	11,32	4,87	14,48	6,86	12,95	10,74	15,30	17,39	24,03	28,42
E.faecium/durans	1,49	7,03	5,81	13,86	7,49	17,00	9,26	23,15	30,42	47,21
Lb. casei	9,43	8,83	16,46	19,09	17,04	20,81	18,44	21,73	22,85	33,84
Lb. casei	3,25	1,96	9,95	13,09	15,55	16,82	22,23	21,70	32,70	38,95
Lb.paracasei	9,57	8,93	16,22	13,39	20,80	16,79	27,77	22,48	31,64	25,45
Lb. paracasei B41	5,07	2,97	3,43	11,50	7,61	17,11	4,76	23,91	7,75	31,30
Lb.plantarum Bom 816	3,40	4,67	4,81	6,97	8,31	15,33	13,59	12,78	20,91	17,98
Lb. pentosus N3	9,02	4,61	13,16	8,28	14,31	9,72	17,86	14,75	18,09	18,40

Results showed that strains exhibited a strong autoaggregating phenotype. Autoaggregation phenotype was similar in PBS and in MRS broth, with slightly better autoaggregation of cells resuspended in PBS buffer (pH 7.2) compared to the aLAB autoaggregation in MRS broth. Kos *et al*. (2003) reported similar results for the autoaggregation ability of *Lb. acidophilus* M92, resuspended in PBS and in its own culture supernatant. The autoaggragation percentage of aLAB resuspended in PBS ranged between 1.16% for *Lb. plantarum* and 55.12% for *E. faecium*, with highest levels of autoaggregation obtained after 5 hours of incubation. Autoaggregation of aLAB suspended in their own supernatant (MRS broth) was slightly lower, varying from 1.12% for one of the strains *Lb. plantarum* to 46.66% for *Lb. fermentum*. Anwar *et al*., (2014) reported that *Lb. paracasei*, *Lb. acidophilus* and *Lb. plantarum* show lower autoaggregation suspended in PBS buffer than in MRS broth, which varied from 15.8% to 63.1%. The observed autoaggregation ability of the tested aLAB strains could be related to cell surface component, because it was not lost after washing and suspending of the cells in PBS buffer.

2. Co-aggregation ability of aLAB strains

One of the first suggested modes of probiotic action is the "barrier" effect, also called resistance to pathogen colonization. It is exerted against pathogenic bacteria by preventing or limiting their colonization. In order to quantify interbacterial adherence, co-aggregation between aLAB strains, *Lactobacillus bulgaricus* LBB and two pathogen strains (Salmonella sp. NBIMCC 1425 and *Listeria monocytogenes* NBIMCC 8669) was examined. Results are

expressed as the percentage reduction after 5 h in the absorbance of a mixed suspension compared with the individual suspension (Figure 1).

The amylolytic strains demonstrated high co-aggregation with *Lb. bulgaricus* LBB, *Salmonella* sp. NBIMCC 1425 and *Listeria monocytogenes* NBIMCC 8669, with strongest interactions between aLAB isolates and *Salmonella* sp. Strains *P. pentosaceus*, *Lb. plantarum* and *Lb. casei* showed strong co-aggregation with potentially probiotic strain *Lb. bulgaricus* LBB - 19.25%, 17.46% and 15.65%, respectively. Co-aggregation was not observed between species *Lb. fermentum*, *E. faecium*, *P. acidilactici, Lb. casei, Lb. paracasei* B41 and *Lb. bulgaricus* LBB. It is important to note that co-aggregation with probiotic bacteria could increase the colonization potential of LAB if they are used in mixed cultures as probiotics[6]. The highest co-aggregation was observed between *Lb. fermentum* and *Salmonella* sp. NBIMCC 1425 (27.31%), followed by co-aggreagtion between the same pathogen and *E. faecium* (24.03%). The species *E. faecium* and *Lb. plantarum* did not demonstrate co-aagregation ability with *Salmonella* sp. NBIMCC 1425, and five aLAB strains were not able to co-aggregate with *Listeria monocytogenes* NBIMCC 8669. The strongest interaction with *Listeria monocytogenes* was detected for *Lb. casei* (20.13%), followed by *P. acidilactici* (16.48%).

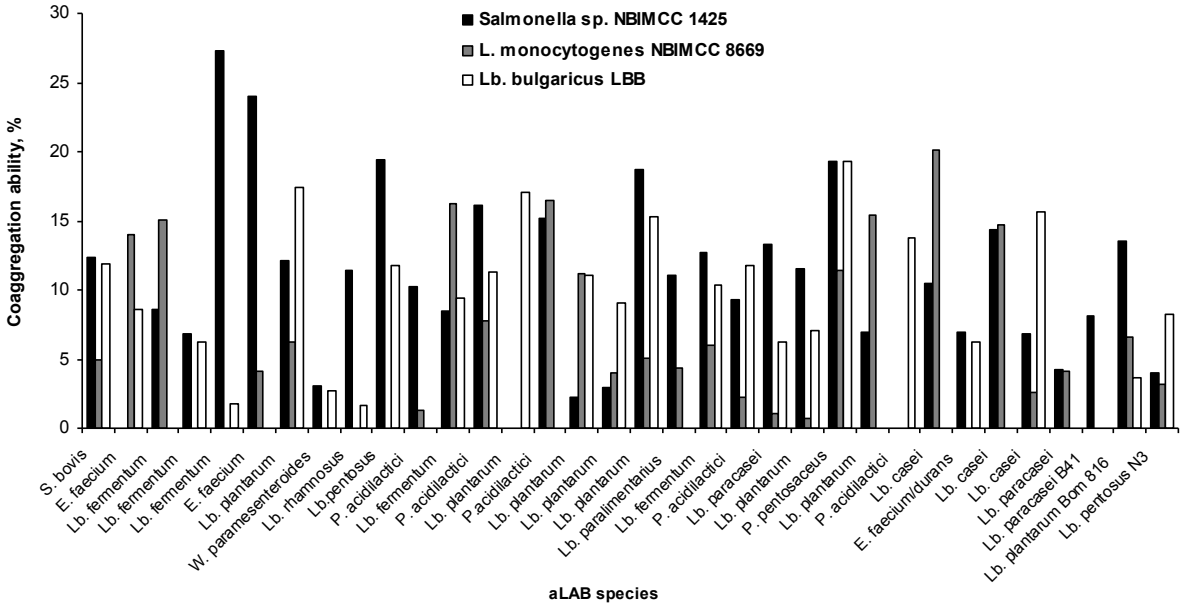

Figure 1. Co-aggregation ability of aLAB strains after 5 h incubation at room temperature in PBS (pH 7.2)

Kos et al. (2003) reported significantly lower percentage of co-aggregation between *Lactobacillus acidophilus* M92 and other probiotic bacteria, such as *Lb. plantarum* L4 (4.36%) and *E. faecium* (19.46%), and also lower co-aggregation of the same strain with pathogens *E. coli* 3014 (15.10%) and *Salmonella ser. typhimurium* (15.70%) in PBS after 5 h incubation at room temperature.

Inhibitor-producing LAB which co-aggregate with pathogens may constitute an important host defence mechanism against infection in the urogenital tract[7]. When present in sufficient numbers in the host, the probiotic lactobacilli are believed to create a healthy equilibrium between beneficial and potentially harmful microflora in the gut[6,10]. Results of the

coaggregation assay show that almost all tested aLAB strains can co-aggregate with a potentially probiotic LAB strain and pathogens, thus showing intriguing probiotic potential.

CONCLUSIONS

The present study revealed the probiotic potential of LAB strains isolated from Bulgarian fermented cereal-based products with regards to their ability to act as a barriers against pathogen colonization in the intestines. Rare starch utilizing species such as *P. acidilactici, P. pentosaceus and E. faecium* showed significant autoaggregation and co-aggregation abilities which are related to bacterial adherence to epithelial cells and mucosal surfaces and therefore represent important probiotic properties. ALAB with probiotic characteristics are not reported yet and the results obtained provide new valuable knowledge on key functional features of LAB with amylolytic activity. Further studies on the probiotic properties of these aLAB strains may result in development of commercially-relevant probiotic starter cultures for production of novel cereal-based functional foods.

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