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Co-aggregation and growth inhibition of probiotic lactobacilli and clinical isolates of mutans streptococci: An *in vitro* study

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Abstract

Objective. Co-aggregation and growth inhibition abilities of probiotic bacteria may play a key role in their interference with the oral biofilm. The aim was to investigate the *in vitro* ability of selected commercial probiotic lactobacilli to co-aggregate and inhibit growth of oral mutans steptococci isolated from adults with contrasting levels of caries. ***Materials and methods.*** Mutans streptococci (MS) strains were isolated from caries-free ($n = 3$) and caries-susceptible ($n = 5$) young adults and processed with eight commercial probiotic lactobacilli strains. One laboratory reference strain (*S. mutans* Ingbritt) was selected as control. Co-aggregation was determined spectrophotometrically and growth inhibition was assessed with the agar overlay technique. ***Results.*** All probiotic lactobacilli showed an ability to co-aggregate with the isolated MS strains. Statistically significant differences ($p < 0.05$) were found between strains from different individuals when compared with the reference strain. The selected lactobacilli inhibited MS growth, but the ability varied between the strains and was clearly related to pH. No differences were observed between the different MS strains from caries-free and caries-susceptible individuals. ***Conclusions.*** The selected lactobacilli displayed co-aggregation activity and inhibited growth of clinical mutans streptococci. The growth inhibition was strain-specific and dependent on pH and cell concentration. The findings indicate that the outcome of lactobacilli-derived probiotic therapy might vary between individuals and depend on the specific strain used.

Key Words: *Dental caries, oral microorganisms, probiotics*

Introduction

There is an emerging interest in the potential capacity of probiotic bacteria to prevent and combat oral diseases. The mode of action is thought to involve both local and systemic interactions; probiotic strains can compete with pathogens for nutrients and binding sites, produce antimicrobial substances and mediate the immune system through cytokine regulation (for reviews, see [1,2]). Up to now, most research has focused on probiotics tailored for gut health and genitourinary infections and much less is known concerning the interactions between the probiotic strains and caries-associated microorganisms [3]. Lactobacilli play a significant role in the oral ecosystem and can be linked to oral disease as well as oral health [4]. Lactobacilli species are mainly found on the mucous surfaces in oral cavity and frequently recovered from deep caries lesions [5,6]. It is previously shown that probiotic lactobacilli strains may survive in saliva and affect the oral ecology by preventing the adherence of other bacteria and by modifying the protein composition of the salivary pellicle [7]. Furthermore, laboratory studies have examined co-aggregation and growth inhibition of both natural occurring and probiotic lactobacilli and mutans streptococci [8–10] and a recent study has shown that probiotic strains can interfere with *S. mutans* biofilm formation *in vitro* [11]. A reduction of salivary mutans strepotococci has been unveiled in

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clinical trials [12,13]. Several lactobacilli species have been shown to produce antimicrobial substances and thereby inhibit the growth of *S. mutans* [14,15], but it is not clear whether this ability differs among the various strains found in commercially available dairy products or between mutans streptococci isolated from individuals with different caries susceptibility.

In order to design clinical studies, there is a need for further research on the abilities of the different probiotic strains to interferere with clinical isolates of mutans streptococci. The aim of the present paper was to investigate the *in vitro* ability of selected probiotic lactobacilli to co-aggregate and inhibit growth of oral mutans steptococci isolated from adults with contrasting levels of caries.

Materials and methods

Bacterial strains

Mutans streptococci strains were isolated from caries-free ($n = 3$) and caries-susceptible ($n = 5$) young adults (mean age 25.5 years). The caries-free subjects had Decayed/Missing/Filled Teeth (DMFT) = 0 and exhibited no early signs of tooth demineralization at any surface. The caries-susceptible persons had a mean DMFT of 15 (range 10–25) and at least five lesions that was considered active according to the ICDAS II criteria [16]. The strains were isolated from saliva using the Dentocult SM strip Mutans (Orion Diagnostica, Helsinki, Finland) and controlled for purity on blood agar plates and Gram-stained smears. The strains were then confirmed as *S. mutans* by addition of α-glycosid and β-glycosid and testing for ammonium production from arginin. Several strains were taken from each saliva sample and the most prevalent isolate identified as *S. mutans* from each subject was selected for the further assays. One laboratory reference strain (*S. mutans* Ingbritt) was used as control. Eight commercially availble probiotic lactobacillus strains, *L. plantarum* 931 (Essum, Umeå, Sweden), *L. plantarum* 299v (Probi AB, Lund, Sweden), *L. paracasei* F19 (Arla, Stockholm, Sweden), *L. rhamnosus GG* (Valio Ltd., Helsinki Finland), *L. rhamnosus* LB21 (Essum, Umeå, Sweden), *L. reuteri* DSM17938 (Biogaia, Stockholm, Sweden), *L. reuteri* ATCC PTA 5289 (Biogaia, Stockholm, Sweden) and *L. acidophilus* La5 (Arla, Stockholm, Sweden) were used for the experiments. The strains were characterized by the API 50 CH system (BioMérieux® SA, Marcy-l 'Etoile, France) as previously described [8].

Measurement of co-aggregation

Cultivation. The lactobacilli strains were cultivated in Man Rogosa Sharpe (MRS) broth (Oxoid Ltd., Basingstoke, Hampshire, UK) in an anaerobic incubator at 37°C for 24 h. The streptococci were grown in Brain Heart Infusion (BHI) broth under similar conditions. After incubation, the bacteria were aerobically harvested by centrifugation at 3000 rpm for 10 min, washed twice in phosphate buffered saline (PBS) and supended in neutral 10 mmol/l PBS buffer (pH 7.2).

Assay. Co-aggregation was determined spectrophotometrically (Genesis 10 uv; Thermo Scientific, Madison, WI) according to Collado et al. [17]. The absorbance was adjusted to OD 0.5 at 600 nm (~10^8 cells/ml) and equal volumes of the probiotic and cariogenic strains were mixed and incubated at 37°C without agitation. The mixed suspensions were monitored at 1, 2 and 4 h of incubation. The co-aggregation ratio was calculated by comparing absorbance of the pathogen suspension with the absorbance of the mixed suspensions. Each experiment was repeated at least three times with nine separate assessments.

Measurement of growth inhibition

Cultivation. The lactobacilli were initially cultured for 16–20 h on MRS agar (Oxoid Ltd., Hampshire, UK). A distinct colony of each bacterium was then transferred to 4.5 ml MRS broth for a further 16–20 h of incubation. The streptococci mutans strains were cultured on blood agar plates (Columbia Blood Agar Base, Alpha BioScience, Baltimore, MD) supplemented with 5% horse blood during 16–20 h and, on the following day, pure colonies of each bacterium were transferred to 2 ml Todd-Hewitt broth (Oxoid Ltd.) and incubated for another 16–20 h. Culturing of lactobacilli and mutans streptococci were performed in anaerobic atmospheres (10% H_2, 5% CO_2 and 85% N_2) in an anaerobe chamber at 37°C.

Agar overlay interference tests. The broth cultures of lactobacilli were serial diluted in 10-fold steps. The optical density (OD) was measured at 630 nm using a spectrophotometer at dilution 10^{-1} (Ultrospec 100 pro, Visible Spectrophotometer, Biochrom Ltd., Cambridge, UK). Undiluted suspension and cell suspensions corresponding to ~10^9, 10^7, 10^5 and 10^3 CFU/ml were used in the inhibition experiments. One millilitre of each lactobacilli suspension was added into 24 ml molten sterile MRS agar (ca 45°C) and plates were casted. When the agar was set, the plates were incubated at 37°C overnight in an anaerobic atmosphere. The next day, a second agar layer of 23 ml melted M17 agar supplemented with 10% sterile filtered lactose (May and Baker, Dagenham, UK) was casted on top of the MRS agar with grown lactobacilli. The plates were allowed to

dry for 3 h at room temperature. Broth cultures of MS grown for 16–20 h in Todd-Hewitt broth were diluted in the same medium and the OD was measured at 500 nm and adjusted to 0.2. The suspensions of MS were stamped on the plates with Steer's replicator (CMI-Promex ICN, Pedricktown, NJ, USA). The plates were left at room temperature for 1 h to dry and were subsequently incubated overnight at 37°C in the anaerobe chamber. The results of the agar overlay tests were categorized according to Simark-Mattsson et al. [9]: Score 0 = complete inhibition (no visible colonies), Score 1 = slight inhibition (at least one visible colony but definitely smaller amounts then in the control plate) and Score 2 = no inhibition (the same growth as on the control plate). The evaluation of the plates was performed by two independent observers, in cases of disagreement a consensus was reached after discussion. All growth inhibition assays were made in duplicate and each experiment was repeated on three different occasions.

pH measurements. As an estimation of the lactobacilli acid production, the surface pH of the MRS plates with M17 was determined before and after the final incubation of each lactobacilli strain devoid of mutans streptococci. A pH-meter (Seven Easy pH, Mettler-Toledoo GmbH, Schwerzenbach, Switzerland) equipped with a flat electrode was used and the mean of two measurements was calculated.

Statistical method

The data obtained were processed with the SPSS software (version 17.0, Chicago, IL) and subjected to one-way analysis of variance (ANOVA). A p-value less than 0.05 was considered statistically significant.

Results

Co-aggregation

All lactobacilli strains showed co-aggregation abilities with the clinical isolates and the behaviour was essentially the same for the different combinations. The co-aggregation ratio increased with time and the results after 120 min incubation are summarized in Figure 1. There were small but significant differences in co-aggregation ratio between the probiotic strains and the various clinical mutans streptococci, but no clear and systematic pattern appeared. *L. acidophilus* La5 was generally most prone to co-aggregate with all eight clinical isolates of mutans streptococci, while the two *L. rhamnosus* strains displayed the lowest values for five of the eight clinical isolates. The relative activity with respect to isolates from caries-free and

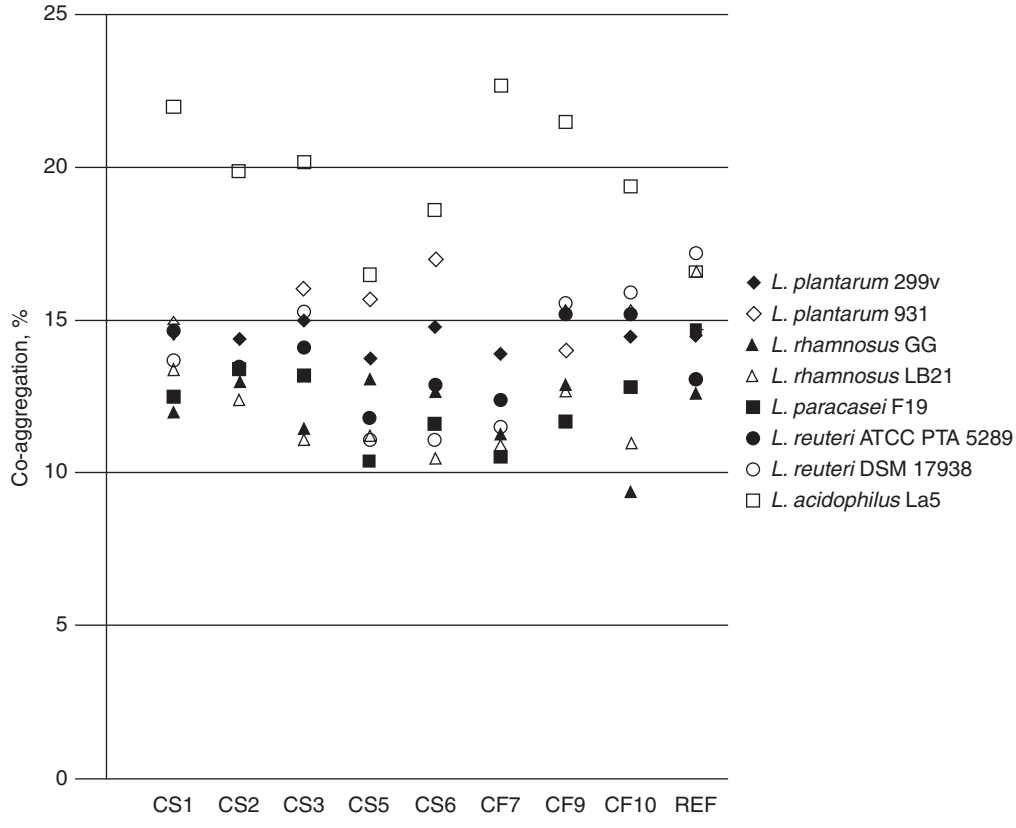

Figure 1. Co-aggregation ratio (%) between selected probiotic lactobacilli and eight clinical strains of salivary mutans streptococci isolated from caries-susceptible (CS) and caries-free (CF) adults after a 120 min assay. *S. mutans* Ingbritt was included as a reference (REF) strain.

caries-susceptible subjects vs the reference strains is shown in Table I. Four strains (*L. rhamnosus* LB21, *L. paracasei* F19, *L. reuteri* DSM17938 and *L. acidophilus* La5) displayed a weaker activity with all the isolates than with the reference strain. Statistically significant differences ($p < 0.05$) in co-aggregation activity between the caries-prone and caries-free isolates were obtained for five of the lactobacilli (Table I); the ability to co-aggregate was lower in strains from caries-free subjects in two cases (*L. plantarum* 931 and *L. rhamnosus* GG) and higher in three (*L. reuteri* PTA5289, *L. reuteri* DSM17938 and *L. acidophilus* La5).

Growth inhibition

The growth of all mutans streptococci strains was totally inhibited by the probiotic lactobacilli at cell concentrations of $\geq 10^7$ CFU/ml, but marked differences were displayed at lower concentration. The results of the growth inhibition assay with a bacterial density corresponding to 10^3 CFU/ml are summarized in Table II. Both *L. plantarum* 299v and *L. plantarum* 931 showed a complete inhibition, while *L. acidophilus* La5 did not seem to affect the growth of any of the *S. mutans* strains. The remaining lactobacilli displayed slight inhibition. The growth inhibition pattern of the clinical mutans streptococci isolates was identical with the reference strain, but no significant differences were observed between the different clinical strains.

pH measurements

The initial pH of the surface of the M17 agar plates were 6.8. After incubation, the pH decreased in all plates inoculated with lactobacilli and varied between 4.2–5.7 in the plates containing 10^3 CFU/ml. *L. acidophilus* La5 displayed the highest pH values while the lowest values were recorded for the *L. plantarum* strains (data not shown). Thus, the ability to inhibit growth of mutans streptococci was clearly dependant on final pH of the agar.

Table I. Relative co-aggregation between probiotic lactobacilli and clinical strains of mutans streptococci isolated from caries-susceptible and caries-free subjects. The co-aggregation ratio with the reference strain (*S. mutans* Ingbritt) was indexed at '100'.

Probiotic strain	Caries-susceptible (CS)	Caries-free (CF)	p*
L. plantarum 299v	100	103	NS
L. plantarum 931	105	98	<0.05
L. rhamnosus GG	99	89	<0.05
L. rhamnosus LB21	71	69	NS
L. paracasei F19	83	80	NS
L. reuteri PTA5289	102	109	<0.05
L. reuteri DSM17938	75	83	<0.05
L. acidophilus La5	117	128	<0.05

*Significant difference between strains isolated from caries-susceptible and caries-free subjects ($p < 0.05$). NS = not significant.

Discussion

A complementary strategy for caries prevention could be to strengthen the oral biofilm diversity and support the beneficial microbiota. Aggregation and growth inhibition assays are therefore useful tools for screening probiotic candidates with possible anti-caries properties. Any clinical conclusions must, however, be drawn with caution since laboratory conditions cannot mirror the complex ecological community in the oral biofilm. The co-aggregation ability of the probiotic strains is central in biofilm formation and competition with pathogens for binding sites [18]. The methods used in this study have previously been used for screening purposes and are proven simple and robust in comparison with more sophisticated techniques [17]. The advantage with the agar overlay method is that the assay enables assessment of different cell concentrations and multiple strains on a single plate [19]. It should, however, be stressed that the sample size with regards to differences in caries susceptibility was too small to allow any firm conclusions.

In the present study, all selected lactobacilli strains displayed co-aggregation activity with the clinical isolates, although of varying degree. The ratio after the 2-h assay ranged between 9.3–22.7%. There were also significant variations in the co-aggregation between bacteria from different individuals. For example, the ratio between *L. rhamnosus* GG and *S. mutans* varied from 9.4% in one caries-free subject to 13.1% in a caries-active. It was not possible to find a clear pattern or relationship to caries-susceptibility which probably illustrates the individual nature of the oral biofilm with respect to microbial composition and response to environmental stress. This indicates that there might not be one single probiotic strain that is optimal for all subjects with a given disease. Lang et al. [20] have recently investigated the specific co-aggregation between *L. paracasei* DSMZ16671 and oral streptococci. They found that this event was heat-stable and protease-resistant and not affected by pH values within the range of 4.0–8.0. Interestingly, the selected *L. plantarum* strain did not co-aggregate with common non-mutans members of the dental biofilm which could suggest that an administration of lactobacilli-derived probiotics has no harmful effects on the beneficial and protective bacteria in the oral cavity [21,22].

All lactobacilli strains showed a varying inhibitory effect to the clinical isolates and the reference strains.

Table II. Growth inhibition of eight clinical isolates of salivary mutans streptococci from caries-susceptible (CS) and caries-free (CF) young adults by probiotic lactobacilli at cell concentration 10^3 CFU/ml. The scores denote: 0 = total inhibition; 1 = slight inhibition; 2 = no inhibition.

	Streptococcus mutans (clinical isolates)								
Probiotic strain	CS1	CS2	CS3	CS5	CS6	CS7	CS9	CF10	REF
L. plantarum 299v	0	0	0	0	0	0	0	0	0
L. plantarum 931	0	0	0	0	0	0	0	0	0
L. rhamnosus GG	1	1	1	1	1	1	0	1	0
L. rhamnosus LB21	1	1	1	1	1	1	1	1	1
L. paracasei F19	1	1	1	1	1	1	1	1	1
L. reuteri PTA5289	1	1	1	1	1	1	1	1	1
L. reuteri DSM17938	2	2	1	2	1	2	1	1	1
L. acidophilus La5	2	2	2	2	2	2	2	2	2

REF = *S. mutans* Ingbritt.

Simark-Mattsson et al. [9] found a difference in the inhibitory potential of naturally occurring lactobacilli from patients with no caries experience as opposed to lactobacilli from persons with either arrested caries or active caries lesions, but no such tendencies were revealed here. It has also been shown that lactobacillus strains from individuals not harbouring mutans streptococci exerted an increased inhibition of a test panel of *S. mutans* strains [9]. The findings suggest that the presence of specific beneficial lactobacilli strains in the oral environment may interfere with and affect the colonization of caries-associated mutans streptococci in the oral biofilm. In the present study, both *L. plantarum* strains showed a maximum interference with the mutans streptococci, while *L. rhamnosus* GG, *L. rhamnosus* LB21 and *L. paracasei* F19 showed slightly impaired ability. This was in agreement with the *in vitro* assays of Simark-Mattsson et al. [9], but, interestingly, clinical studies have reported contrasting findings. Näse et al. [13] demonstrated reduced levels of salivary mutans streptococci in pre-school children after daily intake of milk containing *L. rhamnosus* GG during a 7-month period, while no such suppression was reported by Stecksén-Blicks et al. [22] after long-term use of milk supplemented with *L. rhamnosus* LB21 and fluoride.

The pH of the agar is important for the antibacterial capacity of lactobacilli [8,11,23]. In our experiments, the final agar pH was lowered with increasing cell concentrations and ranged from 4.1–5.7. One explanation for the enhanced growth inhibition at low pH is likely due to the bacteriocin production for which a maximum production is found between pH 4.5–5.5 [23]. This range was reached for all strains except one (*L. acidophilus* La5) at the lowest cell concentration in the present experiments. It should, however, be noted that, at high concentrations, the pH for several of the strains fell below 4.5, implying a sub-optimal bacteriocin production. Nevertheless, the generally low pH-values recorded might have obscured any potential differences in growth inhibition between the mutans streptococci isolated from the various subjects.

Conclusions

In conclusion, the results demonstrated that commercial lactobacilli-derived probiotic bacteria were able to co-aggregate with clinical isolates of mutans streptococci, but the ratio varied significantly between strains isolated from different subjects. The ability to inhibit growth was strain-specific, with no apparent relationship to the caries susceptibility of the subject. Thus, the potential outcome of lactobacilli-derived probiotic therapy will vary between subjects and depend on the specific strain used.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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