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Coaggregation between probiotic bacteria and caries-associated strains: An *in vitro* study

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Abstract

Objective. To evaluate the *in vitro* abilities of probiotic bacteria derived from consumer products to coaggregate with caries-associated mutans streptococci. ***Material and Methods.*** Six lactobacillus strains (*L. acidophilus* (CCUG 5917), *L. plantarum* 299v, *L. rhamnosus* GG and LB21, *L. paracasei* F19, *L. reuteri* PTA5289) were cultivated under anaerobic conditions at 37°C in Man Rogosa Sharpe (MRS) broth for 24 h. Four strains of human streptococci (*S. mutans* Ingbritt, *S. mutans* (ATCC 25175), *S. mutans* GS-5, *S. sobrinus* (ATCC 33478) were similarly grown in Brain Heart Infusion (BHI) broth. A gastrointestinal pathogen (*Escherichia coli*) was aerobically cultivated on BHI broth as a positive control. After incubation, the bacteria were aerobically harvested, washed, and suspended in 10 mmol/l phosphate-buffered saline (pH 7.2). The probiotic strains were characterized with the API 50 CH system to confirm their identity. Coaggregation was determined by spectrophotometry in mixtures and bacterial suspensions alone after 1, 2, 4, and 24 h and expressed as the aggregation ratio (%). ***Results.*** All probiotic strains showed coaggregation abilities with the oral pathogens and the results were strain specific and dependent on time. *S. mutans* GS-5 exhibited a significantly higher ability to coaggregate with all the probiotic strains than the other mutans streptococci and *E. coli*. The differences among the probiotic strains were modest with *L. acidophilus* being the most prone and *L. rhamnosus* LB21 the least prone to coaggregate with the oral streptococci. ***Conclusions.*** The results demonstrated different abilities of lactobacilli-derived probiotic bacteria to coaggregate with selected oral streptococci. Aggregation assays may be a useful complement for screening of probiotic candidates with possible anti-caries properties.

Key Words: *Dental caries, lactobacilli, mutans streptococci, probiotics*

Introduction

Probiotic bacteria are live micro-organisms which, when given in adequate amounts, confer a health benefit for the host by improving its intestinal balance [1]. It is commonly accepted that a daily intake of dairy products containing live lactobacilli and bifidobacteria may reduce the risk of some gastrointestinal diseases [2–4] and there is growing interest in its potential to improve oral health [5–7]. The key event is that non-pathogenic micro-organisms can occupy a space in a human biofilm that otherwise would be colonized by a pathogen resulting in local and systemic effects. Probiotic bacteria can survive and grow in saliva *in vitro* [8] and affect the oral ecology by preventing the adherence of other bacteria and by modifying the protein composition of the salivary pellicle [9]. Permanent colonization, however, seems unlikely in an established oral biofilm [10]. Previous studies have suggested that a regular intake of various strains of the species *Lactobacillus reuteri* and *L. rhamnosus* can reduce the levels of salivary mutans streptococci in children and young adults on a short-term basis [11–13].

Coaggregation is a key mechanism in biofilm formation and a significant factor in dental plaque development [4,9]. The coaggregation abilities of probiotic strains might enable the formation of a barrier that prevents colonization by pathogenic bacteria. It is, however, possible that probiotic strains with a proven efficacy in the gastrointestinal

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tract may not be optimal in the oral ecosystem. Further clinical studies with relevant clinical outcome measures are therefore required. Before the designing and performance of such projects, more knowledge about the mechanism of action and strain differences is called for. Therefore, studies on which probiotic lactobacilli, or combinations of probiotic lactobacilli, are the most suitable for bacteriotherapy in the oral cavity are needed. It was therefore of interest to investigate the coaggregation abilities of probiotic bacteria with some oral pathogens. The aim of the present paper was to investigate the *in vitro* coaggregation between probiotic lactobacilli strains, available in over-the-counter products, with bacteria associated with dental caries.

Material and methods

Probiotic bacterial strains

Five strains of lactobacilli used in commercial dairy products were selected: *L. acidophilus* (CCUG 5917, acidophilus milk), *L. plantarum* 299v (ProViva; Skånemejerier, Malmö, Sweden), *L. rhamnosus* GG (Valio Ltd., Helsinki, Finland), *L. rhamnosus* LB21 (Essum, Umeå, Sweden), and *L. paracasei* subsp. *paracasei* F19 (Arla Ltd., Stockholm, Sweden). *L. reuteri* PTA5289 (BioGaia, Stockholm, Sweden), a strain found in chewing gums and tablets, was also included. All strains were obtained from the provider in pure form, either as frozen suspensions or lyophilized.

Caries-associated strains

The panel of caries-associated bacteria consisted of four laboratory strains of human mutans streptococci representing different serotypes: *S. mutans* Ingbritt (serotype c), *S. mutans* ATCC 25175 (serotype c, e, f), *S. mutans* GS-5 (serotype c), and *S. sobrinus* (ATCC 33478, serotypes d, g). All strains, as well as an intestinal reference strain *(Escherichia coli)* that was selected as a positive control, were obtained from the culture collections at the University of Gothenburg (CCUG) and the Department of Oral Microbiology, University of Copenhagen.

Cultivation

All bacteria were controlled for purity on blood agar plates and Gram-stained smears. The lactobacilli strains were cultivated in Man Rogosa Sharpe (MRS) [14] broth (Oxoid Ltd., Basingstoke, Hampshire, UK) in an anaerobic incubator (modular atmosphere controlled system; DW Scientific, West Yorkshire, UK) at 37°C for 24 h. The oral pathogens as well as the intestinal strain were grown in BHI broth under similar conditions. After incubation, the bacteria were aerobically harvested by centrifugation at 3,000 rpm for 10 min, washed twice in phosphate-buffered saline (PBS) and suspended in neutral 10 mmol/l PBS buffer (pH 7.2). All lactobacilli strains were characterized with the API 50 CH system (BioMérieux® SA, Marcy-l'Etiole, France) to confirm their identity. The *L. paracasei* subsp. *paracasei* strain as well as the species of *L. acidophilus* and *L. plantarum* were identified without difficulty by the API 50 CH system. *L. reuteri* gave a similar biochemical profile as *L. fermentum* in the database. None of the *L. rhamnosus* strains could be fully identified by the API 50 CH.

Measurement of coaggregation

Coaggregation was determined spectrophotometrically (Genesis 10uv; Thermo Scientific, Madison, Wis., USA) according to Collado et al. [15]. In brief, the absorbance was adjusted to 0.25 (SD 0.05) at 600 nm (approximately 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, 4, and 24 h of incubation. The coaggregation ratio (%) was calculated by comparing the absorbance of the pathogen suspension with the absorbance of the mixed suspensions. All assays were done in triplicate in at least four independent experiments in order to correct for intra-assay variation.

Statistical method

The data obtained were processed with the SPSS software (version 17.0, Chicago, Ill., USA) and subjected to one-way analysis of variance (ANOVA).

Results

All probiotic strains showed coaggregation abilities with the selected pathogens and the results were strain specific and dependent on time. As a typical example, the coaggregation ratios between *L. acidophilus* CCUG 5917 and *S. mutans*, *S. sobrinus* and *E. coli* after 1, 2, 4, and 24 h of incubation are shown in Figure 1. The ratio increased by time for all combinations but *S. mutans* GS-5 exhibited a significantly elevated ability to coaggregate ($p < 0.05$) at all designated time-points. The reactions with the gastrointestinal reference strain were of the same magnitude during the initial hours, but after 24 h the *E. coli* strain displayed significantly ($p < 0.05$) lower values than the oral strains, with the exception of *S. sobrinus*. The 2-h findings are compiled in Table I. Again, *S. mutans* GS-5 showed a higher tendency to coaggregate with all the probiotic strains compared with the other mutans streptococci and *E. coli*, a difference that was statistically significant ($p < 0.05$).

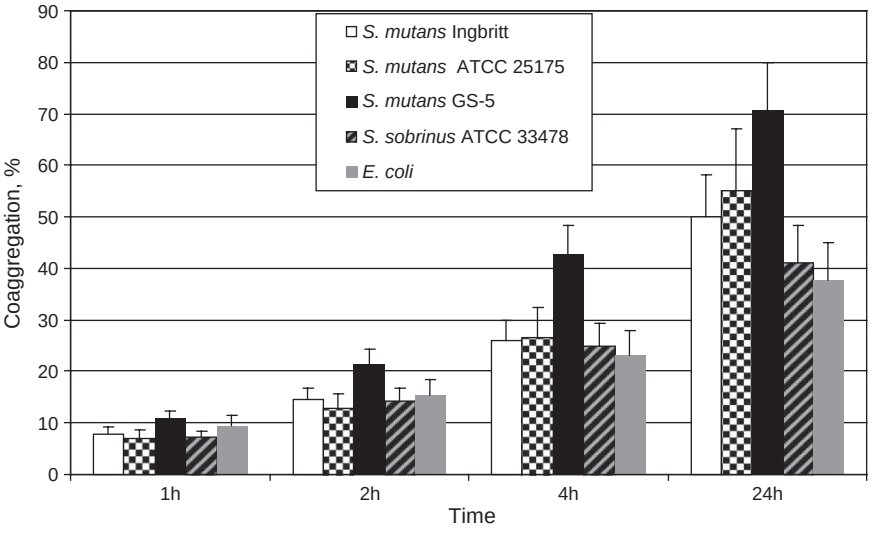

Figure 1. Coaggregation ratio (%) between *L. acidophilus* (CCUG 5917) and the selected caries-associated and gastrointestinal strains at 1, 2, 4, and 24 h of incubation. Vertical bars indicate SD. The difference between *S. mutans* GS-5 (black bars) and the rest of the oral bacteria was statistically significant ($p < 0.05$) at all time-points.

The ability to coaggregate with a given pathogen differed generally in a relatively modest way between the various probiotic strains. For example, the coaggregation ratio between *S. mutans* Ingbritt and the selected probiotic lactobacillus strains ranged from 18.8% to 25.9% after 4 h of incubation, with *L. acidophilus* being the most and *L. rhamnosus* LB21 the least prone to react. The differences between those contrasting strains were, however, statistically significant ($p < 0.05$).

Discussion

Probiotic therapy has recently gained massive interest world-wide owing to the potentially beneficial effects on general and oral health as well as being an important complement to antibiotic treatment. Furthermore, the administration is simple, inexpensive, and safe. Although the mechanisms of action are not fully understood [4], it is generally accepted that the ability of probiotics to coaggregate with pathogens is a desired property. To be effective against oral infections, probiotic bacteria need to adhere to the oral mucosa and dental tissues as a part of the oral biofilm and to compete with the growth of cariogenic bacteria and/or periodontal pathogens [8,16]. The ability of probiotics to adhere and coaggregate with intestinal pathogens has been thoroughly investigated [17–19] and the present study was an attempt to elucidate this event with laboratory strains associated with dental caries. We used a simple and robust spectrophotometric method that has been shown to correspond well to more sophisticated radioactive labeling techniques, albeit with a slightly impaired sensitivity [15]. All the probiotic strains were commercially available in dairy consumer products or in chewing gums and tablets (*L. reuteri*). Notably, the strain *L. reuteri* was closely related to *L. fermentum* and could not be distinguished by biochemical analysis only. Final confirmation of the identity of these two species as well as that of *L. rhamnosus* requires molecular genetic methods [20].

First of all, the results established that the general coaggregation pattern between probiotic and oral strains was similar to the gastrointestinal control; the

Table I. Coaggregation ratio (%) between some caries-associated bacteria and selected probiotic lactobacillus strains derived from consumer products after 2 h of incubation. An intestinal *Escherichia coli* strain was used as reference.

	S. mutans				
Probiotic strain	Ingbritt	25175	GS-5	*S. sobrinus*	*E. coli*
L. acidophilus CCUG 5917	14.5 (2.4)	12.8 (2.8)*	21.6 (2.8)$^{\star,\#}$	14.2 (2.6)	15.3 (3.1)
L. plantarum 299v	11.8 (2.0)*	14.3 (4.9)	18.6 (5.2)$^{\star,\#}$	11.6 (2.9)*	13.3 (2.5)
L. rhamnosus GG	11.0 (4.3)	12.0 (2.5)	18.7 (3.1)$^{\star,\#}$	10.5 (6.2)	13.5 (5.4)
L. rhamnosus LB21	10.1 (5.7)	11.0 (3.7)	19.3 (3.3)$^{\star,\#}$	9.1 (4.2)	11.0 (2.3)
L. paracasei F19	14.2 (4.4)	13.2 (5.2)	20.4 (6.6)$^{\star,\#}$	12.0 (5.3)	12.3 (5.5)
L. reuteri PTA5289	11.0 (5.0)	9.4 (6.0)	20.3 (3.9)$^{\star,\#}$	11.7 (4.4)	11.2 (2.6)

Data are expressed as mean values (SD).

*Statistically significant difference compared with the intestinal reference strain *E. coli* ($p < 0.05$).

$^{\#}$Statistically significant difference compared with the other caries-associated mutans streptococci ($p < 0.05$).

ratio was at least as high for oral bacteria after 4 h of incubation. Moreover, the findings indicated a more pronounced difference in coaggregation properties among the oral pathogens than between the various lactobacilli strains. This was interesting in the light of a recent *in vitro* study from our research group that demonstrated significant differences in the sugar fermenting capacity of the various probiotic strains [21]. Thus, from a coaggregation point of view, none of the commercial strains seemed to have a clear advantage over the others under the present experimental conditions. The contrasting coaggregation properties found between the various streptococci strains were not unexpected since it is well known that the number and activity of glucosyltransferase (GTF) enzymes vary within species of streptococci [22]. For example, significant GTF-A activity has been demonstrated on the cell surface of the serotype c strain GS-5, which likely contributes to its high disposition to coaggregate with other bacteria. In contrast, other findings suggest that, unlike other strains of *S. mutans*, the strain Ingbritt (serotype c) does not exhibit cell-wall anchoring of certain surface proteins, such as surface protein antigen (PAc), glucan-binding protein C, and dextranase [23], which may contribute to a lowered affinity and cariogenicity. Any clinical conclusions must, however, be drawn with caution since laboratory conditions with monocultures by far are representative of the complex ecological community in the oral environment. Moreover, laboratory-maintained strains of mutans streptococci may display altered characteristics compared with the original isolate [24] so we are planning further investigations with fresh isolates from subjects with contrasting levels of caries. Nevertheless, the results from this study indicate that a simple coaggregation assay may be a useful complement to metabolic activity measurements in the initial screening procedure for the selection of probiotic strains with a potential to interfere with bacteria associated with dental caries. Moreover, our results indicate that a case-by-case assessment may be needed in order to select strains with the ability to inhibit or displace a specific pathogen.

In conclusion, the results demonstrated different abilities of commercial lactobacilli-derived probiotic bacteria to coaggregate with selected oral streptococci. Aggregation assays may be a useful complementary tool for the screening of probiotic candidates with possible anti-caries properties.

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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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