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Biofilm growth of *Lactobacillus* species is promoted by *Actinomyces* species and *Streptococcus mutans*

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The ability of oral bacteria to integrate within a biofilm is pivotal to their survival. A dependence on the amount of biofilm growth by noncoaggregating *Lactobacillus rhamnosus* and *Lactobacillus plantarum* on coculture with *Actinomyces naeslundii*, *Actinomyces gerencseriae*, *Streptococcus mutans* and *Veillonella parvula* was investigated using an artificial-mouth culture system. Biofilm formation by the lactobacilli in mono-culture was poor. In coculture with *Actinomyces* species the amount of *L. rhamnosus* increased 7–20 times and *L. plantarum* 4–7 times compared to its mono-culture biofilm. *S. mutans* also promoted substantial biofilm growth of lactobacilli but *V. parvula* had no effect. We conclude that these *Actinomyces* species promoted growth of key *Lactobacillus* species in a biofilm, as did *S. mutans* to a smaller extent, and that the ability of individual bacteria to form mono-culture biofilms is not necessarily an indicator of their survival and pathogenic potential in a complex multispecies biofilm community.

Key words: lactobacilli; *Actinomyces*; *Streptococcus mutans*; biofilm growth promotion; biofilm coculture

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Dental plaque is a complex and dynamic microbial ecosystem comprising hundreds of species that exist in a biofilm (13, 17). Biofilm formation is key to the existence and survival in the oral cavity with numerous inter- and intra-species interactions (12). Some bacterial interactions are potentially beneficial to one or more species of the biofilm, for example through enzyme complementation (18) and aggregation (8, 10, 15), whereas other interactions are antagonistic, such as generation of low pH (4, 11, 20) and bacteriocin production (9). The net result of all these interactions is a highly organized, heterogeneous biofilm consisting of microniches that supports the coexistence of microbial complexes of functionally similar and dissimilar bacteria.

We have previously reported that *Lactobacillus rhamnosus* demonstrated a poor ability to form a mono-culture biofilm but that in the presence of *Actinomyces naeslundii* it predominated in a substantial biofilm (19). *Actinomyces* are major commensal species on both tooth and mucosal surfaces (11–13). The aim of the present study was to establish a specific biofilm growth promotion of lactobacilli by *Actinomyces* species.

Material and methods

Bacterial strains and inoculation procedure

The bacteria investigated were *Lactobacillus plantarum* SA-1, *L. rhamnosus* ATCC 7469, *A. naeslundii* ATCC 12104, *Actinomyces gerencseriae* ATCC 23860 *Streptococcus mutans* NZCDC 3362 and *Veillonella parvula* ATCC 10790. Prior to biofilm inoculation, 10 ml cultures were grown in TSBYK medium in an anaerobic hood at 35°C supplied with 80% N_2, 10% CO_2, and 10% H_2, except for *S. mutans,* which was incubated in a 10% CO_2 in air atmosphere at 35°C. TSBYK medium contains (per liter): 15 g tryptic soy broth (Beckton Dickinson and Co., Sparks, MD); 18.5 g brain heart infusion (Difco; Detroit, MI); 10 g yeast extract (Difco) and 5 mg hemin (Sigma Chemical Co., St. Louis, MO) autoclaved and supplemented with Vitamin K (4 μg/ml final concentration) (25).

Overnight cultures of the test bacteria were harvested by centrifugation (1500 × *g*, 20°C, 30 min) and resuspended in 2 ml sterile water. The selected

cocultures were prepared by mixing equal volumes from the mono-culture suspensions. Coverslips, prepared as described below, were inoculated directly in the multiplaque 'artificial mouth' (MAM) with 0.5 ml of the bacterial suspension 30 min prior to the flow of medium commencing.

General biofilm growth conditions

Details of the MAM have previously been described (19–21, 27). In brief, the biofilms were cultured on 25 mm diameter Thermanox™ coverslips (Nunc Inc., Naperville, IL), 17 mm under a fluid head assembly with lines for nutrient supply and inoculation. The culture chamber, housed in a custom-built Perspex air incubator at 35°C, was supplied with humidified gas (5% CO_2 in N_2) for 30 min every 2 h. Each biofilm was continuously supplied (2.5 ml/h) with a chemically defined analog of saliva (defined medium mucin) (27) and a periodic supply of 5% w/v (146 mM) sucrose (1.5 ml/h, 6 min every 8 h). Defined medium mucin contains (in mM): $CaCl_2$ (1.0), $MgCl_2$ (0.2), KH_2PO_4 (3.5), K_2HPO_4 (1.5), NaCl (10.0), KCl (15.0), NH_4Cl (2.0), urea (1.0); pig gastric mucin (2.5 g/l), amino acids equivalent to 5 g/l casein (total 43.12 mM), and at salivary concentrations, 21 basal amino acids and 17 vitamins or growth factors, pH 7.0.

Experimental protocol

In Experiment I, biofilms of *L. rhamnosus* in mono- and coculture with *A. naeslundii* and *A. gerencseriae* and biofilm monocultures of *A. gerencseriae* and *A. naeslundii*, were established in the MAM and grown undisturbed for 19 days. In Experiment II, biofilm mono-cultures of *L. rhamnosus, L. plantarum* and in coculture with *A. gerencseriae*, *A. naeslundii*, *S. mutans* and *V. parvula* were established in the MAM and grown undisturbed for 14 days.

Biofilm analysis

At the end of each experiment, the biofilm wet weight was measured. The average biofilm depth was estimated as the total wet weight divided by the area of the coverslip (490 mm^2). Photographs of whole biofilms were taken using a digital camera (Olympus DP10, Olympus Australia, Melbourne, Australia) fitted on a binocular microscope (Olympus SZX12, Olympus Australia).

For each biofilm, an 8.0 mg/ml suspension was prepared by homogenizing (Ika-UltraTurrax, Janke and Kunkel GmbH & Co., Staufen, Germany) approximately 0.4 g (wet weight) of biofilm in sterile water for 90 s. Portions were taken for the different analyses below.

To determine total bacterial colony-forming units (CFU), serial 10-fold dilutions in 1% peptone (Difco Laboratories, Detroit, MI) of the homogenized plaque were plated in triplicate using a spiral plater (Model D, Spiral Biotech Inc., Norwood, MA). TSBYK supplemented with 5% defibrinated sheep blood (Invitrogen, Auckland, New Zealand) (TSBYK-B) was used to determine total CFU, and Rogosa SL (Difco) to obtain lactobacilli CFU. Both were incubated anaerobically for 3 days at 35°C. Due to the selective nature of the Rogosa medium, more lactobacilli were recovered on the TSBYK-B plates; a plating efficiency coefficient for Rogosa agar was therefore derived for the lactobacilli mono-culture biofilms: lactobacilli TSBYK-B count divided by the lactobacilli Rogosa count. The Rogosa CFU data of the cocultures were adjusted accordingly and differences in the amount of lactobacilli were analyzed for significance using an independent two-sample *t*-test with SPSS (v12.0.1 for Windows, SPSS Inc., Chicago, IL). The number of non-lactobacilli CFU was calculated by subtracting the Rogosa count adjusted for plating efficiency from the total TSBYK-B count. Where appropriate, other CFU comparisons were analyzed for significance using independent two-sample *t*-tests. The number of CFU per plaque for each species was calculated.

Biofilm Folin-protein was measured (C.V. 2%) (22) and its percentage of wet weight calculated. Direct potential metabolic interactions of the lactobacilli with the coculture species were examined by cross-streaking on TSBYK-B plates and by a deferred antagonism assay (2). The degree of *in vitro* coaggregation between bacterial pairs of the lactobacilli with *A. gerencseriae*, *A. naeslundii*, *S. mutans* or *V. parvula* was assessed visually and assigned a score from 0 (homogeneous suspension) to 4 (clear suspension and large settled cell aggregates) (5).

Results

L. rhamnosus formed a pallid (Fig. 1), very thin (average depth <20 μm/mm^2) biofilm (Table 1). The mono-culture biofilms of *A. naeslundii* and *A. gerencseriae* (in Experiment I) and the coculture biofilms for both *Lactobacillus* species were substantial, with well-defined 'microcolony' clumps giving a cauliflower-like appearance (Fig. 1). The amount of *L. rhamnosus* in the coculture with each of the *Actinomyces* species in both experiments was 7–20 × greater than in its mono-culture biofilm. Biofilm cell protein concentration was also higher in the cocultures. The *L. plantarum* mono-culture biofilm was 5 × larger (average depth ~100 μm/mm^2), than the *L. rhamnosus* mono-culture (Fig. 1, Table 1). *L. plantarum* biofilm growth increased in the presence of the *Actinomyces* species by 4–7-fold. Again, biofilm cell protein concentration increased. *A. naeslundii*, and to a lesser extent *A. gerencseriae*, were inhibited by *L. rhamnosus* (Experiment I). Both lactobacilli caused slight inhibition of each *Actinomyces* species when examined by cross-streaking on plates and in the deferred antagonism assay.

In Experiment II, the biofilm growth-stimulation of *Lactobacilli* species by *Actinomyces* species coculture was compared with the effects of coculture with *S. mutans* and *V. parvula* (Fig. 1, Table 1). The *S. mutans* cocultures produced smaller biofilms than did *Actinomyces* cocultures ($P < 0.01$). *S. mutans* induced at least a 5-fold increase in the total amount of *L. rhamnosus* present, comparable to *A. naeslundii*, and increased the amount of *L. plantarum* 2–4-fold, also increasing biofilm cell protein concentration. Coculture with *V. parvula* had almost no effect on the amount of either *Lactobacilli* species present and *V. parvula* dominated the biofilm. Both lactobacilli caused growth promotion of *V. parvula* when examined by cross-streaking on plates and in a deferred antagonism assay. No coaggregation was observed between the lactobacilli and any of the coculture species.

Discussion

Under the conditions described, *L. rhamnosus* and *L. plantarum* failed to form substantial biofilms in mono-culture. When present with either *A. naeslundii* or *A. gerencseriae* they were able to proliferate and accrue in a large biofilm and, as evident from the biofilm protein concentration, at a higher cell density than the mono-culture. Greater promotion of biofilm growth was seen with *L. rhamnosus* than with *L. plantarum* but the absolute increase in CFU was greater for *L. plantarum*. Overall, *A. gerencseriae* produced greater growth promotion than *A. naeslundii*. The *Lactobacillus* species

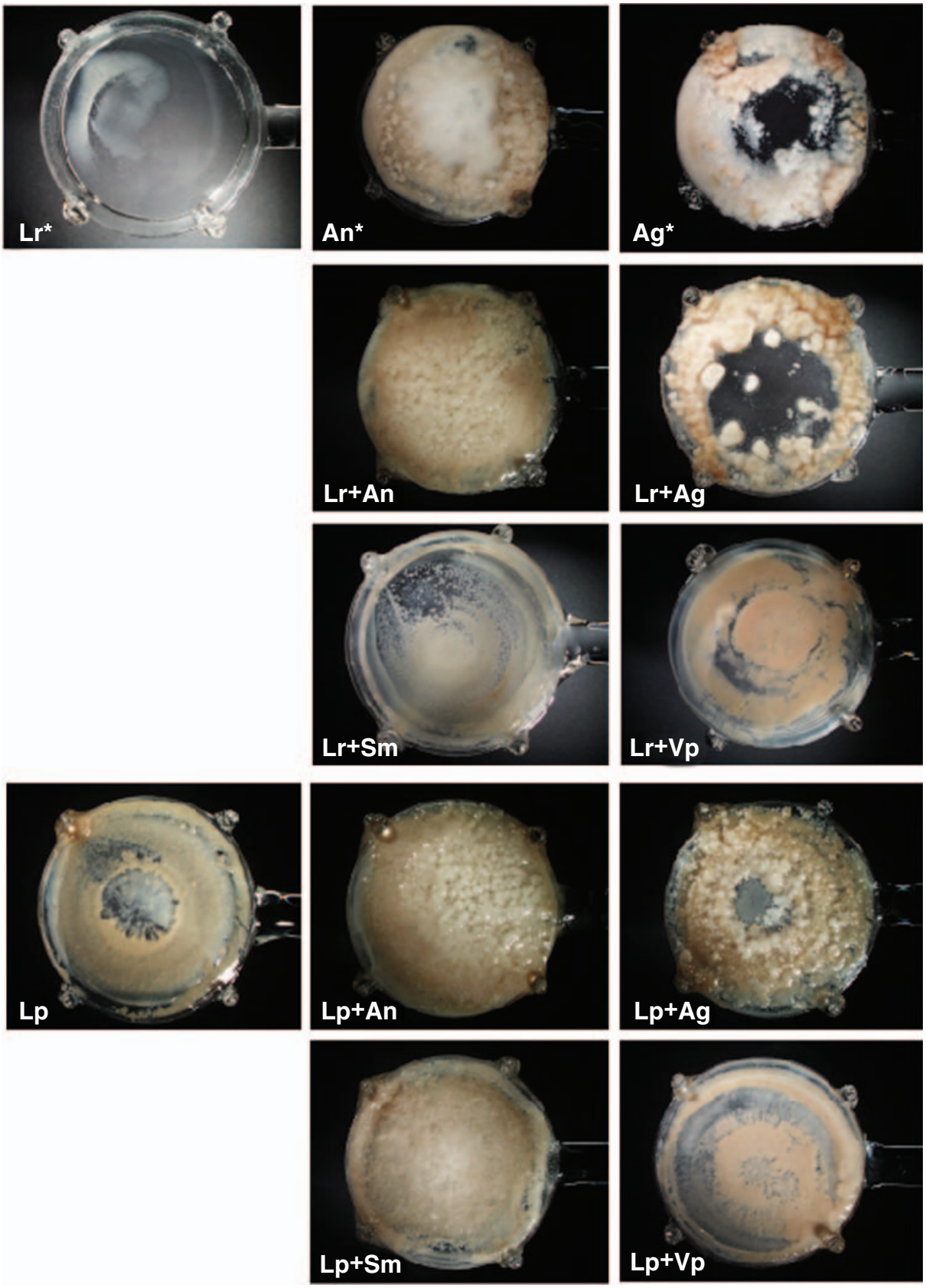

Fig. 1. Photographs of biofilm mono-cultures of *L. rhamnosus* (Lr), *L. plantarum* (Lp), *A. naeslundii* (An), *A. gerencseriae* (Ag), and the co-cultures of the Lactobacilli with *A. naeslundii* (An), *A. gerencseriae* (Ag), *S. mutans* (Sm) and *V. parvula* (Vp). The asterix denotes biofilms from Experiment I, the rest were from Experiment II. The diameter of the coverslip was 25 mm.

Table1. Comparison of biomass and cells number between the mono- and cocultured *Lactobacillus* species

Biofilm species[a]	Biofilm wet weight, mg	Biofilm protein, mg	Biofilm protein, % to wet weight	Total anaerobic TSBYK species[a,d] CFU × 10^{-7}/plaque	*Lactobacillus* species[b,d] CFU × 10^{-7}/plaque	Non-*Lactobacillus* species[c,d] CFU × 10^{-7}/plaque
Experiment I						
Lr	7.1	0.13	1.8	5.3 (0.07)	5.3 (0.07)	0
Lr+An	349	11.1	3.2	127 (13.6)	88.6 (2.37)***	38.4 (27.1)***
An	338	7.8	2.3	111 (5.3)	0	111 (5.3)
Lr+Ag	207	6.9	3.3	63.8 (0.6)	61.9 (1.97)***	2.17 (0.07)***
Ag	283	7.1	2.5	4.42 (0.3)	0	4.42 (0.3)
Experiment II						
Lr	9.9	0.11	1.1	5.3 (0.08)	5.3 (0.08)	0
Lr+An	443	14.1	3.2	437 (16.4)	41.8 (0.85)***	395 (16.9)
Lr+Ag	290	8.7	3.0	99.4 (2)	95.3 (3.17)***	4.12 (2.78)
Lr+Sm	34	1.3	3.8	71.3 (24.5)	31 (3.17)**	40 (6.6)
Lr+Vp	72	3.3	4.6	520 (24.8)	4.57 (0.09)	515 (24.8)
Lp	50	1.2	2.4	27.6 (13.8)	27.6 (13.8)	0
Lp+An	448	13.4	3.0	699 (30.2)	109 (6.63)***	590 (36.4)
Lp+Ag	242	6.9	2.9	672 (57.3)	177 (2.2)***	495 (49.1)
Lp+Sm	199	2.0	1.0	51.8 (2.49)	66.4 (3.12)***	188 (1.07)
Lp+Vp	96	5.5	5.8	741 (36.1)	31.1 (2.03)	710 (36.9)

[a]Lr: *L. rhamnosus*; Lp: *L. plantarum*; An: *A. naeslundii*; Ag: *A. gerencseriae*; Sm: *S. mutans*; Vp: *V. parvula*.
[b]The plating efficiency coefficient (Lactobacilli TSBYK count divided by the Lactobacilli Rogosa count) for the Rogosa agar in Experiment I was 1.2 and 1.3 for *L. rhamnosus* and 1.4 for *L. plantarum* in Experiment II.
[c]The number of non-Lactobacilli CFU was calculated by subtracting the Rogosa count adjusted for plating efficiency from the total TSBYK count.
[d]The figures in parentheses represent SE.
Asterisks indicate alpha level of significance of the difference between species in mono- and coculture: ** $P \leq 0.01$; *** $P \leq 0.001$.

did not coaggregate with the *Actinomyces* species and hence other mutualistic mechanisms were involved. It is possible that the *Actinomyces* species provided a biofilm matrix that encouraged growth of the lactobacilli.

Successful coculture biofilms following failure to establish a substantial monoculture biofilm with human saliva as the growth medium has been reported for other oral bacteria, including *Porphyromonas gingivalis* and *Streptococcus gordonii* DL1 (6). However, in coculture, *S. gordonii* provided coadherence attachment sites that enabled *P. gingivalis* to colonize and accumulate. Species-specific coculture biofilm growth has also been demonstrated with coaggregating species of *A. naeslundii* and *Streptococcus oralis*, which were only able to grow with human saliva as the sole nutrient source when in coculture (16). The other test species, *S. gordonii* DL1, grown in mono- and coculture was not affected by the presence of either of the other species (16). In the present investigation some growth inhibition of *A. naeslundii* by the lactobacilli was observed, which may have been linked to bacteriocins and/or low pH tolerance of organic acids (4, 7).

The ability to produce, tolerate or utilize lactic acid is important in plaque biofilms (4, 26). *A. naeslundii* can metabolize lactate aerobically (4). In this study the biofilm cultures were grown in an anaerobic environment. Coculture with the lactate metabolizing bacterium *V. parvula* produced no increase in biofilm growth of the lactobacilli, hence it is unlikely that lactate removal by *Actinomyces* accounted for biofilm growth promotion of the lactobacilli. Like the lactobacilli, *S. mutans* produces lactate as a major end product of metabolism, but it also promoted biofilm growth of *L. rhamnosus* and *L. plantarum*. These results suggest that lactate dynamics were not involved in the biofilm growth promotion of the *Lactobacillus* species.

In general, lactobacilli are major odontopathogens (12, 24). Our preliminary studies using checkerboard DNA–DNA hybridization analysis, however, suggests that *L. rhamnosus* may be associated with oral health rather than dental caries (23). *L. rhamnosus* GG is also a well known 'pro-biotic' strain and recent evidence suggests that it may promote and maintain oral health in children and adults (1, 14). In the present investigation *L. rhamnosus* was able to establish as a biofilm with both *A. gerencseriae* and *A. naeslundii* the latter having been implicated in root caries (3, 12). Enhanced biofilm growth was also observed for *L. plantarum*, which is implicated in caries (12, 23, 24). The role of lactobacilli in plaque ecology and disease may differ and intraspecies modifications of their prevalence in plaque biofilm may prove important.

This study demonstrates a species-specific biofilm growth promotion of important plaque species. There may be potential for modulating the biofilm prevalence of particular plaque species by targeting partner species. This study also suggests that the ability of individual bacteria to form mono-culture biofilms is not necessarily an indicator of their survival and pathogenic potential in complex multispecies plaque biofilm communities.

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