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Bifidobacteria inhibit the growth of *Porphyromonas gingivalis* but not of *Streptococcus mutans* in an in vitro biofilm model

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There is growing interest in the use of probiotic bifidobacteria for enhancement of the therapy, and in the prevention, of oral microbial diseases. However, the results of clinical studies assessing the effects of bifidobacteria on the oral microbiota are controversial, and the mechanisms of actions of probiotics in the oral cavity remain largely unknown. In addition, very little is known about the role of commensal bifidobacteria in oral health. Our aim was to study the integration of the probiotic *Bifidobacterium animalis* subsp. *lactis* Bb12 and of oral *Bifidobacterium dentium* and *Bifidobacterium longum* isolates in supragingival and subgingival biofilm models and their effects on other bacteria in biofilms in vitro using two different in vitro biofilms and agar-overlay assays. All bifidobacteria integrated well into the subgingival biofilms composed of *Porphyromonas gingivalis, Actinomyces naeslundii,* and *Fusobacterium nucleatum* and decreased significantly only the number of *P. gingivalis* in the biofilms. The integration of bifidobacteria into the supragingival biofilms containing *Streptococcus mutans* and *A. naeslundii* was less efficient, and bifidobacteria did not affect the number of *S. mutans* in biofilms. Therefore, our results suggest that bifidobacteria may have a positive effect on subgingival biofilm and thereby potential in enhancing gingival health; however, their effect on supragingival biofilm may be limited.

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The oral cavity is inhabited by hundreds of bacterial species. Most are commensal and are required to maintain a healthy equilibrium in the oral microbial ecosystem (1). Interference with this microbial equilibrium may lead to an ecological shift, which may result in clinical manifestations, such as dental caries, gingivitis, or periodontitis (2, 3). Although these diseases are polymicrobial, specific species of bacteria, such as mutans streptococci (MS) in dental caries (4, 5) and bacteria of the 'red complex' (e.g. *Porphyromonas gingivalis*) in periodontitis, are associated with their development (6, 7).

There is a constantly growing interest in preventing or enhancing treatment of oral microbial diseases by introducing beneficial bacteria, such as probiotics, into oral biofilms and thereby modulating the oral microbiota. Probiotics are defined as 'Live micro-organisms which when administered in adequate amounts confer a health benefit to the host' (8). The most common probiotic bacterial strains belong to the genera *Lactobacillus* and *Bifidobacterium*. The safe use of these bacteria is supported by their long history of consumption in fermented milk products and growing knowledge of their taxonomy and physiology (9). The numerous mechanisms of actions proposed for probiotics seem to be similar in the oral cavity and in the gut. These actions include competition with pathogens for adhesion sites on host tissues, modulation of the host's immune system, inhibition of the growth of pathogens through the production of bacteriocins or other products, such as acids, as well as modulation of the pathogenic potential of the biofilm (10, 11). The probiotic effects are strain dependent (12, 13).

From a cariological point of view, studies on probiotic bifidobacteria have mainly focused on their potential antagonistic effect on MS and lactobacilli (14). Several studies indicate that short-term supplementation with probiotic products leads to a decrease in the salivary MS counts but has no effect on the number of lactobacilli. The effect has been demonstrated for bifidobacteria used alone and in combination with probiotic lactobacilli (15–18). On the other hand, after an intervention with *Bifidobacterium animalis* subsp. *lactis* Bb12 (Bb12) and *Lactobacillus rhamnosus* GG (LGG),

which are well-characterized probiotics for general health, no difference in the salivary MS counts was observed (19), and long-term consumption of probiotic Bb12 did not affect the caries incidence or MS counts in children (20).

Probiotics have also been proposed as a therapy option for gingivitis and periodontitis, but studies on the effects of probiotic bifidobacteria on periodontal health are scarce. To our knowledge, there is only one clinical study on the effects of probiotic bifidobacteria on gingival health. In that study, the combination of Bb12 and LGG resulted in a decrease of gingival and plaque indices, but had no effect on salivary microbial composition (19). Probiotic bifidobacteria may still have antimicrobial activity against the periodontal pathogens *P. gingivalis* and *Fusobacterium nucleatum* (21).

Bifidobacteria are generally regarded as commensal and they exert numerous positive effects on human health (22). Still, the role of commensal bifidobacteria in oral health is controversial. On the one hand, they have been proposed as associated with the caries process in children, adults, and the elderly (23–25). In addition, bifidobacteria were found only at the sites of active caries when the microbial ecology of the occlusal caries lesions was studied (26). *Bifidobacterium dentium* and *Bifidobacterium longum*, found in caries lesions together with *Streptococcus mutans*, have good resistance to acidic conditions (27), and a genome analysis of *B. dentium* indicates genetic adaptation to the oral cavity (28). On the other hand, oral bifidobacteria have been associated with periodontal health (29).

To summarize, clinical studies have shown some beneficial effects of probiotics on oral health, but the mechanisms are still largely unknown. In addition, whether the observed effects are specific for the probiotic strains or are more general is largely unknown. Our aim was to study the integration of probiotic Bb12 and oral *B. dentium* and *B. longum* isolates in cariogenic and subgingival biofilm models, as well as their effects on other bacteria in the biofilms in vitro. The methods included two different biofilm models and agar-overlay assays.

Material and methods

The bacterial strains used in the biofilm studies and in the agar-overlay assay included *S. mutans* MT8148, *S. mutans* Ingbritt, *P. gingivalis* ATCC 33277, *F. nucleatum* ATCC 10953, *Actinomyces naeslundii* ATCC 12104, Bb12, as well as nine independent oral *Bifidobacterium* isolates listed in Table 1. The origin and isolation procedure of the *Bifidobacterium* isolates has previously been described in detail (29, 30).

Streptococcus mutans MT8148 and *S. mutans* Ingbritt were grown on Mitis Salivarius Agar (Difco; Becton Dickinson, Sparks, MD, USA or Le Pont de Claix, France), in biofilm assays, and on Brain Heart Infusion (BHI; Becton Dickinson, Sparks, MD, USA) medium, supplemented with 13.5 g of agar l^{-1}, in agar-overlay assays. Blood agar containing 5% sheep blood (Difco) was used for culture of *A. naeslundii*, and Brucella agar (Difco) was used for the preculture of *F. nucleatum* and *P. gingivalis*. The number of colony-forming units (CFUs) of *P. gingivalis* in biofilms was counted after growth on Tryptic Soy Agar (Difco) containing yeast extract (Scharlau, Barcelona, Spain), hemin, and vitamin K (Sigma-Aldrich, St Louis, MO, USA), and the number of CFUs of *F. nucleatum* was

Table 1

Number of bifidobacteria, Fusobacterium nucleatum, *and* Actinomyces naeslundii *in the subgingival* biofilm model after 18 or 42 h of incubation with oral* Bifidobacterium *isolates or* Bifidobacterium animalis *sp.* lactis *Bb12*

	Bifidobacteria				*F. nucleatum* ATCC 10953				*A. naeslundii* ATCC 12104			
	18 h		42 h		18 h		42 h		18 h		42 h	
		$P^{\dagger}$		$P^{\dagger}$		$P^{\ddagger}$		$P^{\ddagger}$		$P^{\ddagger}$		$P^{\ddagger}$
B. dentium group	5.8 ± 1.2	0.017	6.5 ± 0.4	<0.001	6.0 ± 0.4	0.123	5.1 ± 1.0	<0.001	6.1 ± 0.6	0.591	6.0 ± 2.1	0.001
B.d. AJ 32-1	5.9 ± 0.3	0.313	6.1 ± 0.2	<0.001	5.6 ± 0.4	0.270	4.8 ± 0.4	<0.001	5.5 ± 0.4	0.433	6.6 ± 0.0	0.885
B.d. AJ 47-1	6.9 ± 0.3	0.005	6.1 ± 0.9	0.050	6.3 ± 0.1	1.000	4.9 ± 0.5	<0.001	6.2 ± 0.0	1.000	6.6 ± 0.2	0.904
B.d. NH 4-1	3.8 ± 1.2	<0.001	6.8 ± 0.4	0.063	5.6 ± 0.5	0.274	6.5 ± 1.4	0.727	5.5 ± 0.7	0.347	6.3 ± 0.1	0.668
B.d. NH 6-1	6.5 ± 0.1	1.000	6.4 ± 0.4	0.003	6.3 ± 0.1	1.000	4.4 ± 0.7	<0.001	6.5 ± 0.8	1.000	3.6 ± 5.1	<0.001
B.d. RC-12	6.1 ± 0.0	0.769	6.2 ± 0.7	<0.001	6.2 ± 0.2	0.999	4.8 ± 0.4	<0.001	6.6 ± 0.4	0.999	6.9 ± 0.2	0.998
B. longum group	4.9 ± 0.4	<0.001	5.9 ± 0.7	<0.001	5.3 ± 0.6	<0.001	5.3 ± 1.4	<0.001	6.2 ± 0.6	0.991	7.2 ± 0.3	0.949
B.l. MU 57-1	4.6 ± 0.0	<0.001	5.4 ± 0.6	<0.001	4.9 ± 0.1	0.003	4.2 ± 0.7	<0.001	6.2 ± 0.1	1.000	6.8 ± 0.0	0.983
B.l. MU 86-7	5.0 ± 0.1	0.001	5.7 ± 0.2	<0.001	4.8 ± 0.1	0.001	4.3 ± 0.9	<0.001	5.9 ± 0.9	0.981	7.4 ± 0.2	1.000
B.l. MU 92-2	5.4 ± 0.0	0.010	6.7 ± 0.4	0.122	6.0 ± 0.1	0.948	5.5 ± 1.4	0.002	6.9 ± 0.4	0.810	7.2 ± 0.0	1.000
B.l. MU 93-4	4.5 ± 0.3	<0.001	5.7 ± 0.4	<0.001	5.7 ± 0.5	0.517	7.1 ± 0.2	1.000	6.0 ± 0.0	0.998	7.2 ± 0.2	1.000
Bb12	6.5 ± 0.2		7.6 ± 0.1		5.7 ± 0.6	0.024	6.8 ± 0.4	0.756	5.9 ± 0.3	0.633	7.3 ± 0.3	1.000
Control	–		–		6.4 ± 0.6		7.2 ± 0.5		6.3 ± 0.6		7.3 ± 0.3	

Data were calculated in log (CFU+1) and are given as mean ± SD log (CFU+1). CFU, colony-forming units.
*For the number of *P. gingivalis*, see Fig. 1 and the text.
†Compared with Bb12 biofilm (Dunnett's two-sided *t*-test). There were significantly more bifidobacteria in the *B. dentium* biofilms than in the *B. longum* biofilms at the 18-h time point ($P = 0.046$) but no difference at the 42-h time point ($P = 0.187$) (Student's *t*-test).
‡Compared with the control (Dunnett's two-sided *t*-test).

counted after growth on Brucella agar (Difco) containing 4 mg l^{-1} of vancomycin (Sigma-Aldrich). In the agar-overlay assays, blood agar was used for culture of *P. gingivalis*. Bifidobacteria were grown on deMan Rogosa and Sharpe agar (MRS; Difco). In the biofilm studies with Bb12, the plates were supplemented with 0.05% (wt vol^{-1}) cysteine hydrochloride and 5 mg ml^{-1} of mupirocin (Merck, Darmstadt, Germany).

Fluid universal medium (FUM; 32), or FUM supplemented with 3% (wt vol^{-1}) sucrose (S-FUM; 33), were used for all cultures in liquid phase and in biofilm assays. All incubations were done in anaerobic atmosphere (90% N_2, 5% CO_2, and 5% H_2) at 37°C.

Saliva preparations

Paraffin wax-stimulated whole saliva was collected from several healthy volunteers at least 1 h after eating, drinking, or tooth cleaning. Saliva was collected in sterile polypropylene tubes on ice, pooled, centrifuged, and pasteurized as described earlier (31). The efficacy of the pasteurization was assessed by plating processed saliva onto blood agar containing 5% sheep blood (Difco). The saliva samples were stored in 2-ml aliquots at −20°C.

Biofilm assays

Two different biofilm models, both modified from the 'Zurich biofilm model' (32), were used. The supragingival cariogenic biofilms included *S. mutans* (MT8148 or Ingbritt), *A. naeslundii,* and one of the *Bifidobacterium* strains listed in Table 2 and the subgingival biofilms included *P. gingivalis, F. nucleatum, A. naeslundii,* and one of the *Bifidobacterium* strains listed in Table 1. Control biofilms were prepared without bifidobacteria.

The biofilms were prepared as described by Marttinen *et al*. (31). Briefly, the stock cultures were plated on agar medium and grown first for 3–5 d. Loopfuls (10 μl) of plate-grown bacteria were inoculated into separate tubes each containing 5 ml of FUM each and precultures were incubated for 15 h. Seed cultures were prepared by adding 400-μl aliquots of preculture into 1,600 μl of FUM (all bacterial species except *F. nucleatum*) and 2.5 ml of preculture into 5 ml of FUM (*F. nucleatum*). For the supragingival cariogenic biofilm model (containing *S. mutans*, *A. naeslundii*, and bifidobacteria), S-FUM was used as culture medium. Seed cultures were grown for 5 h, to early logarithmic phase, washed once with 8 ml of PBS, and resuspended in 3 ml of FUM or S-FUM. Bacterial suspensions were adjusted independently to approximately 10^8 CFU ml^{-1} by dilution with fresh FUM or S-FUM [to give an optical density at 550 nm (OD_{550}) of: 0.1 (±0.05) for *S. mutans*; 0.5 (±0.05) for *A. naeslundii*, *P. gingivalis*, and bifidobacteria; and 1.0 (±0.05) for *F. nucleatum*]. Adjusted suspensions were stored on ice until the biofilms were prepared.

Biofilms were prepared on circular hydroxyapatite (HA) discs (Dense Hydroxyapatite Discs, 5 mm × 2 mm; Clarkson Chromatography Products, South Williamsport, PA, USA), which were sterilized and refreshed as described earlier (30, 33). First of all, the number of needed HA discs were placed in the same 10-ml Falcon tube containing 2 ml of saliva preparation (see above) and 2 ml of PBS, and were mixed for 30 min to allow the salivary pellicle to form. Then, the HA discs were washed with PBS and placed each in a separate well of a sterile 48-well polystyrene cell-culture plate (Greiner Bio-One, Frickenhausen, Germany) and covered with 250 μl of saliva and 250 μl of the mixture of bacterial suspensions containing equal amounts of each bacteria or FUM, as follows: for the supragingival cariogenic biofilm model, the bacterial suspension contained one of the *S. mutans* strains, *A. naeslundii*, and one of the *Bifidobacterium* strains (62 μl of each and 62 μl of S-FUM); and for the subgingival biofilm model, the bacterial suspension contained *P. gingivalis*, *A. naeslundii, F. nucleatum*, and one of the *Bifidobacterium* strains (62 μl of each). In the control biofilms, the *Bifidobacterium* suspension was replaced with 63 μl of FUM or S-FUM.

After 18 h of incubation, the biofilm on the top of the HA disc and a sample of the surrounding medium, representative of 18-h biofilms, were collected as described by Marttinen *et al*. (31), and the HA discs to provide 42-h biofilms were transferred into a new well containing 250 μl of saliva and 250 μl of FUM or S-FUM, as appropriate, and incubated for an additional 24 h, after which the samples (biofilm and surrounding medium) were collected. The

Table 2

Number of bifidobacteria, Streptococcus mutans *MT8148, and* Actinomyces naeslundii *in the cariogenic biofilm model containing oral* Bifidobacterium dentium *isolates or* Bifidobacterium animalis *sp.* lactis *Bb12*

	Bifidobacteria*				*S. mutans* MT8148†				*A. naeslundii* ATCC 12104			
	18 h		42 h		18 h		42 h		18 h		42 h	
		P‡		P‡		P§		P§		P§		P§
B. dentium group	2.7 ± 2.1	0.064	4.3 ± 2.0	0.062	5.9 ± 0.5	0.927	7.1 ± 0.6	0.581	4.5 ± 1.8	0.005	1.9 ± 3.0	0.989
B.d. AJ 32-1	5.3 ± 0.2	0.978	6.4 ± 0.2	1.000	6.2 ± 0.1	1.000	7.7 ± 0.0	0.349	6.0 ± 0.1	0.082	3.0 ± 4.2	0.980
B.d. AJ 47-1	0.0 ± 0.0	0.001	3.0 ± 0.4	0.024	6.2 ± 0.0	0.996	7.5 ± 0.2	0.706	4.1 ± 0.2	0.541	6.4 ± 0.2	0.103
B.d. NH 4-1	1.5 ± 2.1	0.008	1.9 ± 2.7	0.005	6.1 ± 0.5	1.000	6.7 ± 0.7	0.912	2.5 ± 3.6	0.984	0.0 ± 0.0	0.912
B.d. NH 6-1	3.3 ± 0.1	0.259	6.0 ± 0	0.986	5.7 ± 0.2	0.969	6.3 ± 0.5	0.912	5.7 ± 1.6	0.115	0.0 ± 0.0	0.912
B.d. RC-12	3.5 ± 0.7	0.315	3.9 ± 0.4	0.097	5.5 ± 1.2	0.757	7.3 ± 0.2	0.912	4.3 ± 0.4	0.482	0.0 ± 0.0	0.912
Bb12	4.9 ± 0.7		6.5 ± 0.7		5.8 ± 1.2	0.819	6.6 ± 0.8	0.684	3.3 ± 2.3	0.256	0.0 ± 0.0	0.466
Control	–		–		6.0 ± 0.3		6.9 ± 0.6		2.4 ± 0.6		2.9 ± 0.8	

Data were calculated in log (CFU+1) and are given as mean ± SD log (CFU+1). CFU, colony-forming units.
**B. longum* isolates did not integrate into the biofilm.
†None of the bifidobacteria survived in the biofilm containing *S. mutans* Ingbritt.
‡Compared with Bb12 biofilm [Student's two-sided *t*-test (*B. dentium* group)] or Dunnett's two-sided *t*-test (strain level comparisons)].
§Compared with the control (Dunnett's two-sided *t*-test).

samples were serially diluted and cultured on the corresponding agar plates, and the colonies were counted after 2-3 d (*S. mutans* and bifidobacteria) or 7 d (*A. naeslundii, F. nucleatum*, and *P. gingivalis*) of incubation. pH was measured in the culture medium after sample collection.

Agar-overlay interference assays

Based on the results of the biofilm assays, the ability of *S. mutans* to inhibit the growth of bifidobacteria, and the ability of bifidobacteria to inhibit the growth of *P. gingivalis*, were studied using agar-overlay assays. The assays were performed as described by HASSLÖF *et al.* (34), with the following modifications. Bacteria from frozen stocks were plated on blood (*S. mutans* and *P. gingivalis*) or MRS (bifidobacteria) agar and the plate-grown bacteria were inoculated into 5 ml of FUM and incubated overnight. Then, the bacteria were washed once with 5 ml of PBS and bacterial suspensions corresponding to approximately 10^9, 10^7, and 10^5 CFUs ml^{-1} were prepared in FUM. On the first day, bifidobacteria were suspended in molten (approximately 45°C) sterile MRS agar and *S. mutans* (MT8148 or Ingbritt) were suspended in molten (approximately 45°C) sterile BHI agar and the plates were poured. After overnight incubation, a second layer of either MRS agar (for bifidobacteria) or blood agar (for *P. gingivalis*) was poured on top of the agar containing *S. mutans* or bifidobacteria. A 50-μl aliquot of bacterial suspension (*Bifidobacterium* or *P. gingivalis*), containing approximately 10^8 CFUs ml^{-1}, was plated on the top agar and plates were incubated for 2–3 d (*S. mutans* + *Bifidobacterium)* or 5–7 d (*Bifidobacterium* + *P. gingivalis)* in an anaerobic atmosphere.

Statistical analysis

Subgingival biofilm experiments with the probiotic Bb12 were performed four times with two independent biofilms, and other biofilm experiments were run twice. The results of the biofilm assays are reported as the mean values and SD of either $\log_{10}$ (CFU+1) or the end pH of the biofilm medium. To compare the differences between control biofilms and biofilms containing bifidobacteria, the data were first of all subjected to one-way ANOVA, and subsequent pairwise comparisons (either each *Bifidobacterium* strain separately or groups according to the species compared with controls, as well as the comparisons between oral *Bifidobacterium* isolates and Bb12) were made using Dunnett's two-sided *t*-test. The comparisons between 18- and 42-h biofilms, and between *B. dentium* and *B. longum* groups, were carried out using the Student's *t*-test for independent samples. The results of the agar-overlay tests were categorized as: complete inhibition (score 1); slight inhibition (at least one visible colony but definitely smaller amounts than in the control plate, score 2); and no inhibition (score 3). All statistical analyses were performed using IBM SPSS statistics version 22.0 for Windows (SPSS, Chicago, IL, USA) and the level of statistical significance was set at $P < 0.05$.

Results

Subgingival in vitro biofilm

Probiotic Bb12 and all nine oral *Bifidobacterium* isolates integrated into the biofilms with *A. naeslundii, F. nucleatum*, and *P. gingivalis* (Table 1). At the species level, there were significantly more CFUs of Bb12 than of *B. dentium* or *B. longum* in the biofilms ($P = 0.017$ and $P < 0.001$, respectively), but individual strains behaved differently (Table 1). There was a significant increase in the number of *B. longum* strains and of Bb12 during the incubation (i.e. there were significantly more CFUs in the 42-h biofilms than in the 18-h biofilms), but not in the number of *B. dentium* strains ($P < 0.001$ for Bb12, $P = 0.228$ for the group of *B. dentium* strains, and $P = 0.002$ for the group of *B. longum* strains).

The number of *P. gingivalis* was significantly lower in the biofilms containing Bb12 than in the control biofilms, at both 18- and 42-h time points (Fig. 1). Three oral isolates of *Bifidobacterium* (*B. dentium* NH 4-1, *B. longum* MU 92-2, and *B. longum* MU 93-4) did not have any effect on the level of *P. gingivalis* at the 18-h time point. Other oral species of bifidobacteria inhibited the growth of *P. gingivalis* significantly. In the control biofilms, the mean ± SD log CFU ml^{-1} of *P. gingivalis* was 5.99 ± 0.95. With *B. longum* MU 57-1, the mean ± SD log CFU ml^{-1} of *P. gingivalis* was 2.8 ± 4.2 ($P = 0.001$, compared with the controls), and in all other biofilms containing bifidobacteria the number of *P. gingivalis* was under the detection limit (100 CFU ml^{-1}, $P < 0.001$) at the 18-h time point. At the species level, the mean ± SD log CFU ml^{-1} of *P. gingivalis* was 1.1 ± 2.4 in biofilms containing *B. dentium* ($P < 0.001$) and 3.5 ± 2.9 in biofilms containing *B. longum* ($P = 0.001$, compared with the control). At the 42-h time point, *P. gingivalis* was uncultivable in all biofilms containing oral *Bifidobacterium* isolates.

During the first 18 h of incubation, the number of *F. nucleatum* remained unaffected in biofilms containing any of the *B. dentium* strains and two of the four *B. longum* strains, but decreased in biofilms containing

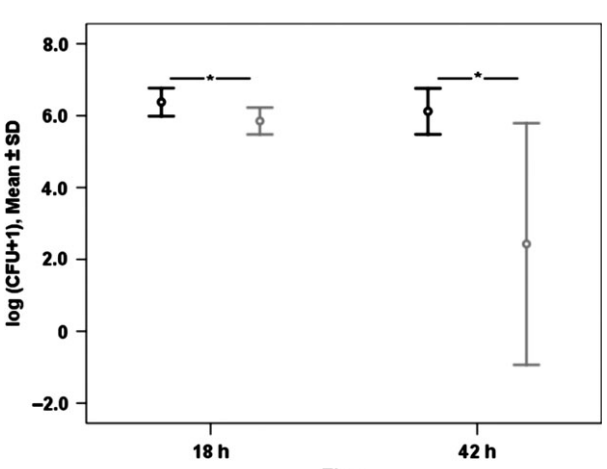

Fig. 1. Effect of *Bifidobacterium animalis* subsp. *lactis* Bb12 on the growth of *Porphyromonas gingivalis* in the subgingival biofilm model. Data represent growth of *P. gingivalis* [presented as mean log (CFU+1) ± SD] in the presence (grey) or absence (black; control) of Bb12. *$P < 0.05$ (Student's *t*-test).

Bb12 or the *B. longum* strains MU 57-1 and MU 86-7 compared with the control biofilms (Table 1). At the 42-h time point, all except two of the oral *Bifidobacterium* isolates (*B. dentinum* NH 4-1 and *B. longum* MU 92-2) resulted in a decreased number of *F. nucleatum* ($P < 0.001$), but in biofilms containing Bb12 the number of *F. nucleatum* was the same as in the controls. In control biofilms, the number of *F. nucleatum* increased significantly between the 18 h and the 42 h time points ($P < 0.001$).

The number of *A. naeslundii* was not affected by any of the bifidobacteria at the 18-h time point, but there were significantly fewer CFUs of *A. naeslundii* in 42-h biofilms containing *B. dentium* compared with the controls ($P = 0.001$, Table 1). The number of *A. naeslundii* increased significantly in control biofilms from 18 to 42 h of incubation ($P < 0.001$).

The pH remained unaffected in biofilms containing Bb12 when compared with the controls (mean pH ± SD: 6.5 ± 0.4 at the 18-h time point and 6.2 ± 0.5 at the 42-h time point), but decreased significantly in the biofilms containing oral *Bifidobacterium* isolates when studied at species level [mean pH ± SD: 6.0 ± 0.4 ($P = 0.022$) for the *B. dentium* group and 5.9 ± 0.5 ($P = 0.004$) for the *B. longum* group at the 18-h time point and 5.7 ± 0.4 in *B. dentium* and 5.6 ± 0.3 in *B. longum* biofilms ($P = 0.001$ for both groups) at the 42-h time point, compared with the control biofilms].

Supragingival cariogenic in vitro biofilm

The integration of bifidobacteria into the biofilms containing *S. mutans* and *A. naeslundii*, and their effects on the supragingival cariogenic biofilm, was studied with *S. mutans* Ingbritt and *S. mutans* MT8148. None of the bifidobacteria studied survived in biofilms containing *S. mutans* Ingbritt, nor did they have any effect on the other bacteria in the biofilms. Probiotic Bb12 and all oral *B. dentium* strains, but none of the *B. longum* isolates, integrated into the biofilm containing *S. mutans* MT8148 and *A. naeslundii* (Table 2). Figure 2 represents the number of CFUs of Bb12 in biofilms containing *A. naeslundii* and two different *S. mutans* strains. After 18 h of incubation, the number of CFUs of one *B. dentium* isolate (AJ 47-1) was below the detection limit (100 CFUs ml^{-1}) and there was a significantly lower number of CFUs of *B. dentium* NH 4-1 than of probiotic Bb12 in the 18-h biofilms (Table 2). An increase in the number of CFUs was observed for most *B. dentium* strains between 18 h and 42 h of incubation, but the number of CFUs of *B. dentium* AJ 47-1 and NH 4-1 was lower than that of Bb12 also in the 48-h biofilms (Table 2).

None of the bifidobacteria affected the growth of *S. mutans* (Table 2). The growth of *A. naeslundii* was poor in control biofilms, and the presence of Bb12 did not affect the numbers of *A. naeslundii*. The presence of *B. dentium* first enhanced the growth of *A. naeslundii* (i.e. there was a significantly higher number of *A. naeslundii* CFUs in biofilms containing *B. dentium*) compared with the control at the 18-h time point

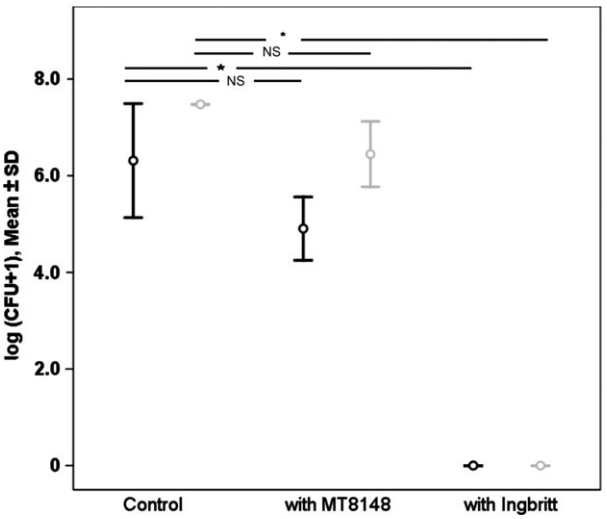

Fig. 2. Number of *Bifidobacterium animalis* subsp. *lactis* Bb12 (Bb12) in biofilms with *Actinomyces naeslundii* (control), with *A. naeslundii* + *Streptococcus mutans* strain MT8148 (with MT8148), or with *A. naeslundii* + *S. mutans* strain Ingbritt (with Ingbritt). Data represent the number of CFUs [expressed as mean log (CFU+1) ± SD] after 18 h (black) and 42 h (grey) of culture. NS, statistically non-significant difference. *$P < 0.001$ (Dunnett's *t*-test).

($P = 0.005$). At 42 h, *A. naeslundii* grew well only in the presence of *B. dentium* 47-1, and grew more slowly in controls and in the presence of *B. dentium* 32-1. In biofilms containing other oral *B. dentium* isolates or Bb12, the number of CFUs of *A. naeslundii* was below the detection limit (100 CFUs ml^{-1}).

The pH varied between 4.1 and 4.6 in control biofilms, between 4.2 and 4.5 in biofilms containing Bb12, and between 4.3 and 4.7 in biofilms containing *B. dentium* isolates; there was no statistically significant difference between the groups. Only at the 42-h time point, in biofilms containing *B. dentium* NH6-1, was the pH slightly higher than in controls (mean difference = 0.3, $P = 0.001$).

Agar-overlay interference assays

Streptococcus mutans strains Ingbritt and MT8148 completely inhibited the growth of all bifidobacteria tested, and all *Bifidobacterium* strains completely inhibited the growth of *P. gingivalis* in the agar-overlay assays, even at the lowest concentration tested.

Discussion

Integration of probiotic bacteria into the oral biofilm, intermicrobial interactions, and colonization in the oral cavity are considered to be of major importance for probiotic actions in the oral cavity (35), although systemic effects are also possible (19, 36, 37). In this study, our aim was to compare the integration of probiotic Bb12 and oral *Bifidobacterium* isolates into the oral

biofilms in vitro and to study their effects on the growth of other oral bacteria.

The integration of oral *B. longum* strains was low when compared with that of the probiotic Bb12 or oral *B. dentium* isolates. This may reflect the origin of the isolates: it has been suggested that the primary source of *B. longum* in the oral cavity might be probiotic foods (29). Studies on bacterial diversity of orally healthy individuals also suggest that bifidobacteria may not be significant members of a healthy oral microbiota (38, 39). On the other hand, in vitro studies suggest that *F. nucleatum* is important for the adherence and biofilm formation of bifidobacteria on hydroxyapatite (40, 41), and the colonization of Bb12 has been poor in clinical studies of children or adults with good oral hygiene (19, 20). In addition, the use of probiotic bifidobacteria has affected the salivary levels of other bacteria in some clinical studies (15, 16), and this suggests oral colonization. Therefore, the role of other bacteria in the microbiota of each individual might be important for the oral colonization of bifidobacteria, and this study supports the idea that the colonization of probiotic bacteria is affected by oral indigenous bacteria.

In our study, the growth of *P. gingivalis* was reduced significantly in biofilms and was completely inhibited in agar-overlay tests with bifidobacteria. In part, this was probably a result of the acidic conditions generated by bifidobacteria, but because some of the *Bifidobacterium* strains did not influence the growth of *P. gingivalis* – and this phenomenon may not be pH-dependent – other reasons are also likely. One reason might be competition for nutrients. The growth inhibition of *P. gingivalis* by oral bifidobacteria may be related to the ability of bifidobacteria to reduce the mutual growth factor vitamin K (42). In the growth-inhibition study performed by Zhu *et al.* 2010, probiotic *Bifidobacterium* inhibited the growth of *P. gingivalis* when inoculated first, but no inhibition was shown when both bacteria were inoculated simultaneously. The inhibition was assumed to be a result of the production of inhibitory substances by probiotic bacteria or competition for nutrients (21). On the other hand, *F. nucleatum* has good resistance to acidic conditions and its ability to neutralize acids may have a role in development of a favourable environment for the periodontal pathogen, *P. gingivalis* (43). In this study, the decreased level of *P. gingivalis* in biofilms containing bifidobacteria may also have been partially a result of the decreased level of *F. nucleatum*.

Bifidobacteria had no effect on *S. mutans* levels in biofilms. In contrast, *S. mutans* Ingbritt inhibited the growth of bifidobacteria, both on agar and in biofilms. This is in accordance with the results from our clinical studies, in which no effects on salivary MS were observed (19, 20), and in cross-sectional clinical studies the number of MS and bifidobacteria in saliva were significant, independent variables associated with caries in children and adults (25, 30). Although in some clinical studies probiotic bifidobacteria have reduced the salivary levels of *S. mutans* (15, 16), the idea of caries control by the use of bifidobacteria might be questionable. Bifidobacteria are present in the advancing front of the caries lesion (26). In addition, while heat-inactivated Bb12 reduced the putative cariogenity of *S. mutans* in vitro, viable Bb12 did not (44). However, data from clinical studies, although limited, does not suggest that the use of probiotic products would have harmful effects on dental health (20, 45–48). In the present study, Bb12 and *B. dentium* strains remained viable at a pH of < 4.61, but *B. longum* did not survive in the biofilms containing *S. mutans*. This may be for reasons other than pH because *B. longum* has previously shown good resistance to acidic conditions (27). One explanation could be the bacteriocins produced by *S. mutans*. For example, *S. mutans* strain C3603, which represents serotype c, such as the strains Ingbritt and MT8148 used in this study, produces a bacteriocin that is very effective against *B. longum* (49).

The idea behind probiotic therapy is the introduction of beneficial bacteria into the biofilm and modulation of its pathogenic potential. Systemic effects are also possible (36, 37), but most likely oral colonization leads to more effective impact (50). In addition, the effect on the adhesion of oral pathogens might be greater than the effect on the biofilm (31), and disruption of the existing oral biofilm may be necessary before the administration of a probiotic (51).

To conclude, our results suggest that the integration of probiotic bifidobacteria into oral biofilms is affected by the oral indigenous bacteria. Bifidobacteria might have an effect on periodontal pathogens in the oral microbiota; however, their effect on cariogenic biofilm seems to be limited.

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