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Prevalence and Adhesion Properties of Oral *Bifidobacterium* species in Caries-active and Caries-free Thai Children

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Abstract

Several publications have reported the association of bifidobacteria with dental caries lesions, but no data for the prevalence or the adhesive properties of oral *Bifidobacterium* spp. have been evaluated in Thai children. The objectives of this study were to compare the prevalence of oral *Bifidobacterium* spp. in caries-active and caries-free Thai children and to characterize the adhesive properties of the predominant bifidobacteria isolated from caries lesions. A total number of 167 strains of oral bifidobacteria were isolated from 50 caries-active and 50 caries-free subjects and identified by molecular biology techniques. The selected bifidobacteria from both groups were examined for adhesive ability, surface properties, and biofilm formation. The prevalence of oral bifidobacteria in caries-active children (48 %) (24/50) was significantly higher than in the caries-free group (24 %) (12/50) ($p < 0.05$), with total counts of 5.8±0.9 Log CFU/ml and 2.7±0.8 log CFU/ml, respectively. The predominant species of bifidobacteria were *B. dentium* (82.9 %) (102/123), *B. breve* (11.4 %) (14/123), and *B. longum* (5.7 %) (7/123) for the caries-active group, and *B. dentium* 100 % (44/44) for the caries-free group. All strains of bifidobacteria were able to adhere to keratinocyte cell line *in vitro*. The adherent strains of *B. dentium* showed higher total adhesive ability in caries-active subjects (66 %) than in the caries-free group (58 %). *B. dentium* showed strain variations in the cell surface characteristics of hydrophobic and hydrophilic surface charges. The strains of *B. dentium* from both groups were able to form biofilm. In conclusion, the predominant strains of *B. dentium* had high adhesive ability with keratinocytes and a biofilm forming capacity, implying a role in the colonization of oral mucosa.

Keywords: Prevalence, adhesion, biofilm, bifidobacteria, dental caries

Introduction

Dental caries is caused by the bacterial acid demineralization of teeth. In addition to *Streptococcus mutans* and *Streptococcus sobrinus*, bifidobacteria are also recognized as acidogenic and aciduric to be able to proliferate in a cariogenic environment [1-3]. The *Bifidobacterium* species is anaerobic, gram-positive rodded, pleomorphic branched, non-motile, non-spore-forming, and possesses fructose-6-phosphate phosphoketolase (F6PPK) to produce lactic acid as well as acetic acid as end products of glucose metabolism [4]. Bifidobacteria in the oral cavity are limited to the genera of *Bifidobacterium*, *Scardovia*, *Parascardovia,* and *Alloscardovia* [5,6]. Recently, Mantzourani *et al*. [7] reported that bifidobacterial species predominating in active occlusal lesions from 87 % of adults and 67 % of children were *Bifidobacterium dentium, Parascardovia denticolens, Scardovia inopicata, Bifidobacterium longum, Scardovia genomosp.* C1, and *Bifidobacterium breve,* whereas no bifidobacteria were detected in supra-

or subgingival plaques from clinically healthy teeth [8]. Other studies in England reported the isolation of bifidobacteria from the saliva of 95 % of caries-active, and from only 9 % of caries-free, children [3], and 96.8 % of bifidobacteria found in the saliva of caries-active older adults [1]. However, information on the prevalence of oral bifidobacteria in Thai children has not yet been evaluated.

Caries-associated bifidobacteria may be incorporated into dental plaques, where acids are responsible for the caries process [9]. Haukioja *et al*. [10] studied the adhesion of bifidobacterial strains (*B. breve*, *B. longum, B. lactis*, and *B. adolescentis*) to human saliva coated hydroxyapatite, but the binding ability was less than 5 %. The adhesive ability of oral bifidobacteria, and the capacity of biofilm formation on the surfaces of teeth coated with saliva, food debris, and bacterial consortia, would be interesting for further investigation. Information on the adhesive property of the oral bifidobacteria and the cell surface charges of hydrophobic and hydrophilic characteristics, reflecting on the colonizing ability of the oral strains, are still limited. It is postulated that the oral bifidobacteria have an ability to adhere to oral epithelial cells for its purpose to maintain in oral cavity. The objectives of this study were to evaluate the prevalence of bifidobacterial strains in Thai children in high risk caries-active and caries-free groups, and to investigate the adhesive ability with keratinocyte cell line *in vitro* and biofilm formation, as well as assessment of hydrophobic and hydrophilic surface charges of the oral bifidobacteria.

Materials and methods

Subjects and clinical examination

Examination of the dental caries status of subjects was performed by dentists using a WHO probe (#621) and mouth mirror under unit light. A scoring system was adapted from the WHO criteria, 1997 [11] . The dental status of each examined set of teeth was categorized as: S = Sound surface, D = Dental caries with cavitated lesions.

A total of 100 children (aged 6 to 9 years old) from the Paediatric dental clinic, Faculty of Dentistry, Prince of Songkla University, Thailand, were included in this study. A total of 100 pool supragingival plaque samples were used for analysis: 50 samples collected from the sound teeth of 50 caries-free children, and 50 samples collected from the carious lesions of 50 caries-active children. The study protocol was approved by the Ethics Committee of the Faculty of Dentistry, Prince of Songkla University.

Bacterial sampling

The plaque samples were collected using a curette and were immediately suspended in 200 µl of reducing transport fluid (RTF). Ten-fold dilution series of each sample was made in phosphate buffer saline (PBS) with 0.05 % L-cysteine hydrochloride (as a reducing agent), and 0.1 ml of the diluted sample was spread on Beerens agar plate. After 2 to 7 days of incubation at 37 °C under anaerobic conditions (10 % H_2, 10 % CO_2 and 80 % N_2), the number of bifidobacteria-like colonies were counted as colony forming units per milliliter (CFU/ml). Then, 2 - 5 colonies, either of the same or different colonial appearance, were collected and were initially identified as bifidobacteria based on being gram-positive, having pleomorphic rods, being catalase negative, and the presence of the key enzyme fructose-6-phosphate phosphoketolase (F6PPK) from the glucose catabolic pathway, as described by Scardovi [12]. After culture purification, all isolates were kept at −80 °C until use.

Identification of *Bifidobacterium* spp. using 16S rRNA genes PCR-RFLP

A total number of 167 strains of oral bifidobacteria were isolated from 50 caries-active and 50 caries-free subjects and were identified to species levels by restriction fragment length polymorphism analysis of PCR-amplified 16S rRNA genes (16S rRNA genes PCR-RFLP), according to the method of Teanpaisan & Dahlen [13]. Briefly, the 16S rRNA genes were amplified by PCR using the universal primers 8UA (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGGTACCTTGTTACGACTT-3'). A 50 µl PCR reaction mixture contained 100 ng of DNA template, 1.0 µM of each primer, 5 µl of 10x Buffer with 2.0 mM $MgCl_2$, 1.0 U of Taq DNA polymerase, and 0.2 mM of each dNTP. Amplification proceeded using a GeneAmp PCR System 2400 (Applied Biosystems, Foster, CA). Initial heat

denaturation at 95 °C for 15 min was followed by annealing at 50 °C for 2 min and a primer extension at 72 °C for 1.5 min. Subsequent cycles of denaturation were at 94 °C for 1 min; after 35 such cycles, the reaction was stopped at 72 °C for 10 min. The PCR products of 16S rRNA genes were individually digested with *HpaII* (New England Biolab, Ipswich, MA) according to the manufacturer's instructions. Digestion products were separated by 7.5 % polyacrylamide and stained with silver staining. The discrimination of uncertain strains was confirmed by denaturing gradient gel electrophoresis (DGGE) and 16S rRNA genes by DNA sequencing. The following reference panel strains were used for comparative identification: *B. longum* CCUG 28903, *B. breve* CCUG 30511A, *B. dentium* CCUG 18367, *Bifidobacterium scardovii* CCUG 13008A, *Alloscardovia omnicolens* CCUG 31649, and *S. inopinata* CCUG 35729.

Adhesion assay

The H357 keratinocyte from the oral squamous carcinoma cell line used in this study was a gift from Professor Paul Speight of the University of Sheffield, UK. The keratinocytes were grown in flasks and maintained in medium containing 3 parts Dulbecco's modified Eagle's medium (DMEM) plus 1 part Ham's F-12 nutrient mixture, supplemented with 10 % fetal calf serum, epidermal growth factor (10 ng/ml), hydrocortisone (0.5 g/ml), penicillin (100 U/ml), streptomycin (100 g/ml), and amphotericin B (2.5 g/ml). The cells were harvested by trypsinization with 0.25 % trypsin - 0.05 % EDTA at 37 °C for 10 to 15 min and collected by centrifugation. The keratinocytes were subcultured in 24-well plates at approximately 10^5 cells/well, and were grown at 37 °C in 5 % CO_2 to confluence over 2 days.

The adhesion assay was performed on fixed keratinocytes by a modification of the methods described by Kintarak *et al*. [14]. Each selected *Bifidobacterium* strain was grown anaerobically overnight in 10 ml MRS broth with 0.05 % L-cysteine hydrochloride at 37 °C under anaerobic conditions (10 % H_2, 10 % CO_2 and 80 % N_2). The bacterial cells were harvested and washed twice with phosphate buffered saline containing 0.05 % L-cysteine hydrochloride. A bacterial inoculum containing approximately 10^8 CFU/ml of each bifidobacteria strain suspended in DMEM was added to each well and incubated at 37 °C in 5 % CO_2 for 1 h. Non adherent bacteria were washed off, and the adherent bacteria plus intracellular bacteria were quantified as the adhesion.

To quantitate internalization, 1 ml of a solution, containing 100 µg/ml of ampicillin in DMEM, was added to each well to kill extracellular bacteria. The plates were incubated with ampicillin for 1 h at 37 °C in a 5 % CO_2 and then washed twice with PBS. To determine the number of bacteria, the keratinocytes were trypsinized with 0.05 % trypsin-EDTA and lysed with 0.1 % Triton X-100, and serial dilutions were plated onto MRS agar to determine the viable bacterial counts. Data were expressed as Log CFU/ml.

Total adhesion or internalization was reported as a percentage from duplicates according to the formula for total adhesion or internalization, as follows: (%) = $(N/N_0) \times 100$, where N_0 and N are $\log_{10}$ number of bacterial cells (CFU/ml) before and after total adhesion or internalization. Adhesion (externalization) was calculated as total adhesion minus internalization.

Bacterial adhesion to solvents

The microbial adhesion to solvents (MATS) test was performed according to the methods of Rosenberg *et al.* [15] with some modifications. The adhesion of bacteria to different hydrocarbon solutions, including xylene (nonpolar neutral solvent), chloroform (polar acidic solvent), and ethyl acetate (polar basic solvent), were measured. Bacterial cells suspended in PBS (pH 7.0) containing 0.05 % L-cysteine hydrochloride were adjusted to A_{600} of 0.5 (approximately 10^8 CFU/ml cell density). A volume of 3 ml bacterial suspension was mixed with 1 ml of hydrocarbon solution by vortexing for 60 s and after allowing the phases to separate for 30 min of incubation at room temperature, the absorbance of the aqueous phase was measured at 600 nm. The results were reported as a percentage from triplicates, according to the formula MATS (%) = $1 - (A_t / A_0) \times 100$, where A_t represents the absorbance at time t = 30 min and A_0 the absorbance at t = 0. The bifidobacteria were classified into 3 groups: those with low hydrophobicity (0 - 35 %), moderate hydrophobicity (36 - 70 %), and high hydrophobicity (71 - 100 %).

Biofilm assay

Biofilm formation was examined in 96-well flat bottom plates, as previously described [16]. The selected 2-strains of high adhesive property of *B. dentium* from caries-active and caries-free subjects were used for biofilm assay, as well as a reference strain of *B. dentium* CCUG 18367. Fresh bacterial suspensions were prepared in BHI broth from overnight cultures and adjusted to A_{600} of 0.5 (approximately 10^8 CFU/ml cell density). Aliquots of 200 µl bacterial suspension were inoculated into individual wells of a 96-well flat-bottomed polystyrene plate and incubated overnight at 37 °C for 24 h. Following overnight incubation, plates were gently washed with phosphate buffered saline (PBS; pH 7.0) and air-dried for 1 h. Biofilms were stained with 200 µl crystal violet for 30 min, the wells washed gently to remove the crystal violet, and the plates air-dried. After the biofilms had been visually analyzed and imaged using a flatbed scanner, the crystal violet was solubilized with 100 µl of 33 % (v/v) acetic acid per well. For quantitative results, the A_{600} of the solubilized crystal violet was measured, using the 96-well plate reader.

Statistical analysis

Data were expressed as mean ± standard deviation (SD). The Chi-square test was used to assess the difference of the prevalence of each studied group. The distribution of *Bifidobacterium* spp. was calculated as a percentage. The comparative differences of adhesion properties and surface charges between caries-active and caries-free groups were evaluated using the Mann-Whitney U-test. All analyses were performed with the Statistical Package for Social Sciences version 17.0 (SPSS Inc., Chicago, IL, USA) software package. The differences were considered significant when $p < 0.05$.

Results and discussion

Prevalence of oral *Bifidobacterium* spp. in caries-active and caries-free subjects

The prevalence of bifidobacteria were 48 % (24/50) from caries-active children, which was significantly higher than the 24 % (12/50) from the caries-free group ($p < 0.05$), as shown in **Table 1**. The quantity of bifidobacteria in caries-active subjects exhibited a bacterial count of 5.8 ± 0.9 Log CFU/ml, whereas the lower number of bacteria found in the caries-free group was 2.7 ± 0.8 Log CFU/ml. The most frequently found species of bifidobacteria were *B. dentium* (82.9 %) (102/123), *B. breve* (11.4 %) (14/123), and *B. longum* (5.7 %) (7/123) for caries-active subjects, and *B. dentium* (100 %) (44/44) for the caries-free group (**Table 2**).

Table 1 Prevalence of oral *Bifidobacterium* spp. in caries-active and caries-free groups.

Characteristic	No. of Subjects	Prevalence, N (%)	Total count (Log CFU/ml)
Caries free	50	12 (24)	2.70 ± 0.79
Caries active	50	24[1] (48)	5.77[2] ± 0.88

[1]Chi-square test indicates a statistical significance ($p < 0.05$) in the prevalence of bifidobacteria from caries-active and caries-free groups.
[2]Mann-Whitney U Test indicates a statistical significance ($p < 0.05$) in the total count from caries-active and caries-free groups.

Table 2 Distribution of oral *Bifidobacterium* spp. in caries-active and caries-free groups.

Species	All subjects, N = 36		Caries-free group, N = 12		Caries-active group, N = 24	
	No. of subjects (%)	No. of isolates (%)	No. of subjects (%)	No. of isolates (%)	No. of Subjects (%)	No. of isolates (%)
B. dentium	34 (94.4)	146 (87.4)	12 (100)	44 (100)	22[1] (95.5)	102 (82.9)
B. breve	5 (13.9)	14 (8.4)	ND	ND	5 (22.7)	14 (11.4)
B. longum	2 (5.6)	7 (4.2)	ND	ND	2 (9.1)	7 (5.7)

[1]Chi-square test indicates a statistical significance ($p < 0.05$) in *B. dentium* from caries-active and caries-free groups. ND = Not detected.

Adhesion abilities of oral *Bifidobacterium* spp. to keratinocyte cells

The adhesion rates of the isolated oral bifidobacteria to keratinocytes are shown in **Figure 1**. All isolated bifidobacteria were able to adhere to culture cells. There were strain variations in the adhesive properties of total adhesion, externalization, and internalization abilities. The adherent strains expressed as percentages of total adhesion, externalization, and internalization are per the following: *B. dentium* (76, 12 and 64 %), *B. breve* (60, 6 and 55 %), and *B. longum* (55, 4 and 52 %) from caries-active subjects, respectively (**Figure 1(a)**), while *B. dentium* from the caries-free group showed 66, 15 and 50 % (**Figure 1(b)**).

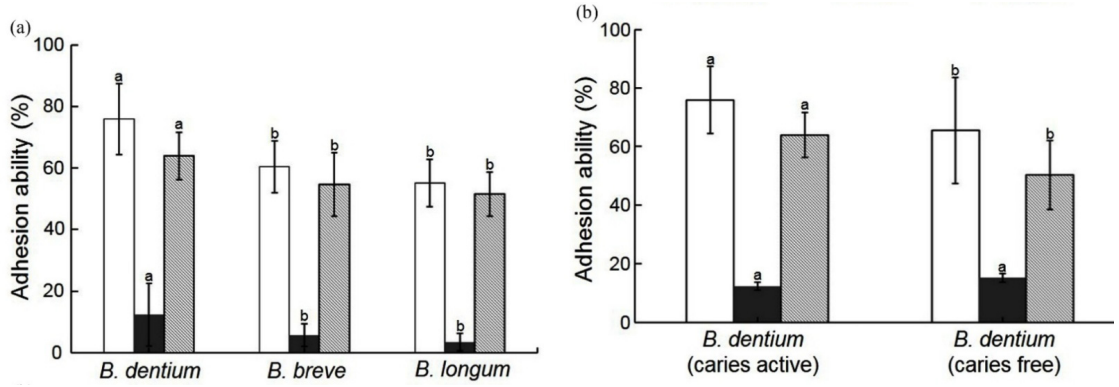

Figure 1 Adhesive ability with keratinocyte cells of (a) *B. dentium*, *B.breve*, and *B.longum* from caries-active subjects; (b) *B. dentium* from caries-active and caries-free groups. The adhesive ability represents total adhesion (□), externalization (■), and internalization (▨). Data are expressed as mean ± SD. Different superscript letters indicate significant differences of each parameter at $p < 0.05$.

Physicochemical cell surface properties of oral *Bifidobacterium* spp.

The cell surface hydrophobicity and surface charges of the isolated oral bifidobacteria are shown in **Figure 2**. The adhesive characteristics of bifidobacteria were analyzed by measuring adhesion to xylene (hydrophobicity), chloroform, and ethyl acetate (surface charge to describe electron donor (basic) and electron acceptor (acidic) characteristics of bacterial surface, respectively). The bifidobacteria showed strain variations for adhesive characteristics and exhibited a moderate to high degree of hydrophobic (affinity to xylene) and hydrophilic (affinity to chloroform and affinity to ethyl acetate) surface charges as

follows: *B. dentium* (81, 96 and 56 %), *B. breve* (25, 63 and 44 %), and *B. longum* (82, 98 and 46 %) for caries-active subjects, and *B. dentium* (86, 98 and 59 %) for the caries-free group, respectively. Moreover, the strain *B. dentium* showed no significant differences in surface properties in both groups.

Biofilm formation of oral *Bifidobacterium* spp.

Biofilm-forming bifidobactetia assessed by biofilm assay are shown in **Figure 3**. All selected strains of oral *B. dentium* were able to form biofilm. The predominant *B. dentium* from caries-active subjects and *B. dentium* CCUG 18367 showed biofilm formation higher than that of *B. dentium* from the caries-free group.

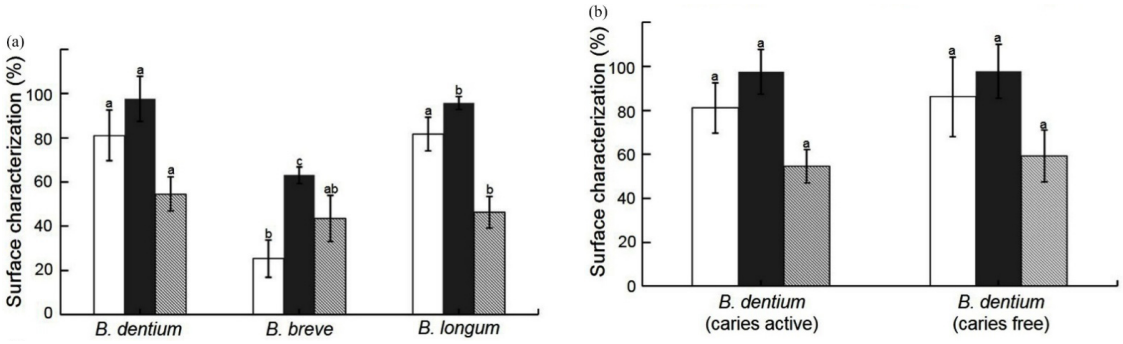

Figure 2 Adhesive characteristics in different solvents of (a) *B. dentium*, *B.breve* and *B.longum* from caries-active subjects; (b) *B. dentium* from caries-active and caries-free groups. The solvents represent xylene (□), chloroform (■), and ethyl acetate (▨). Data are expressed as mean ± SD. Different superscript letters indicate significant differences of each parameter at $p < 0.05$.

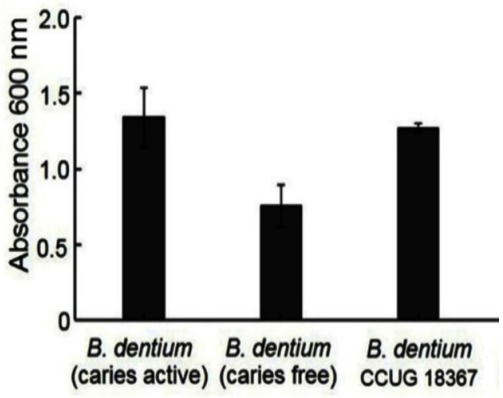

Figure 3 Biofilm formation capacity of *B. dentium* isolated from caries-active and caries-free subjects and reference strains of *B. dentium* CCUG 18367. Data are expressed as mean ± SD.

Discussion

The prevalence rate of bifidobacteria in Thai children (48 % in caries children) was lower than previous reports from occlusal lesions (67 %) [7] and caries saliva (95 %) [3]. However, some reports showed that bifidobacteria were undetected in the mouths of healthy individuals [8] and in active root caries lesions [17]. Interestingly, in this study, the species of *B. dentium* was the major strain isolated from the oral route in both groups. The results are in agreement with previous reports, in that the

predominant bifidobacteria is *B. dentium*, found in children in root surface plaque (80 %), infected root caries lesions (100 %), and soft active lesions (80 %) [2]. Other analyses identified caries-associated bifidobacteria as follows: *B. dentium* (83 %), *B. longum* (12.8 %), *P. denticolens* (3.9 %), *S. inopinata* (4.6 %), *B. scardovii* (1.3 %), and *A. omnicolens* (0.7 %) [1]. The differences in the occurrence of bifidobacterial strains are not surprising, owing to the methods of isolation, identification, individual races, age group, diversity of microbial flora, habit of food consumption, and personal hygiene. Importantly, in a host habitat, the particular pH in such an environment would determine the dominant strain of bifidobacterial species. The *B. dentium* grows well in around neutral pH, and could maintain viability with mutans streptococci [18], since saliva pH is around 7.0 for the caries-free group and 6.5 for the caries-active group [19]. The frequently found strains of *B. dentium* are strongly associated with dental caries in children [3] and adults [7]. The other bifidobacterial strains, such as *B. breve* and *B. longum*, were sporadically isolated in caries lesions in this study, suggesting that they were not significantly involved in the caries process. It is obvious that *B. dentium* shows higher numbers of pilus-like appendages around the cell surface than other bifidobacterial species [20], indicating that *B. dentium* is the strain with the most potential to adhere to host tissue. Previous reports demonstrated that bifidobacteria had variations in adhesive ability and cell surface property [21]. These properties were assessed in an intestinal bifidobacterial strain of *B. longum* B6 and several probiotic lactobacilli using a Caco-2 cell line originated from a human colonic adenocarcinoma, indicating a good relationship between *in vitro* adhesion and *in vivo* colonization [21].

In dental caries, the association of bifidobacteria in caries lesions arises from the crucial colonization ability to form biofilm, as well as dental plaque and the production of invasive acids. The findings that *B. dentium* strains showed a high ability of adhesion (both internalization and externalization) may explain the high prevalence of *B. dentium* found in the plaque samples used in this study. Such an ability may also affect the capability of biofilm formation of the *B. dentium* strains. Biofilm formation occurs from a few minutes up to few months, mediated by the adsorption of protein and the carbohydrate intake, followed by the immobilization of multispecies bacteria and the consolidation of exopolysaccharide production from bacteria, and finally colonization to form biofilm [22]. The accumulation of microbial consortia generates acids when sugar and/or carbohydrate is supplied, which results in dental disease [23]. In oral environments, bifidobacteria (*B. breve* and *B. longum*) may not attach to the hard tissue of the teeth surface, as evidenced by the low affinity to hydroxyapatite [10], whereas the strains of *B. dentium* as demonstrated in this study showed high adhesive ability with keratinocytes. Additionally, the *B. dentium* possessed a high affinity to xylene and chloroform, indicating the properties of bacterial cell surface exhibited hydrophobic and hydrophilic electron donor (basic) characteristics in the adherence to host cells. Interestingly, *B. dentium* was able to adhere to keratinocytes, representing the epithelium of oral mucosa of gingival, cheek bulge and palate in the oral cavity; this may facilitate the proliferation of *B. dentium.*

Conclusions

The prevalence of oral bifidobacteria was significantly higher in caries-active Thai children than in the caries-free group. The predominant strain of *B. dentium* was found in both groups. *B. dentium* from caries-active subjects showed a high degree of adhesive ability with keratinocytes, and was able to form biofilm, implying an important role in the colonization of oral mucosa.

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