# Prevalence and Adhesion Properties of *Bifidobacterium* Species in the Oral Cavity and Vagina

**Parada Utto**

**A Thesis Submitted in Fulfillment of the Requirements for the Degree of Doctor of Philosophy (Oral Health Sciences)**

**Prince of Songkla University**

**2016**

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I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

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**ชื่อวิทยานิพนธ์** ความชุกและสมบัติการเกาะติดของสปีชีส์ไบฟีโดแบคทีเรียในช่องปากและช่องคลอด

**ผู้เขียน** นางสาวภารดา อุทโท

**สาขาวิชา** วิทยาศาสตร์สุขภาพช่องปาก

**ปีการศึกษา** 2558

## บทคัดย่อ

ได้มีรายงานการตรวจพบสปีชีส์ไบฟีโดแบคทีเรียในช่องปากของคนที่เป็นโรคฟันผุและในช่องคลอดของสตรีผู้เป็นช่องคลอดอักเสบแบคทีเรียผสม แต่บทบาทของเชื้อไบฟีโดแบคทีเรียยังไม่ทราบแน่ชัดในความสัมพันธ์กับการก่อให้เกิดโรคและยังไม่มีข้อมูลความชุกการพบเชื้อในประชากรไทย วัตถุประสงค์ของการศึกษาครั้งนี้ คือ 1) การตรวจหาความชุกของไบฟีโดแบคทีเรียที่แยกจากช่องปากและช่องคลอด และ 2) ศึกษาความสามารถการเกาะติด สมบัติของผิวเซลล์ และประเมินความสามารถการสร้างไบโอฟิล์มและความเป็นพิษต่อเซลล์ของไบฟีโดแบคทีเรียที่แยกได้จากช่องปากและช่องคลอด

โรคฟันผุเกิดจากการย่อยสลายฟันด้วยกรดที่ผลิตจากกิจกรรมของแบคทีเรียที่ทำให้เกิดโรคฟันผุ โดยมีรายงานหลายฉบับพบว่า ไบฟีโดแบคทีเรียมีความสัมพันธ์กับการก่อให้เกิดโรค แต่ยังไม่มีข้อมูลความชุกและการเกาะติดของเชื้อนี้ในกลุ่มเด็กไทย จึงได้ทำการทดลองโดยใช้ไบฟีโดแบคทีเรีย จำนวน 167 สายพันธุ์ ที่แยกได้จากเด็กที่เป็นโรคฟันผุ 50 ราย และเด็กสุขภาพดี 50 ราย แล้วนำเชื้อมาจำแนกสายพันธุ์โดยเทคนิคชีววิทยาโมเลกุล และศึกษาความสามารถในการเกาะติด สมบัติของผิวเซลล์ การสร้างไบโอฟิล์มและความเป็นพิษต่อเซลล์ พบว่าความชุกของเชื้อไบฟีโดแบคทีเรียในเด็กโรคฟันผุ (48%) (24/50) มีปริมาณสูงกว่าเด็กผู้มีสุขภาพดี (24%) (12/50) อย่างมีนัยสำคัญทางสถิติ ($p<0.05$) โดยมีปริมาณแบคทีเรียทั้งหมด 5.8±0.9 Log CFU/ml และ 2.7±0.8 Log CFU/ml ตามลำดับ ไบฟีโดแบคทีเรียที่พบบ่อยในเด็กโรคฟันผุ ได้แก่ สายพันธุ์ *B. dentium* (82.9%) (102/123), *B. breve* (11.4%) (14/123), และ *B. longum* (5.7%) (7/123) ส่วนเด็กผู้มีสุขภาพดีพบสายพันธุ์ *B. dentium* (100%) (44/44), ไบฟีโดแบคทีเรียทุกสายพันธุ์ ที่แยกได้จากทั้งสองกลุ่มตัวอย่าง มีความสามารถในการเกาะติดเซลล์เพาะเลี้ยง keratinocyte ในหลอดทดลอง โดยสายพันธุ์ *B. dentium* จากเด็กฟันผุ (66%) มีความสามารถในการเกาะติดสูงกว่าสายพันธุ์ที่แยกได้จากเด็กสุขภาพดี (58%) และ *B. dentium* แต่ละสายพันธุ์แสดงความแตกต่างของสมบัติผิวเซลล์ในการชอบน้ำหรือไม่ชอบน้ำ นอกจากนี้ *B. dentium* ยังมีความสามารถในการสร้างไบโอฟิล์ม แต่

ไม่มีความเป็นพิษต่อเซลล์ โดยสรุป *B. dentium* เป็นสายพันธุ์ที่เด่นที่สามารถแสดงศักยภาพในการเกาะติดผิวเซลล์และสามารถสร้างไบโอฟิล์มได้ดี ซึ่งแสดงถึงบทบาทสำคัญของความสัมพันธ์ในการคงอยู่ของการทำให้เกิดโรคฟันผุ

ส่วนโรคช่องคลอดอักเสบแบคทีเรียผสมเป็นโรคที่พบในสตรีวัยเจริญพันธุ์ และได้มีรายงานการตรวจพบสกุลไบฟิโดแบคทีเรียในสตรีผู้เป็นช่องคลอดอักเสบแบคทีเรียผสม แต่อย่างไรก็ตามข้อมูลเกี่ยวกับความชุกและความสามารถในการเกาะติดของสกุลไบฟิโดแบคทีเรียที่แยกได้สตรีไทยผู้เป็นช่องคลอดอักเสบแบคทีเรียผสมยังมีอยู่อย่างจำกัด จึงได้ทำการทดลองเชื้อไบฟิโดแบคทีเรียจำนวน 139 สายพันธุ์ จากสตรีผู้เป็นช่องคลอดอักเสบแบคทีเรียผสม 20 ใน 60 ราย และผู้มีสุขภาพดี 7 ใน 60 ราย ได้นำมาจำแนกสายพันธุ์โดยเทคนิคชีววิทยาโมเลกุล และศึกษาความสามารถในการเกาะติดและสมบัติของผิวเซลล์ การสร้างไบโอฟิล์มและความเป็นพิษต่อเซลล์ พบว่า ความชุกของเชื้อไบฟิโดแบคทีเรียในสตรีผู้เป็นช่องคลอดอักเสบแบคทีเรียผสม (33.3%) (20/60) มีปริมาณสูงกว่าสตรีผู้มีสุขภาพดี (11.7%) (7/60) อย่างมีนัยสำคัญทางสถิติ ($p<0.05$) โดยมีปริมาณแบคทีเรียทั้งหมด 8.9 ± 3.4 Log CFU/ml และ 5.7 ± 2.9 Log CFU/ml ตามลำดับ ไบฟิโดแบคทีเรียที่พบบ่อยในสตรีผู้เป็นช่องคลอดอักเสบแบคทีเรียผสมได้แก่ สายพันธุ์ *B. bifidum* (52.5%) (53/101), *B. longum* (37.6%) (38/101), *B. breve* (5.9%) (6/101) และ *B. dentium* (4.0%) (4/101), ส่วนสตรีผู้มีสุขภาพดีพบสายพันธุ์ *B. bifidum* (55.3%) (21/38), *B. longum* (28.9%) (11/38), and *B. breve* (15.8%) (6/38) ไบฟิโดแบคทีเรียทุกสายพันธุ์ ที่แยกได้จากสตรีผู้เป็นช่องคลอดอักเสบแบคทีเรียผสมและผู้มีสุขภาพดี มีความสามารถในการเกาะติดเซลล์เพาะเลี้ยง HeLa cells ในหลอดทดลอง โดย *B. bifidum* และ *B. dentium* ที่แยกได้จากสตรีผู้เป็นช่องคลอดอักเสบแบคทีเรียผสม มีความสามารถในการเกาะติดสูง และความสามารถในการเกาะติดสัมพันธ์กับสมบัติของผิวเซลล์ของเชื้อไบฟิโดแบคทีเรีย โดยเฉพาะ สายพันธุ์ *B. dentium* ที่แยกได้จากสตรีผู้เป็นช่องคลอดอักเสบแบคทีเรียผสม สามารถสร้างไบโอฟิล์มได้ดี และเป็นพิษต่อเซลล์เพาะเลี้ยงสูง สรุป ความชุกของไบฟิโดแบคทีเรีย ในสตรีผู้เป็นช่องคลอดอักเสบแบคทีเรียผสม สูงกว่าผู้มีสุขภาพดีอย่างมีนัยสำคัญ *B. bifidum* และ *B. dentium* ที่แยกได้จากสตรีผู้เป็นช่องคลอดอักเสบแบคทีเรียผสม มีความสามารถในการเกาะติดสูง แสดงถึงบทบาทสำคัญของการคงอยู่ในช่องคลอดสตรีผู้เป็นช่องคลอดอักเสบแบคทีเรียผสมและ ที่น่าสนใจคือ *B. dentium* จากช่องคลอดมีความสามารถในการเกาะติด HeLa cells ได้ดีกว่าเซลล์ keratinocyte ซึ่งแสดงถึงความคงอยู่ในช่องคลอดได้ดีกว่าในช่องปากและในทางกลับกัน *B. dentium* จากช่องปากชอบอยู่ในสภาวะช่องปากมากกว่าช่องคลอด

| | |
|---|---|
| **Thesis Title** | Prevalence and Adhesion Properties of *Bifidobacterium* Species in the Oral Cavity and Vagina |
| **Author** | Miss Parada Utto |
| **Major Program** | Oral Health Sciences |
| **Academic Year** | 2015 |

## ABSTRACT

*Bifidobacterium* species have been reported in oral cavity with dental caries and in vagina with bacterial vaginosis. However, the role of bifidobacteria in the association with pathogenesis of both routes is unclear and there are no prevalence data available for Thai population. The aims of the present thesis were therefore: 1) to investigate prevalence of bifidobacteria isolated from oral cavity and vagina. 2) to evaluate adhesion ability, hydrophobic and hydrophilic surface charges as well as assessment of biofilm formation and cytotoxicity of the oral and vaginal bifidobacteria.

Dental caries is the consequence of demineralization of teeth caused by acids due to cariogenic bacteria. Several publications have reported the association of bifidobacteria with dental caries lesions but no data of prevalence and adhesion properties of oral *Bifidobacterium* spp. have been evaluated in Thai children. A total number of 167 strains of oral bifidobacteria were isolated from 50 caries-active children and 50 caries-free subjects and identified by molecular biology techniques. The bifidobacteria of both groups were examined for adhesion ability, surface properties, biofilm formation and cytotoxicity. The prevalence of oral bifidobacteria in caries-active children (48%) (24/50) was significantly higher than caries-free group (24%) (12/50) ($p<0.05$) with total count of 5.8±0.9 Log CFU/ml and 2.7±0.8 log CFU/ml, respectively. The 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 and *B. dentium* 100% (44/44) for caries-free group. All strains of bifidobacteria were able to adhere keratinocyte cell line *in vitro*. The adherent strains of *B. dentium* showed higher total adhesion ability in caries-active subjects (66%) than caries-free group (58%). The *B. dentium* showed strain variations in cell surface characteristics of hydrophobic and hydrophilic surface charges. The strains of *B.*

*dentium* from both groups were able to form biofilm but none of them had cytotoxic activity. In conclusions, the predominant strains of *B. dentium* had high adhesion ability and biofilm forming capacity implying a role of colonization to oral mucosa and support to the prevalence of bifidobacteria in the association with caries process.

Bacterial vaginosis (BV) is the common cause of abnormal vaginal discharge among women of reproductive age. *Bifidobacterium* spp. have been reported in women with BV, nevertheless data of prevalence and adhesion properties of the vaginal bifidobacteria in Thai women with bacterial vaginosis (BV) is still limited. A total number of 139 bifidobacterial were isolated from 20 of 60 women with BV and 7 of 60 healthy women. The isolated strains were identified by molecular biology techniques and were examined for adhesion properties, surface charges, biofilm formation and cytotoxicity. The prevalence of vaginal bifidobacteria in women with BV (33.3%) (20/60) was significantly ($p<0.05$) higher than healthy women (11.7%) (7/60) with total counts of 8.9 ± 3.4 Log CFU/ml and 5.7 ± 2.9 Log CFU/ml, respectively. The frequent species of *B. bifidum* (52.5%) (53/101), *B. longum* (37.6%) (38/101), *B. breve* (5.9%) (6/101) and *B. dentium* (4.0%) (4/101) were found in women with BV, while healthy women harbored *B. bifidum* (55.3%) (21/38), *B. longum* (28.9%) (11/38) and *B. breve* (15.8%) (6/38). All vaginal bifidobacteria from BV and healthy subjects were able to adhere HeLa cell line *in vitro*. The adhesion ability of *B. bifidum* and *B. dentium* from BV subjects showed high degree of adhesion property and was in correlation with cell surface characteristics. Particularly, the strains of *B. dentium* from BV subjects had high biofilm forming capability and high cytotoxic activity. In conclusions, the prevalence of vaginal bifidobacteria occurred significantly higher in women with BV than healthy group. The strains of *B. bifidum* and *B. dentium* showed high adhesion properties which implied an important role of vaginal colonization in the association with BV. Interestingly, the vaginal *B. dentium* had higher adhesion ability to HeLa cells than keratinocyte cell line implying a better localization in vagina than oral environment and vice versa, the oral *B. dentium* was more in favor of colonization in the oral cavity than vaginal habitat.

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Parada Utto

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# LIST OF ABBREVIATIONS AND SYMBOLS

| | |
|---|---|
| ATCC | American Type Culture Collection |
| *B.* | *Bifidobacterium* |
| BHI broth/agar | Brain heart infusion broth/agar |
| BV | Bacterial vaginosis |
| CAB | Columbia agar base |
| CCUG | Culture Collection, University of Göteborg |
| CFU | Colony forming unit |
| DGGE | Denaturing gradient gel electrophoresis |
| DNA | Deoxyribonucleic acid |
| FBS | Fetal bovine serum |
| FISH | Fluorescence in situ hybridization |
| F6PPK | Fructose-6-phosphate phosphoketolase |
| MRS broth/agar | de Man, Rogosa and Sharpe broth/agar |
| PBS | Phosphate-buffered saline |
| PCR | Polymerase chain reaction |
| RAPD | Randomly amplified polymorphic DNA fingerprinting |
| RFLP | Restriction fragment length polymorphism |
| rRNA | Ribosomal RNA |
| RTF | Reducing transport fluid |
| spp. | Species |

# LIST OF PAPERS AND PROCEEDING

**International Journal Papers**

1. Parada Utto, Rawee Teanpaisan and Supatcharin Piwat. Prevalence and Adhesion Properties of Oral *Bifidobacterium* species in Caries-active and Caries-free Thai Children. ***Walailak J. Sci. & Tech.*** Accepted for publication. August 11, 2016.
2. Parada Utto, Rawee Teanpaisan, Supatcharin Piwat and Verapol Chandeying. Assessment of Prevalence, Adhesion and Surface Charges of *Bifidobacterium* spp. Isolated from Thai Women with Bacterial Vaginosis and Healthy Women. ***J Med Assoc Thai.*** Accepted for publication. June 7, 2016.

**Conference Proceeding**

1. Utto P. and Teanpaisan R. Screening of bifidobacteria from oral cavity to use as probiotic. The 2nd Current Drug Development International Conference; 2012 May 2-4; Phuket Graceland Resort & Spa Hotel, Phuket, Thailand. Quintessence Publishing; 2012. p. 187 -188.

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With respectively,
Parada Utto

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February 17 th, 2012

Dear: Parada Utto,

The license agreement from the 2nd Current Drug Development International Conference

On behalf of the organizing committee, we confirmed that Parada Utto's paper has been accepted and can use published article for any academic purpose barring commercial venture with appropriate citation (Title: Screening of bifidobacteria from oral cavity to use as probiotic, Poster No. PA-13).

With best regards,

Teerapol Srichana

Teerapol Srichana
Chair of CDD 2012

# 1. INTRODUCTION

## Background and Rationale

The bifidobacteria are generally present in healthy gastrointestinal tract of humans and animals[1] as well as they can be found in the oral cavity [2] and vagina of women.[3] However, data of prevalence and adhesion property of the oral bifidobacteria in Thai children with dental caries and the vaginal bifidobacteria in Thai women with bacterial vaginosis (BV) is still limited. An attention of this study was performed into two parts: part one was the study of prevalence and adhesion properties of *Bifidobacterium* species from caries-active and caries-free Thai children and part two was the study of prevalence and adhesion properties and surface charges of vaginal *Bifidobacterium* species from women with bacterial vaginosis and healthy women.

In oral cavity, dental caries is the consequence of demineralization of teeth caused by acids due to bacterial activity. The cariogenic bacteria actively involved in caries process are *Streptococcus mutans*, *S. sobrinus,* and Lactobacilli.[4] However, bifidobacteria are also recognized as acidogenic and aciduric to be able to proliferate in a cariogenic environment of caries pathogenesis.[4-6] The bifidobacteria in oral cavity are limited in number which includes genus of *Bifidobacterium*, *Scardovia*, *Parascardovia,* and *Alloscardovia*.[7, 8] Recently, Mantzourani *et al*. [2] have reported predominant bifidobacteria from active occlusal lesion of 87% in adults and 67% in children as *Bifidobacterium dentium, Parascardovia denticolens, Scardovia inopicata, Bifidobacterium longum, Scardovia genomosp.* C1 and *Bifidobacterium breve,* while no bifidobacteria was found in supra and subgingival plaques from clinical healthy teeth.[9] Recent studies indicate the isolation of bifidobacteria from saliva of 95% of the caries-active and from only 9% of the caries-free children [4] and 96.8% of bifidobacteria found in saliva of caries-active older adults.[5] The salivary levels of bifidobacteria are significantly correlated with amount of sugar in the diet and oral hygiene practice.[5]

In addition, the caries-associated bifidobacteria may adhere to dental plaques which participate colonization in caries process.[10] However, Haukioja *et al*. [11] have studied the oral adhesion *in vitro* of the bifidobacterial strains of *B. breve*, *B. longum, B. lactis* and *B. adolescentis* using human saliva coated on hydroxyapatite imitating tooth hard tissue but the binding ability of the bifidobacteria were low of less than 5%. The adhesion ability of oral bifidobacteria and the capability of biofilm formation on the surfaces of the teeth coating with saliva, food debris and bacterial consortia would be interesting for further investigation. At present, the biofilm-forming bifidobacteria have not yet been studied and the cytotoxic role of the oral bifidobacteria is still not known. It is postulated that the oral bifidobacteria may possess high adherence to oral buccal cavity referring maintenance to colonize dental plaque. Therefore, the aims of this study were to evaluate prevalence of bifidobacterial strains in Thai children of high risk caries-active and caries-free group and to investigate adhesion ability to keratinocyte cell line *in vitro*, cell surface properties, biofilm-forming capability, and cytotoxicity the oral bifidobacteria which would shed light on colonization process in dental caries.

In the human vagina, *Bifidobacterium* spp. are found in healthy women or women with bacterial vaginosis such as *B. infantis, B. bifidum, B. breve, B. longum* and *B. adolescentis.*[12] However, no epidemiological data are available on vaginal bifidobacteria in bacterial vaginosis and healthy Thai women. Bacterial vaginosis (BV) is one of the most common cause of abnormal vaginal discharge among women of reproductive age in the association with obstetric and gynaecologic complications.[13-16] The BV appears as a change in vaginal microbes, due to a decrease in the number of lactobacilli and overgrowth of various facultative or anaerobic bacterial species such as *Gardnerella vaginalis*, *Mobiluncus* spp., *Prevotella* spp.,[17] and *Bifidobacterium* spp.[18, 19] Preliminary studies have indicated that *Bifidobacterium* spp. were found more common in women with BV and abnormal vaginal flora than in healthy subjects.[3] The bifidobacteria were reported to be associated with BV in African[20] and Western women.[3] The prevalence of vaginal bifidobacteria varied from 12% in healthy controls, 41% to 58% of those with abnormal microflora and up to 83%[20] to 94% in women with BV.[3] However, prevalence of bifidobacteria have not yet been reported for Thai women with BV and healthy women. Moreover, the role of bifidobacteria in women with BV is still unclear. An attention is now focused on adhesion property of vaginal bifidobacteria. Since adhesion to host

tissues is the first step in bacterial colonization and also influences the subsequent phases leading to commensalism or infection. Previous studies have reported that the adhesion properties of the intestinal bifidobacterial strains using mucin and Caco-2 cell line.[21-23] However, the information of the adhesion properties of the vaginal bifidobacteria is still limited and cell surface charges of hydrophobic and hydrophilic characteristics reflecting on colonization ability of the vaginal strains have not yet been studied. Additionally, there are no data on the other factors as biofilm formation capability and cytotoxic activity of the vaginal bifidobacteria. Therefore, the aims of this study were to determine prevalence of vaginal *Bifidobacterium* spp. and to evaluate adhesion ability, cell surface properties, biofilm-forming capability and cytotoxicity of vaginal bifidobacterial isolates from Thai women with BV, compared to healthy subjects which may indicate a role in the association with BV pathogenesis.

## Literature Reviews

### 1. Bifidobacteria

The bifidobacteria are presumed to establish in gut before weaning period.[24] The gut microbiota commences at birth with colonization by bacteria from a variety of sources such as vaginal and fecal microbiota of mother during delivery, maternal milk of breast-feeding infants as well as well as other environmental microbes.[25] The bifidobacteria could adapt in human gut and survive the stressful conditions of bile salts, acidic or osmotic stress during diet variation. The bifidobacteria are generally present in the healthy gastrointestinal tract of humans and animals[1]. They are believed as probiotic microorganisms because they benefit the host through the regulatory effect of the gut microbiota and may produce bacteriocin [26] and vitamin.[27]

#### 1.1. Taxonomy

The family Bifidobacteriaceae consists of 7 genera, *Bifidobacterium, Aeriscardovia, Falcivibrio, Gardnerella, Parascardovia, Scardovia* and *Alloscardovia,* which have been isolated from human and animal sources.[28] The species of *Bifidobacterium* are gram-positive, catalase-negative, non-spore-forming, non-motile and polymorphic rods with a high G+C content (55 to 67%). The morphology of bifidobacterial ranges from uniform to branched

bifurcated Y and V forms which are spatula or club shaped (Fig. 1). The branching nature of bifidobacteria is dependent on strain and culture media.[29, 30]

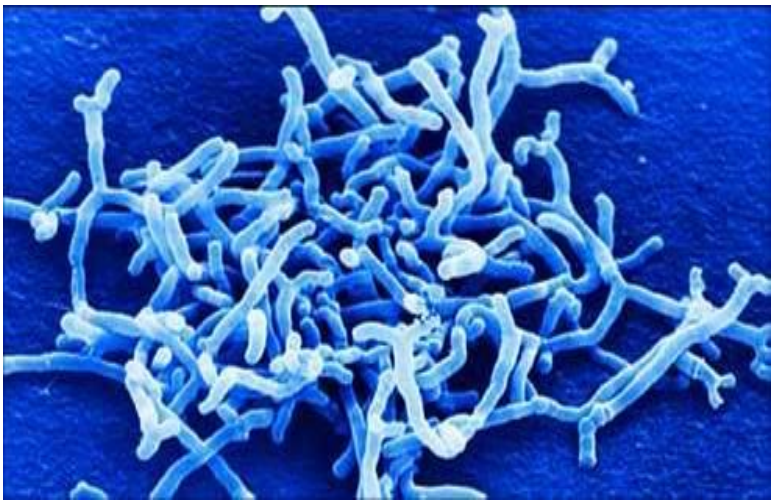

**Fig. 1.** Typical morphology of bifidobacteria.[31]

There are around 32 species are currently known in the genus of *Bifidobacterium*. The isolated strains of bifidobacteria are from the host specificity such as *B. pseudolongum* subsp. *globosum, B. thermophilum,* and *B. bourn* are present in ruminant feces,[32] *B. suis* in swine, *B. cuniculi* and *B. magnum* in rabbit, *B. pullorum* in chicken. While in the human intestine could be found various species as *B. adolescentis, B. dentium, B. bifidum, B. breve, B. catenulatum, B. infantis, B. longum* and *B. animalis subsp. lactis*.[33] Recently, the bifidobacteria have been isolated from oral cavity [34] as the species of *B. dentium*, *B. bifidum, B. breve, B. longum, B. adolescentis* and *B. scardovii.*[35] Whereas fewer strains of bifidobacteria are found in women vagina such as *B. infantis, B. bifidum, B. breve, B. longum* and *B. adolescentis*.[36] The bacterial cell envelope habors proteins and carbohydrates that facilitate bacterial attachment and colonization. The bifidobacteria strain of *B. breve* produces cell surface-associated exopolysaccharides and the pilus-like structure to attach between the bacterial cell and the host cell surface.[37] Atomic force microscopy (AFM) of various human intestinal bifidobacterial strains belonging to *B. bifidum, B. adolescentis, B. animalis* subsp. *lactis*, *B. dentium* and *B. longum* subsp. *infantis* reveal the presence of cell surface-located pilus-like appendages as shown

in (Fig. 2).[38] Bacterial metabolic end-products, such as short-chain fatty acids, vitamins, and linoleic acid (polyunsaturated fatty acids) mediate host–microbe interactions.

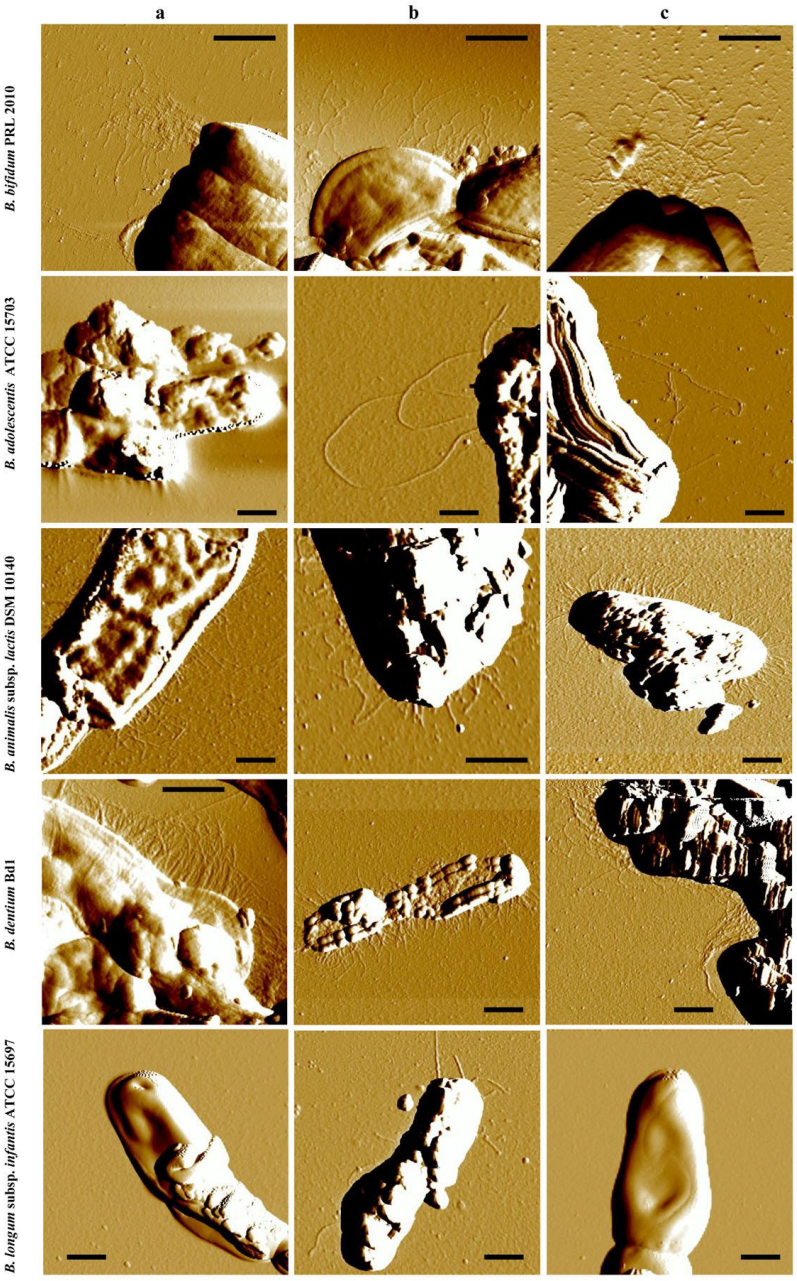


**Fig. 2.** Presence of pilus-like structures in various bifidobacterial species viewed by Atomic force microscope. Panels a, b and c represent bifidobacteria cultivated in MRS medium containing glucose, lactose or fructooligosaccharides, respectively, as a sole carbon source. Scale bar 0.5 μm.[38]

### 1.2. Physiology

The bifidobacteria are chemoorganotroph that uses organic compounds as carbon and energy source. Most of the bifidobacteria are not able to grow in a completely synthetic medium. The bifidobacteria require complex biological substances such as casein, bovine serum albumin, casein or yeast extract.[39, 40] The common selective medium used for primary isolation bifidobacteria are the following: CAB (Columbia agar base) plus propionic acid and non-selective medium of MRS (de Man Rogosa and Sharpe) plus L-cysteine hydrochloride as a reducing agent.[41] The bifidobacteria are saccharolytic which produce acids but not gas from a variety of carbohydrates. The optimum temperature of growth is 37-41 °C whereas the temperature of 25-28 °C and 43-45 °C are the minimum and maximum growth, respectively. The optimum pH at the beginning of growth is between 6.5-7.0.[42-47] No growth has been recorded at pH lower than 4.5 or higher than 8.5 with the exception of *B. thermacidophilum*, which is able to grow at pH 4.0. [1, 48]

### 1.3. Metabolism

In the genus *Bifidobacterium*, hexoses are degraded only and exclusively by the fructose 6-phosphate pathway, as shown in (Fig. 3).[49] The "bifid shunt" [50] or glucose catabolism through the fructose 6-phosphate phosphoketolase, generates acetyl phosphate and erytrose 4-phosphate. The final products of the fermentation route are formed by the sequential action of the enzymes (transaldolase, transketolase and xylulose 5-phosphate phosphoketolase) which generates glyceraldehyde 3-phosphate that gives rise to lactic acid and acetic acid in approximately ratio 3:2.

Cleavage of pyruvate to formic acid and acetyl phosphate, and the reduction of acetyl phosphate to ethanol can often alter the fermentation balance to a highly variable extent.[51] Different bifidobacterial species produce different amounts of acetic, lactic and formic acid, and ethanol under the same conditions. Furthermore, variations of growth conditions, such as type and quantity of the carbon source, may result in the production of varying amounts of fermentation products. During metabolism of hexose, there is no production of carbon dioxide, except during degradation of gluconate. The modified of fructose-6-phosphate phosphoketolase (F6PPK) test is commonly used for presumptive identification of bifidobacteria due to its simple and inexpensive

assay. For identification of bifidobacteria, the conventional biochemical test is used. However, at present, the most powerful methods for identification of bifidobacteria are the molecular biology techniques. The most applied techniques are the PCR-based methodologies, which could target the genome using universal primers or specific primers.

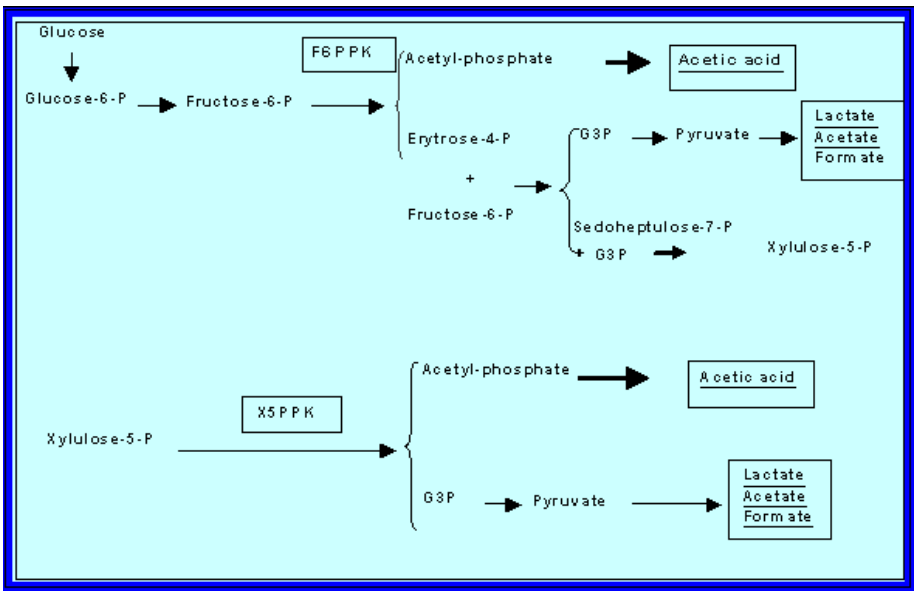


**Fig. 3.** The "bifid shunt" (bifidobacteria fermentation route). F6PPK = Fructose 6-phosphate phosphoketolase; X5PPK = Xylulose 5-phosphate phosphoketolase.[52]

**2. Methods of identification for *Bifidobacterium* spp.**

Traditionally, identification methods for bifidobacteria are relied on the morphological and biochemical characteristic as well as the selective culture plating. All of these methods are culture-dependent, labour-intensive, time consuming and inaccurate.[30, 53, 54] The most commonly used to identify bifidobacteria to the genus level based is based on the presence of F6PPK, the key enzyme of bifidobacterial hexose metabolism. Genus *Bifidobacterium* can be distinguished from other bacterial groups such as *Lactobacillus* and *Actinomycetaceae* [50] by a particular metabolic pathway that can be used in an initial identification of the genus level. However, phenotypic identification of members of the genus *Bifidobacterium* is difficult due to the intraspecies variability of differentiating physiological–biochemical characteristics.[55] In addition, many morphological, cultural, and physiological–biochemical properties of

bifidobacteria greatly depend on the composition of the medium, cultivation conditions, culture age, and some other factors.[44, 45] One of the very first identification schemes developed for *Bifidobacterium* species was based on a simple carbohydrate fermentation pattern.

Molecular biologists and microbiologists have recognized how molecular techniques could be used to answer major questions limiting the progress in strain differentiation. However, this approach does not allow identification at the species level. Several methods based on the polymerase chain reaction (PCR) have been developed for classification of bifidobacteria. Nucleic acid analysis is one of the most extensively practical techniques. This technique is usually performed by the comparison of DNA-DNA homology or 16S rRNA gene sequences. Many molecular typing methods have been adapted to the *Bifidobacterium* species, either requiring prior cultivation in order to separate colonies of bacterial strains or culture-independent direct detection from bacterial communities.

PCR is a technique, which uses a DNA polymerase enzyme to make a huge number of copies of any given piece of DNA or gene. In microbiology, using these techniques to amplify the 16S rRNA gene, approximately sixteen hundred nucleotides in length, leads to a new approach for microbial detection and identification.[56]

In PCR-RFLP (Restriction Fragment Length Polymorphism) analysis, chromosomal fragments resulting from a selective PCR reaction are digested by restriction enzymes, resulting in the various banding patterns. The discriminatory power of PCR-RFLP methods is high,[57, 58] however it could not distinguish some species of *Bifidobacterium.* Although this technique is expensive, more time-consuming and has some limitations, PCR-RFLP is still used for identifying *Bifidobacterium* species due to its non-complex of its patterns and being straightforward to interpret and compare between species.

**3. Dental caries**

Dental caries is the consequence of demineralization of susceptible dental hard tissues caused by organic acids (Fig. 4).[59] There are three major hypotheses for the etiology of dental caries: the specific plaque hypothesis, the nonspecific plaque hypothesis, and the ecological plaque hypothesis.[60-62] The specific plaque hypothesis has proposed that only a few specific species, such as *S. mutans* and *S. sobrinus*, are actively involved in the disease. On the

other hand, the nonspecific plaque hypothesis maintains that caries is the outcome of the overall activity of the total plaque microflora, which is comprised of many bacterial species.[62] The ecological plaque hypothesis suggests that caries is a result of a shift in the homeostatic balance of the resident microflora driven by changes in local environmental conditions, particularly pH shift to acid (Fig. 5).[61] The demineralization begins at pH range between 5.5-5.0.[63]

Caries-associated bacteria traditionally have been identified by using culture-based methods, which exclude not-yet-cultivated species. Molecular methods are now performed for identification and enumeration of bifidobacterial species that are associated with dental caries.[64, 65] A number of publications have reported on the bacterial species other than *S. mutans*, likely play important roles in caries progression e.g., species of the genera *Veillonella*, *Lactobacillus*, *Bifidobacterium*, *Propionibacterium*, low-pH non-*S. mutans* streptococci, *Actinomyces*, and *Atopobium*.[66, 67] Dental plaque is the material that adheres to the teeth and consists of bacterial cells (mainly *S. mutans* and *S. sanguis*), salivary polymers and bacterial extracellular products.

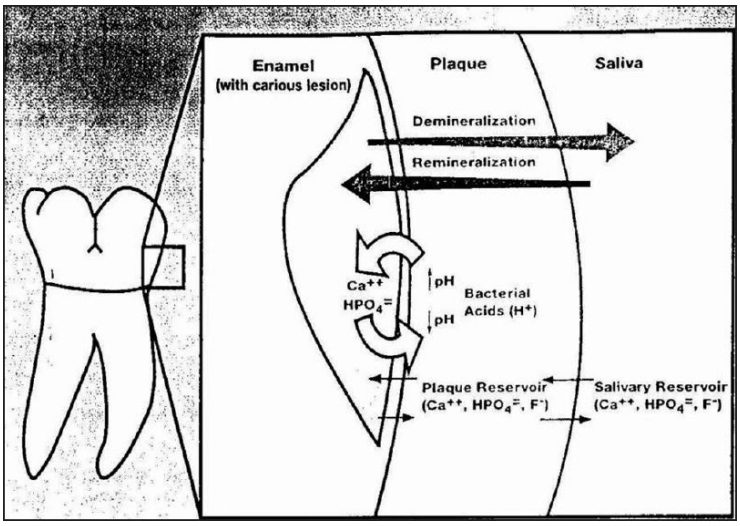


**Fig. 4.** Cycle of demineralization and remineralization in enamel.[68]

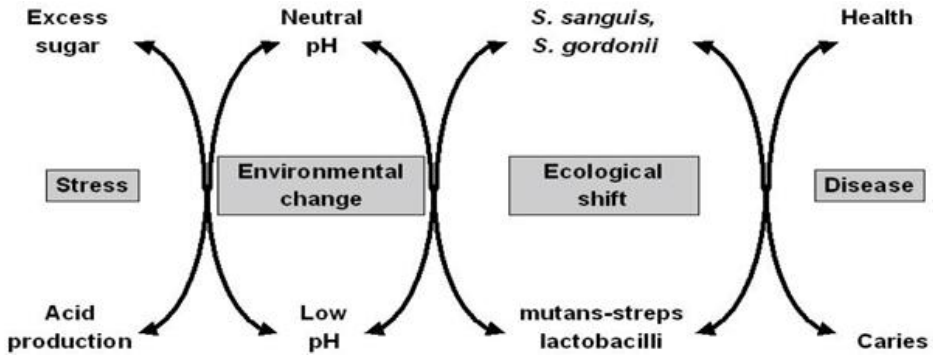


**Fig. 5.** Ecological plaque hypothesis in the pH shift to acids by microbes.[69]

**4. Prevalence and type species of *Bifidobacterium* in oral cavity**

Bifidobacteria have been isolated from saliva, supra-gingival dental plaque, carious dentine, endodontic infections, and infected dentin,[9, 67, 70-73] in which they accounted 6-83% of caries subjects.[4] Bifidobacterial species isolated from oral samples include *B. dentium*, *S. inopicata*, *P. denticolens, A. omnicolens*, [7, 8, 35] *S. genomospecies* strain C1, *Scardovia* sp. strain T01-04, *B. bifidum, B. breve, B. longum, B. adolescentis, B. scardovii* and *S. wiggsiae.* [8, 33, 70, 72, 74, 75] They have been detected in high numbers from caries lesions in children.[67] *Bifidobacterium* species are also involved in the development of carious lesions and have been shown to be associated with the initiation of dental caries in humans.[71, 76-78] However, the association between dental caries and the prevalence of *Bifidobacterium* has been recognized for several decades. Most common *Bifidobacterium* species in the oral cavity as *B. dentium*, *S. inopinata*, *P. denticolens*, *B. scardovii* and *A. omnicolens* are shown in (Table 1).

**Table 1.** *Bifidobacterium* species reported in various studies

| **Authors** | **Subject** | **Sample** | **Identification technique** | ***Bifidobacterium* species** |
|---|---|---|---|---|
| Modesto et al., 2006[73] | 34 adults | Plaque | DNA-DNA homology | *B. dentium*, *P. denticolens* and *S. inopicata* |
| Hojo et al., 2007[77] | 46 dental students | Saliva | Species-specific primer and PCR | *B. dentium*, *B. adolescentis*, *A. omnicolens* and *B. longum* |
| Beighton et al., 2008[78] | 192 healthy dentate adults | wax-stimulated saliva | REP-PCR | *B. dentium*, *S. inopinata*, *P. denticolens*, *B. scardovii* and *A. omnicolens* |
| Mantzourani et al., 2009[6] | 44 adults | Plaque | 16S rRNA sequencing | *B. dentium, B. subtile, P. denticolens, S. inopicata, S. genomosp. C1, B. breve,* and *B. longum* |
| Mantzourani et al., 2009[2] | 24 childrens or 15 adults | Plaque and saliva | 16S rRNA sequencing | *B. dentium, P. denticolens, S. inopicata, B. longum, S.* genomosp. C1 and *B. breve* |
| Beighton et al., 2010[5] | 156 dentate patients | stimulated saliva | 16S rRNA sequencing | *B. dentium*, *B. longum*, *P. denticolens*, *S. inopicata*, *B. scardovii* and *A.omnicolens* |
| Kaur et al., 2012[4] | 60 childrens | Saliva | 16S rRNA sequencing | *B. dentium*, *P. denticolens*, *S. inopicata*, *S. wiggsiae*, *B. longum*, *B. scardovii*, *A. omnicolens, B. adolescentis* and *A.omnicolens* |

**5. Association of bifidobacteria with health and disease condition in the oral cavity**

Members of the family Bifidobacteriaceae have both acidogenic and aciduric characteristics relevant to the progression of dental caries, including root caries.[6, 79, 80] They also exhibit a range of abilities to degrade complex carbohydrates, including dextrans.[81] Despite these phenotypic properties, the reports of the isolation of bifidobacteria from healthy or diseased intraoral sites have generally been sporadic and usually in relation to active progressing caries. [9, 67, 71, 73, 75, 82] However, Caglar et al. [83] found that the bifidobacteria could reduce prevalence of caries-associated mutans streptococci in the saliva of young adults after daily intake of yogurt containing the probiotic strain *Bifidobacterium* DN-173010.

**6. Bacterial vaginosis**

Bacterial vaginosis (BV) occurs as a disturbance in this vaginal ecosystem in which the lactobacilli are replaced by an overgrowth of vaginal commensal organisms. Under the influence of estrogen, stratified squamous epithelium develops in the vagina. Lactobacilli become the dominant organism and lactic acid is produced by both bacterial metabolism and that of the epithelium. The vaginal pH falls to a level usually between 4.0 and 4.5. Physiological discharge consists of mucus, desquamated epithelial cells, and lactobacilli. The pH may rise above 4.5 at the time of menstruation, when the concentration of lactobacilli is reduced as well as cervical mucus and semen have pH between 7 and 8. If BV develops, the pH rises to a level between 4.5 and 6.0 to 7.0. The anaerobic or facultative anaerobic organisms which are usually present in low numbers increase by between 100- and 1,000-fold, to considerably outnumber the lactobacilli, which may eventually disappear. The loss of protective $H_2O_2$-producing lactobacilli has been hypothesized that lactobacilli are killed by bacteriophage infection. The anaerobic metabolism are thought to produce a number of amines such as trimethylamine, the polyamines, putrescine and cadaverine to give the fishy smell. The symptoms of BV show vaginal fluid of a thin, white or yellow discharge. Microscopy of vaginal smear shows multiple small bacteria and epithelial cells with large numbers of adherent gram-positive and gram-negative bacteria recognized as “clue cells”, as a clue to the diagnosis of nonspecific vaginitis. Treatment with antibiotics (metronidazole, clindamycin, doxycycline) to suppress the anaerobic overgrowth is usually successful in eradicating BV.

**7. Prevalence and type species of *Bifidobacterium* in the human vagina**

The human vagina is complex ecosystem containing an abundance of microorganisms. The member of bifidobacteria have been reported to increase gradually from normal vagina to bacterial vaginosis. From the available data, *Bifidobacterium* spp. were found between 12% to 94% of subjects.[3] There are several factors such as the host's state of health, pregnancy, age, or geographical variations that may influence the distribution of *Bifidobacterium* spp. with strain variations. Type species included *B. adolescentis*, *B. bifidum*, *B. breve, B. longum, B. denticolens, B. dentium,* and *B. inopinatum*.[12, 36] While *B. breve* and *B. adolescentis* have been the most frequently isolated species, followed by *B. longum* and *B. bifidum* (Table 2).[12]

**Table 2.** *Bifidobacterium* species reported in the vagina

| **Authors** | **Subject** | **Identification technique** | ***Bifidobacterium* species** |
|---|---|---|---|
| Rosenstein et al., 1996[3] | 174 pregnant women | Gram stain | *Bifidobacterium* sp. |
| Korshunov et al., 1999[12] | 56 clinical | - | *B. bifidum, B. breve*, *B. adolescentis* and *B. longum* |
| Burton et al., 2003[84] | 55 women | DGGE analysis | *B. adolescentis, B. angulatum, B. animalis, B. bifidum, B. breve, B. catenulatum, B. infantis, B. longum, B. lactis B. pseudocatenulatum* and *B. scardovi* |
| Hyman et al., 2005[85] | 20 women | PCR Amplification | *B. breve, Bifidobacteriumsp. oral* strainH6-M4, and *B. urinalis* |
| Verhelst et al., 2005[36] | 197 pregnant women | Gram stain and culture | *B. biavatii, B. bifidum, B. breve, B. dentium, B.longum* and *Bifidobacterium* sp. |
| El Aila et al., 2009[86] | 132 pregnant women | tRNA intergenic length polymorphism analysis (tDNA-PCR) and RAPD-analysis. | *B. bifidum* and *B. longum* subsp. *longum* |
| Swidsinski et al., 2010[87] | 10 women | FISH analysis | *B. adolescentis, B. longum, B. breve, B. bifidum* and *B. catenulatum* |
| Pepin et al., 2011[20] | 1555 participants | PCR assays | *Bifidobacterium* sp. |

**8. Association of bifidobacteria with healthy and diseased conditions in the vagina**

The association of bifidobacteria with healthy and diseased status in vagina is still vague. The member of bifidobacteria have been reported to increase gradually from normal vagina to bacterial vaginosis.[3] However, Lazarenko et al. [88] found that the bifidobacteria could produce antimicrobial activity against the bacterial vaginosis in association with *Staphylococcus aureus*, an opportunistic commensal pathogens. Korschunov et al. [89] proposed the use of bifidobacteria strains as probiotic in the correction of the microflora of the urogenital tract in females. In this habitat, bifidobacteria could play a role in maintaining vaginal homeostasis by producing organic acids and antagonistic bacteriocin towards pathogens.[90]

**9. Microbial colonization**

Microbial colonization is the first stage to establish microbial cells at appropriate site of host tissues. The process of colonization requires microbial adhesion ability, biofilm formation and properties of cell surface and also influences the subsequent phases leading to commensalism or infection by means of invasiveness or toxigenesis.

9.1. Bacterial adhesion or attachment to a tissue surface requires the participation of two factors: a receptor and a ligand.[10] The receptors are usually specific carbohydrate or peptide residues on the eukaryotic cell surface. The bacterial ligand, called an adhesin, is typically a macromolecular component of the bacterial cell surface which interacts with the host cell receptor (Fig. 6). Adhesins and receptors usually interact in a complementary and specific fashion with specificity comparable to enzyme-substrate relationships and antigen-antibody reactions. The possible interactions and forces involved are hydrophobic interactions, electrostatic attractions of Van der Waals forces which is due to an interaction between oscillating dipoles of the surface molecules.[10] Previous studies have reported on the adhesion properties of the intestinal bifidobacterial strain of *B. longum* B6 using mucin and Caco-2 cell line [21-23] and the oral bifidobacterial strains of *B. breve*, *B. longum, B. lactis* and *B. adolescentis* using human saliva coated on hydroxyapatite imitating tooth hard tissue.[11]

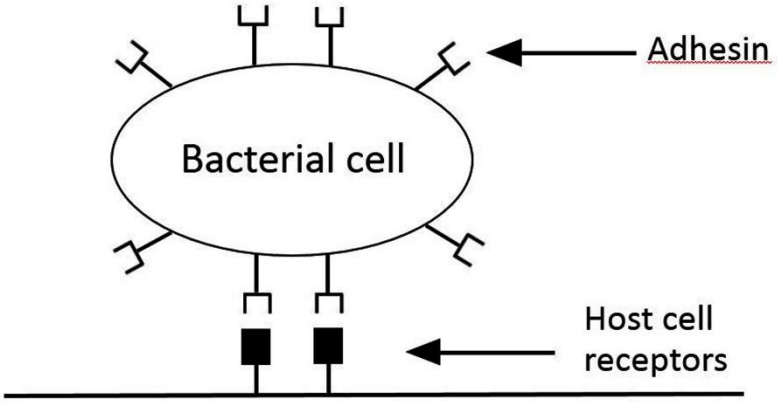


**Fig. 6.** Adherence of bacterial cell to host surface.

9.2. Biofilm formation is an adherence of microbial cells to a surface enclosed in a matrix of primarily polysaccharide material. Biofilm may form on a wide variety of surfaces of living tissues or other aquatic systems. Other characteristics of the aqueous medium, such as pH, nutrient levels, ionic strength, and temperature, may play a role in the rate of microbial attachment. Dental plaque is a biofilm on the surfaces of the teeth, coating with saliva and food debris found on the surface of teeth (Fig. 7). It is a cell-cell contact mediated by specific protein adhesion and often, as in the case of inter-species aggregation, by complementary polysaccharide receptors. Biofilm formation occurs in a few minutes up to few months mediated by adsorption of protein and carbohydrate intake, followed by immobilization of oral bacteria and consolidation of exopolysaccharide production from bacteria and finally colonization to form biofilm.[91] This accumulation of microorganisms subject the teeth and gingival tissues to high concentrations of bacterial metabolites which results in dental disease. To date, biofilm formation of the oral and vaginal bifidobacteria has not been investigated.

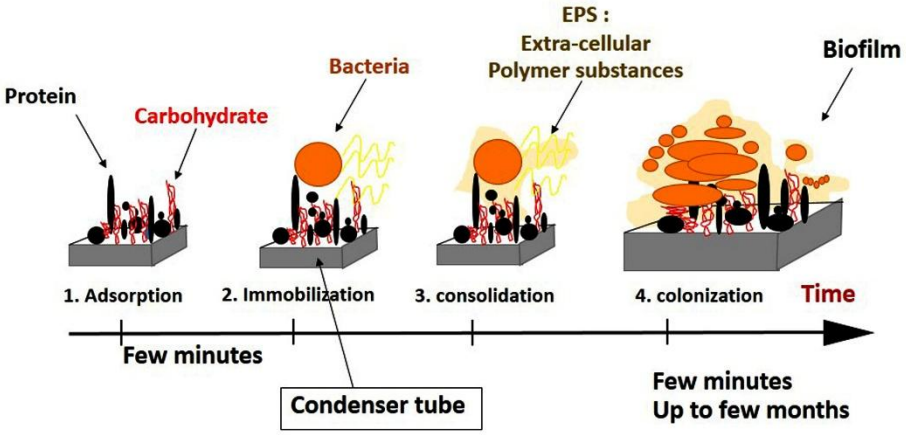


**Fig. 7.** Biofilm formation by adsorption of foods, immobilization of bacteria, consolidation of exopolysaccharides and colonization of biofilm.

9.3. Bacterial cell surface is the outermost layer of cell structure comprising hydrophobic and hydrophilic surface characteristics. Properties of cell surface hydrophobicity, presence of fimbriae and flagella, and production of extracellular polymeric substrates (EPS), influence the rate and extent of attachment of microbial cells. The hydrophobicity of the cell surface is assessed by adherence to hydrocarbon using xylene (non-polar solvent).The hydrophobicity is important in adhesion because hydrophobic interactions tend to increase with an increasing non-polar nature of one or both surfaces.[92] Hydrophobicity of bacterial cell surface is one of important factor that control bacterial adhesion to various surfaces. Hydrophobic interactions define the strong attraction between hydrophobic molecules and surfaces in water. In biological systems hydrophobic interactions are the strongest long-range non-covalent interactions and are considered a determining factor in microbial adhesion to surfaces.[20,21]

Properties of hydrophilic cell surface charge are the study of the surface of bacteria using chloroform (polar acidic solvent) and ethyl acetate (polar basic solvent). In the Lewis theory of acid-base reactions, acids accept pairs of electrons (electron acceptor) and bases donate pairs of electrons (electron donor). A hydrophilic molecule or portion of a molecule is one whose interactions with water and other polar substances are more thermodynamically favorable than their interactions with oil or other hydrophobic solvents. To date, previous studies have accessed cell surface properties of several number of probiotic lactobacilli and a bifidobacterial

strain, *B. longum* B6 indicating that the hydrophobicity was highly correlated with coaggregative abilities suggesting a good relationship between *in vitro* adhesion and *in vivo* colonization.[23]

9.4. Cytotoxicity is the broad qualities of pathogenic bacteria that cause disease in terms of invasiveness or toxigenesis. Invasiveness is the ability of a pathogen to invade tissue by colonization, invasion and multiplication. Invasion is mediated by the production of invasion (protein or enzyme) that facilitates bacterial growth and spreads the pathogen. Toxigenesis is the ability to produce extracellular toxin by certain bacteria and cause cytotoxicity at local or systemic effect. At present, there is still no evaluation of cytotoxic capability of the oral or vaginal bifidobacteria.

**Objectives**

1. To study the prevalence and type of bifidobacteria isolated from the oral cavity and vagina.
2. To evaluate adhesion ability, cell surface properties, biofilm-forming capability, and cytotoxicity of bifidobacterial isolates from the oral cavity and vagina.

# 2. MATERIALS AND METHODS

## Oral investigation

### 1. Subjects

From totally 100 subjects (6 to 9 year old children) from Paediatric dental clinic, Faculty of Dentisty, Prince of Songkla University, Thailand. Fifty pool plaque samples of dental caries and 50 pool plaque samples from sound surfaces were collected for this study. The pilot study was performed by testing 22 caries-active children and 9 subjects were found positive for bifidobacteria (40.91%), while 22 caries-free subjects were not found (0%). The sample size was calculated using a formula illustrated below.

$$n = [2(Z\alpha_{/2} + Z\beta)^2 P(1-P)]/\Delta^2$$

$$= 16$$

$$P = (P_{test} + P_{control}) /2$$

$P_{test}$ = Prevalence of bifidobacteria in caries-active subjects from pilot study was 40.91%; ($P_{test} = 0.41$)

$P_{control}$ = Prevalence of bifidobacteria in caries-free subjects from pilot study was 0%; ($P_{control} = 0$)

$\Delta = (P_{test} - P_{control})$

$\alpha = 0.05$ ($Z_{0.025} = 1.96$)

$\beta = 0.2$ ($Z_{0.2} = 0.84$)

The study protocol was approved by the Ethics Committee of the Faculty of Dentisty, Prince of Songkla University (EC5507-26-L).

### 2. Oral examination

The examination of dental caries status of the subjects was performed by dentists using WHO probe (#621) and mouth mirror under unit light. The scoring system was adapted from the WHO's criteria, 1997. [93] The dental status of each examined teeth was categorized as: S = Sound surface. D = Dental caries with cavitated lesion.

**3. Bacterial sampling and cultivation**

The 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 were 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 spreaded on Beerens agar plate. After 2 to 7 days of incubation at 37°C under an 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).

**4. Isolation of clinical bifidobacteria strains**

At least four colonies looked like bifidobacteria of each primary plate were isolated and purified. The provisional identification of bifidobacteria was based on a colonial morphology, a gram-positive, cell morphology, and a negative catalase reaction. Then all isolates were kept and stored at -80°C in storage media until used.

**5. Presumptive of genus identification for *Bifidobacterium***

The presence of fructose-6-phosphate phosphoketolase (F6PPK), the key enzyme of the glucose catabolic pathway in the genera of the bifidobacteriaceae family, was determined as described by Scardovi.[1] Bacterial cells were culture on MRS agar for 18-24 h. The bacteria cells were washed in 1 ml of sodium phosphate buffer in a microfuge tube, then centrifuged to remove any supernatant. A sodium phosphate buffer was added and disrupted the cells by adding CTAB (hexadecyltrimethylammonium bromide)(0.00045 g/ml). The sample was mixed by vortex and incubated for 10 min. 50 µl aliquots of the cells were transferred to three wells of the microtiter plate. 12 µl of the sodium fluoride (NaF, 6 mg/ml) - iodoacetic iodoacetate solution (NaI, 10 mg/ml) were added to each well. Then 12 µl of fructose-6-phosphate (80 mg/ml) were added to two of the wells (test) but not to the third well, which served as a negative control. The plates were then incubated at 37°C for 30 minutes. The reaction was stopped by adding 75 µl of hydroxylamine solution (13.9 g/ml) to each well and leaving it at 37°C for 10 minutes. Then 50 µl of trichloroacetic acid solution (15% weight/volume) and 50 µl of 4 M hydrochloric acid were added. 50 µl of ferric chloride solution (5% weight/volume in 0.1 M hydrochloride acid) were also added. A reddish-violet colour indicates fructose-6-phosphate

phosphoketolase activity.

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

A total number of 167 strains of oral bifidobacteria were isolated from 50 caries-active children and 50 caries-free subjects. The bacterial DNA samples were prepared using a Genomic DNA Extraction Kit (RBC Bioscience, Taipei, Taiwan) following the manufacturer's protocol for gram-positive bacteria. The *Bifidobacterium* isolates were identified to species levels by restriction fragment length polymorphism analysis of polymerase chain reaction (PCR-RFLP)-amplified 16S ribosomal RNA (rRNA) genes by the methods of Teanpaisan & Dahlen.[58] Briefly, the 16S rRNA gene 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) programmed as follows: initial heat activation at 95°C for 15 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 2 min, then a primer extension at 72°C for 1.5 min, and a final extension step at 72°C for 10 min. The PCR products of 16S rRNA genes were individually digested with *HpaII* (New England Biolap, 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 gene sequencing. The following reference panel strains were used for comparative identification: *Bifidobacterium longum* CCUG 28903, *Bifidobacterium breve* CCUG 30511A, *Bifidobacterium dentium* CCUG 18367, *Bifidobacterium scardovii* CCUG 13008A, *Alloscardovia omnicolens* CCUG 31649 and *Scardovia inopinata* CCUG 35729.

### 7. Identification of *Bifidobacterium* spp. using DGGE

The uncertain bifidobacterial strains such as *B. longum* and *B. breve* were further identified by using methods of Denaturing Gradient Gel Electrophoresis (DGGE). DGGE is a

technique used for separating DNA fragments of PCR products according to their mobilities under increasingly denaturing conditions of gel gradient. The primers were HAD-1-GC (5'CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGGACTCCTACGGGAGG CAGCAGT-3') and HAD-2 (5'-GTATTACCGCGGCTGCTGGCAC-3'), which were designed at V2-V3 region of 16S rRNA gene. The 25 µl PCR reaction mixture contained 100 ng of DNA template, 1.0 µM of each primer, 2.5µl 10xbuffer with 2.0 mM $MgCl_2$, 1.0 U of Taq DNA polymerase, and 0.2 mM of each dNTP. Amplification was proceeded using a GeneAmp PCR System 2400 (Applied Biosystems, Foster, CA) programmed as follows: initial heat activation at 94°C for 4 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 2 30 s, primer extension at 68°C for 1 min and a final extension step at 68°C for 7 min. DGGE analysis was performed on optimized gels, using a 10% polyacrylamide gel with gradient of 35 to (70% v/v) denaturant and electrophoresis running time adjusted to 6 h at 120 V. The reference strains were also used for comparative analysis.

**8. Identification of *Bifidobacterium* spp. using 16S rRNA gene sequencing**

The use of 16S rRNA gene sequence (the most conserved DNA in cells) informatics is to show evolutionary relatedness among microorganism for identification into genus and species. The chromosomal DNA of bifidobacteria were extracted and was kindly performed in DNA sequencing by the Scientific Equipment Center of Prince of Songkla University. DNA sequencing was done by using an ABI PRISM Big Dye Terminator Kit and ABI PRISM 377 genetic analyzer (Applied Biosystems). In a 50-µl volume, PCR mixture consisted of 500 ng template, 0.8 µl Terminator Ready Reaction Mix (Applied Biosystems), and 3.2 pmol of each universal primer (8UA and 1492R primers). PCR was performed at 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min for a total of 25 cycles using the Gene Amp®PCR System 2400 (Applied Biosystems). Analysis of the alignment of percentages homology for the sequences was performed using the blast programs (http://www.ncbi.nlm.nih.gov/ BLAST/).

**9. Adhesion assay for oral *Bifidobacterium* spp.**

Keratinocytes are the predominant outermost layer of cell type in epidermis of the skin. The oral squamous carcinoma cell line of H357 keratinocyte used in this study, was a

kind gift from Professor Paul Speight of the University of Sheffield, UK. The keratinocyte cells were grown and maintained in keratinocyte growth medium containing three parts Dulbecco's modified Eagle's medium (DMEM) plus 1 part Ham's F-12 nutrient mixture supplemented with 10% foetal 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). To harvest cells for experiments, the cells were lifted from a culture plate by trypsinization with 0.25% trypsin–0.05% EDTA at 37°C for 10 to 15 min and collected by centrifugation. The keratinocyte cells were subcultured in 24-well plates at approximately $10^5$ cells/well and were grown at 37°C in 5% $CO_2$ for 2 days to reach confluence.

The adhesion assay was performed on fixed keratinocyte cells was a modification of the methods described by Kintarak *et al*. [94] Each tested *Bifidobacterium* strain was grown anaerobically overnight in 10 ml MRS broth with 0.05% L-cysteine hydrochloride at 37°C under an anaerobic conditions (10% $H_2$, 10% $CO_2$ and 80% $N_2$). The bacterial cells were harvested and washed twice with phosphate buffered saline that contained 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 the plates were incubated at 37°C in a 5% $CO_2$ for 1 h. Non adherent bacteria were washed off and then the adherent bacteria plus intracellular bacteria were quantified as the adhesion.

To quantitate invasion, 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 2 h at 37°C in a 5% $CO_2$ and then washed twice with PBS. To determine the number of bacteria, the keratinocyte cells were trypsinized with 0.05% trypsin-EDTA and lysed with 0.1% Triton X-100. After 15 min of incubation at 37°C, a serial 10-fold dilutions of adherent bacteria were then plated in duplicate on MRS agar for bifidobacteria counting and the counting plates were then incubated at 37°C anaerobically for 48 h. Data were expressed as Log CFU per well.

Total adhesion or internalization was reported as a percentages from duplicates according to the formula of total adhesion or internalization as follow: (%) = ($A_{0\ or}\ A_1$/A) x 100, where A and $A_0$were $\log_{10}$ number of bacterial cells (CFU $ml^{-1}$) before (A) and after total adhesion

($A_{0)}$ or internalization ($A_1$). Adhesion (externalization) was calculated as total adhesion minus by internalization.

**Bacterial vaginosis investigation**

**1. Clinical examination**

Participants were interviewed using the structured questionnaire, regarding medical history, contraception, sexual activity and including vaginal discharge. The normal vaginal discharge is floccular in consistency, white, non-homogeneous and viscous. A small number of PMN leukocytes may be seen. The pH is below 4.5, usually between 3.8 and 4.2. The predominant organisms are lactobacilli, large gram-positive rods. In contrast, the vaginal discharge of women with BV present five criteria: homogeneous discharge, positive amine test, clue cells, pH more than 4.5 and absent lactobacilli. Diagnosis of BV can be made if the patient fulfils 3 out of 5 criteria.[95]

**2. Subjects**

The study sample comprised of 120 subjects (18-to 60-year-old woman) who attended the Gynecology clinic, Songklanagarind Hospital in Hat Yai, Thailand. They had regular monthly cycle or pregnant. The studies groups consisted of 60 healthy women and 60 women with bacterial vaginosis. The diagnosis of bacterial vaginosis was based on at least 3 out of 5 indicators of modified Amsel's criteria : homogeneous discharge, elevated vaginal pH (pH >4.5), production of fishy amine odor upon the addition of 10% potassium hydroxide (KOH), presence clue cells more than 20% of the total vaginal epithelial cells and absent gram-positive rods.[95, 96] The vaginal discharge of healthy women had no more than two of these five characteristics, and none contained clue cells. Exclusion criteria included the following: use of systemic antibiotics within the 1 week prior to sampling, ovariectomized, menstruating and disease HIV. Informed consent was obtained from all subjects. The study protocol was approved by the Ethics Committee of the Faculty of Medicine, Prince of Songkla University.

The pilot study was performed by testing 22 women with BV and healthy group. The subjects of 7 BV (31.82%) and 2 healthy women (9.1%) were found positive for bifidobacteria. The number of subjects (n) was calculated as follows:

**Estimated sample size**

The number of subjects (n) was calculated as follows

$$n = [2(Z\alpha_{/2} + Z\beta)^2 P(1-P)]/\Delta^2$$

$$= 44$$

$$P = (P_{test} + P_{control})/2$$

$P_{test}$= Prevalence of bifidobacteria in BV subjects from pilot study was 31.82%; ($P_{test}$= 0.32)

$P_{control}$= Prevalence of bifidobacteria in healthy groups from pilot study was 9.1%; ($P_{control}$ = 0.09)

$\Delta = (P_{test} - P_{control})$

$\alpha =$ 0.05 ($Z_{0.025}$= 1.96)

$\beta =$ 0.2 ($Z_{0.2}$= 0.84)

**3. Bacterial sampling and cultivation**

A sterile swab was rolled over high vaginal wall and placed in sterile screw cap tubes containing 3 ml of sterile reducing transport fluid (RTF). The specimens were collected from the transport tube by centrifugation at 5,000 g for 5 min and resuspended in 1 ml phosphate buffered saline pH 7.0 (PBS, contained 0.05% L-cysteine hydrochloride). Ten-fold dilution series of each sample was made in PBS, and 0.1 ml of the diluted sample was spread on Beerens agar plate. After 2-7 days of incubation at 37°C under anaerobic condition (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 the same or different colonial appearance were collected and were initially identified as bifidobacteria based on being gram-positive rods, giving catalase negative and presence of the key enzyme fructose-6-phosphate phosphoketolase (F6PPK) from the glucose catabolic pathway as described by Scardovi.[1] After culture purification, all isolates were kept at -80°C until used.

For the isolation of vaginal bifidobacterial strains and presumptive identification for the genus *Bifidobacterium* were performed as previously described in Materials and Methods 1.4 and 1.5, respectively.

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

A total number of 139 isolates of bifidobacteria isolated from 20 of 60 women with BV and 7 of 60 healthy women were identified to species levels by PCR-amplified 16S rRNA genes PCR-RFLP according to the method of Teanpaisan and Dahlen.[97] Detailed of the methods were followed as previously described in Materials and Methods 1.6.

**5. Adhesion assay for vaginal bifidobacteria**

The HeLa cells, a continuous cell line that originated from a human cervical cancer cells, was used to assess adherence ability. The cells were grown and maintained in Dulbecco's modified Eagle's minimal essential medium (DMEM; HyClone Laboratories Inc., Logan, UT, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; HyClone), penicillin (100 U/ml), and streptomycin (100 µg/ml), then kept in the incubator at 37°C with 95% (v/v) humidified air and 5% (v/v) $CO_2$ at 37$^{o}$C. To harvest cells for experiments, the cells were lifted from a culture plate by trypsinization with 0.25% trypsin–0.05% EDTA at 37°C for 7 to 10 min and collected by centrifugation. The HeLa cells were subcultured in 24-well plates at approximately $10^5$ cells/well and were grown at 37°C in 5% $CO_2$ for 2 days to reach confluence.

The adhesion assay was performed with a modification method of Le Blay *et al*.[98] Each tested *Bifidobacterium* strain was grown anaerobically 18-24 h in 10 ml MRS broth supplement with 0.05% L-cysteine hydrochloride at 37°C. The bacterial cells were harvested and washed twice with PBS. A bacterial inoculum containing approximately $10^8$ CFU/ml suspended in DMEM was added to each well, and the plates were incubated at 37°C in a 5% $CO_2$ for 60 min. Non adherent bacteria were washed off and then the adherent bacteria plus intracellular bacteria were quantified as total adhesion. For internalization, the procedure was the same as the one mentioned above. After washing off the non-adherent bacteria, 1 ml of a solution containing 100 g $ml^{-1}$ of ampicillin in DMEM was added to each well to kill extracellular bacteria. The plates

were incubated with ampicillin for 60 min at 37°C in a 5% $CO_2$ and then washed twice with PBS. To determine the number of bacteria, the HeLa cells 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.

Total adhesion or internalization was reported as a percentages from duplicates according to the formula of total adhesion or internalization as follows: (%) = ($A_0$/A) x 100, where A and $A_0$ were $\log_{10}$ number of bacterial cells (CFU $ml^{-1}$) before and after total adhesion or internalization. Adhesion (externalization) was calculated as total adhesion minus by internalization.

**6. Bacterial adhesion to solvents**

The microbial adhesion to hydrocarbon (MATH) test was performed according to the modified methods of Xu *et al*.[23] The bifidobacterial strains used in this study were from dental caries and BV subjects. The adhesion to xylene (apolar solvent) demonstrates the hydrophobic surface characteristic of bacteria. The affinities to chloroform (polar acidic solvent) and ethyl acetate (polar basic solvent) describe electron donor (basic) and electron acceptor (acidic) characteristics of bacterial cell surface, respectively. Bacterial cells were suspended in PBS to concentration of $10^8$ CFU/ml. A volume of 3 ml bacterial suspension was mixed with 1 ml of solvents; xylene, chloroform, or ethyl acetate. The mixture was vortexed for 1 min and allowed to stand for 30 min to separate into two phases. The aqueous phase was measured at room temperature, and its absorbance at 600 nm was measured. The results were reported as a percentage from triplicates according to the formula MATS (%) = 1- ($A_t$ / $A_0$) × 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 three groups: those with low (0-35%), moderate (36-70%), or high (71-100%) hydrophobicity or charge surfaces.

**7. Biofilm assay**

Biofilm formation was examined in 96-well flat bottom plates as previously described by Patterson *et al*.[99] Two strains of bifidobacteria of each species showing high adhesion property were selected from dental caries and BV subjects were used in this study.

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 the plates were air-dried for 1 h. Biofilms were stained with 200 µl crystal violet for 30 min, then wells were washed gently to remove the crystal violet, and the plates were air-dried. After the biofilms had been visually analysed and imaged using a flatbed scanner, the crystal violet was solubilized with 100 µl 33% (v/v) acetic acid per well. For quantitative results, the absorbance of $A_{600}$ of the solubilized crystal violet was measured, using the 96-well plate reader.

**8. Cytotoxicity assay**

The bifidobacterial strains from previously described in Materials and Methods 2.7 were used in this study. The epithelial cells from either keratinocyte cell line or HeLa cell line were seeded into a 96- well tissue culture plate at a density of $10^5$ cells per well as previously described by Patterson *et al.*[99] After 24 h, or when a monolayer of cells had formed, the tissue culture medium was removed, and a suspension containing approximately $10^5$ CFU/ml of bacteria in medium was added to each well. The monolayers were analysed for cytotoxicity by light microscopy of 6 fields under 20x magnification at time interval 1, 2, 3 and 4 h. The tests were performed in duplicate. The cytotoxic activity was determined as cytotoxicity score showing round cells or lysed cells. Cytotoxicity was scored as follows: 0, no difference between the experimental well and the control; 1, < 25 % cells were rounded; 2, 26–50 % cells were rounded; 3, 51-75 % cells were rounded; 4, >75 % cells were rounded, with partial disruption of the monolayer; 5, complete disruption/absence of the monolayer.

**9. Statistical analysis**

Data were expressed as mean ± standard deviation (SD). Chi-square test was used to assess the difference of the prevalence of each studied group. The distribution of *Bifidobacterium* species was calculated as a percentage. The relationship between the presence of *B. dentium* of caries-active and caries-free subjects was evaluated using chi-square. Normality

and homogeneity of variance assumption of bifidobacterial count were assessed by Shapiro-Wilk test, while adhesion properties and surface charges were assessed by kolmogorov-smirnov test. Nonparametric tests were used due to non-normal distribution of the data. The Mann-Whitney U-test was used to compare the difference of bifidobacteria between healthy or disease groups. The comparative differences of adhesion properties and surface charges between species within the same group were evaluated using the Kruskall-Wallis test and the Mann-Whitney U-test. The comparative differences of adhesion properties and surface charges between healthy or disease were evaluated using the Mann-Whitney U-test. The cytotoxicity was scored on the basis of percentage evaluation. The biofilm formation was expressed as Mean $\pm$ SD of absorbance. The correlation between hydrophobicity and surface charges was assessed using Spearman's rho test. All analyses were performed using the Statistical Package for Social Sciences (version 17.0; SPSS Inc, Chicago, IL, USA). The differences were considered significant when $p<0.05$.

# 3. RESULTS

This chapter presents a summary of the thesis results based on Paper I and Paper II.

Paper I: Prevalence and Adhesion Properties of Oral *Bifidobacterium* species in Caries-active and Caries-free Thai Children. ***Walailak J. Sci. & Tech***. Accepted for publication. August 11, 2016.

Paper II: Assessment of Prevalence, Adhesion and Surface Charges of *Bifidobacterium* spp. Isolated from Thai Women with Bacterial Vaginosis and Healthy Women. ***J Med Assoc Thai***. Accepted for publication. June 7, 2016.

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

### 1. Identification of oral *Bifidobacterium* spp.

A total number of 167 strains of oral *Bifidobacterium* spp. isolated from 50 caries-active and 50 caries-free children were used for identification by presumptive methods and molecular biology techniques as shown in (Table 3). The *Bifidobacterium* spp. were positive in the following characteristics as an anaerobe, gram positive, pleomorphic branching of Y or rod shape and catalase negative. All bifidobacteria possessed F6PPK enzyme using fructose-6-phosphate as substrate. The isolated strains showed different PCR products from PCR-RFLP methods, compared to the reference strains as shown in the same mobility of the bands or molecular weight (Fig. 8). The PCR products from *B. dentium* were different from *B. bifidum, B. breve* and *B. longum*, while PCR products from *B. breve* and *B. longum* showed same mobility of the bands. The PCR products without endonuclease digestion of *B. breve* and *B. longum* were further run on DGGE electrophoresis. The fingerprint results showed different mobility pattern DNA mobility on DGGE as compared to the reference strains (Fig. 9). For uncertain one strains with different mobility band from the reference strains were further performed by DNA sequencing of 16S rRNA gene. The DNA sequence was blasted to the DNA gene bank, expressed as percentages homology for the sequence. For oral bifidobacteria, there were B. *dentium* 102 strains, *B. breve* 14 strains and *B.longum* 7 strains from caries-active subjects and B. *dentium* 44 strains from caries-free group.

**Table 3.** Identification of oral *Bifidobacterium* spp. isolated from dental caries-active and caries-free children

Presumptive identification

| **Methods** | ***Bifidobacterium* species** |
|---|---|
| Gram positive | + |
| Pleomorphic Y or rod shape | + |
| Catalase | - |
| F6PPK | + |

Molecular identification

| **Methods** | ***B. dentium* (N)** | ***B. breve* (N)** | ***B. longum* (N)** |
|---|---|---|---|
| PCR-RFLP | 146 | 21 | |
| DGGE | ND | 14 | 7 |
| DNA sequencing | 1 | ND | ND |

ND = Not done

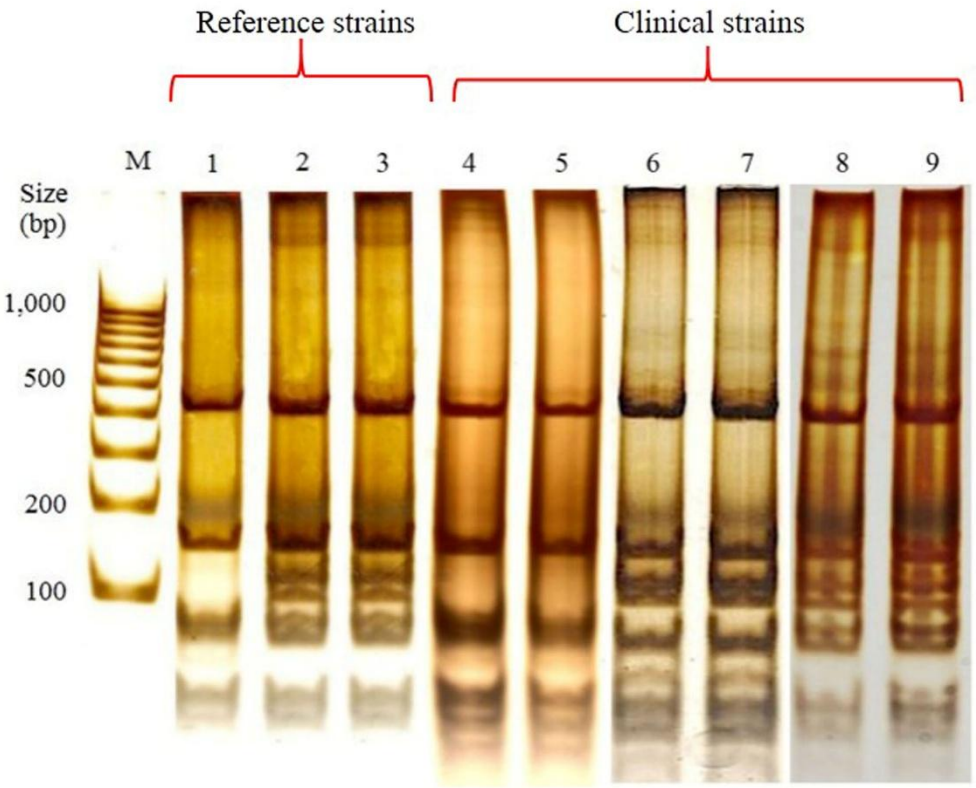


**Fig. 8.** PCR-RFLP products digested with *Hpa*II of *Bifidobacterium* spp. lane M, Molecular weight maker, lane 1, *B. dentium* CCUG 18367, lane 2, *B. breve* CCUG 30511A, lane 3, *B. longum* CCUG 28903, lane 4-5, *B. dentium*, lane 6-7, *B. bifidum* and lane 8-9, *B. breve* or *B. longum*.

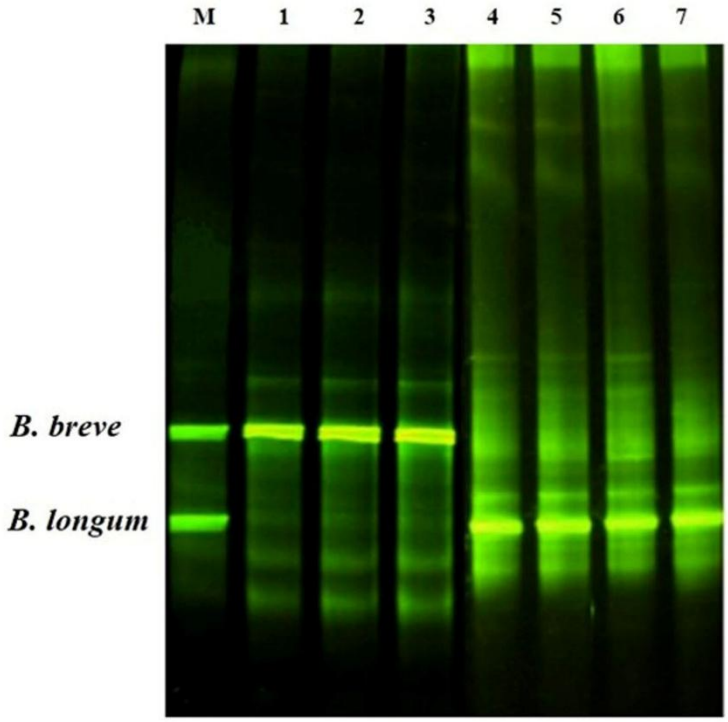


**Fig. 9.** DGGE fingerprints of *B. breve* CCUG 30511A and *B. longum* CCUG 28903 of maker reference strains (M). Lane 1-3, *B. longum* and Lane 4-7, *B. breve*.

**2. 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 showed significantly higher than 24% (12/50) from caries-free group ($p<0.05$) as shown in (Table 4). The quantity of bifidobacteria of caries-active subjects was increased about 1,000 times significant higher than caries-free group ($p < 0.05$). The caries-active subjects exhibited a bacterial count of 5.8 ± 0.9 Log CFU/ml, whereas the lower number of bacteria found in caries-free group was 2.7 ± 0.8 Log CFU/ml.

**Table 4.** 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 ($p < 0.05$) of the prevalence of bifidobacteria from caries-active and caries-free groups. [2]Mann-Whitney U Test indicates a statistical significance ($p < 0.05$) of total count from caries-active and caries-free groups.

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

The distribution of oral bifidobacteria in occlusal teeth of 50 caries-active and 50 caries-free subjects are demonstrated in (Table 5). 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 caries-active and *B. dentium* (100%) (44/44) for caries-free group. The predominant strains were *B. dentium* in both groups. The prevalence of *B. dentium* isolated from caries-active significant higher than *B. dentium* isolated from caries-free.

**Table 5.** Distribution of oral *Bifidobacterium* spp. in caries-active and caries-free groups

| Species | All subjects, N=36 | | Caries-free groups, N=12 | | Caries groups, 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) |

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

**4. Adhesion ability of oral *Bifidobacterium* spp. to keratinocyte cells**

The adhesion ability of the isolated oral bifidobacteria to keratinocyte cells are shown in (Fig. 10) and (Fig. 11). All isolated bifidobacteria were able to adhere and invade culture cells and had higher internalization (invasion) than externalization (adhesion) ability to the tested cells. There were strain variations in adhesion properties of total adhesion externalization and internalization abilities. The adherent strains expressing as percentage of total adhesion, externalization and internalization as 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 (Fig. 10), while *B. dentium* from caries-free group showed 66%, 15% and 50%. Notably, the total adhesion ability of *B. dentium* from caries-active subjects was significantly higher than caries-free group (Fig. 11).

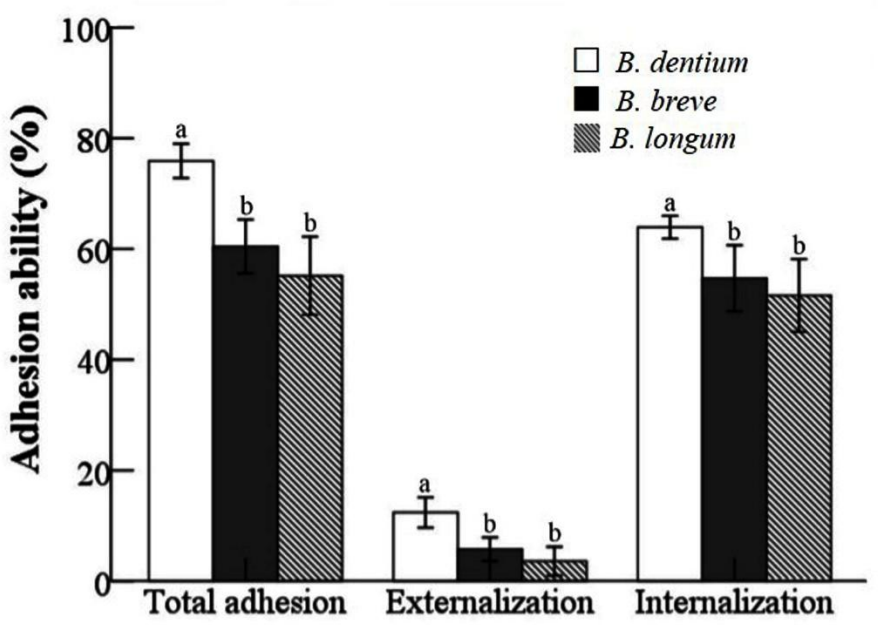


**Fig. 10.** Adhesion ability to keratinocyte cells of oral *B. dentium*, *B. breve* and *B. longum* from caries-active subjects. Shown in figure are the average ± standard deviation (% adhesion ability) of total adhesion, externalization and internalization of *Bifidobacterium* spp. Different superscript letters indicate significant differences of each parameter at $p < 0.05$.

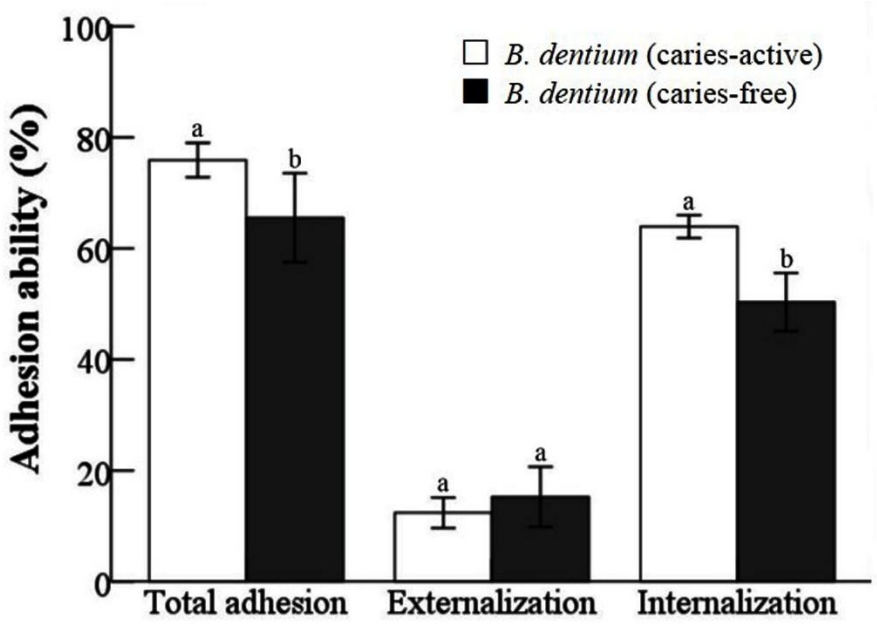


**Fig. 11.** Adhesion ability to keratinocyte cells of oral *B. dentium* from caries-active and and caries-free groups. Shown in figure are the average ± standard deviation (% adhesion ability) of total adhesion, externalization and internalization of *Bifidobacterium* spp. Different superscript letters indicate significant differences of each parameter at $p < 0.05$.

### 5. Physicochemical properties of oral *Bifidobacterium* spp. cell surfaces

The cell surface hydrophobicity and surface charges of the isolated oral bifidobacteria are shown in (Fig. 12) and (Fig. 13). The adhesive characteristics of bifidobacteria were analyzed by measuring adhesion to xylene (hydrophobicity), chloroform and ethyl acetate which describe electron donor (basic) and electron acceptor (acidic) characteristics of bacterial surface, respectively. It is in the accordance with Lewis theory of acid-base reactions, acids accept pairs of electrons (electron acceptor) and bases donate pairs of electrons (electron donor). The bifidobacteria showed strain variations for adhesive characteristics and exhibited a moderate to high degree of hydrophobicity (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%), *B. longum* (82%, 98% and 46%) for caries-active subjects and *B. dentium*

(86%, 98% and 59%) for caries-free group, respectively (Fig. 12). Notably, the strain *B. dentium* from both groups showed no significant differences in the surface properties (Fig. 13).

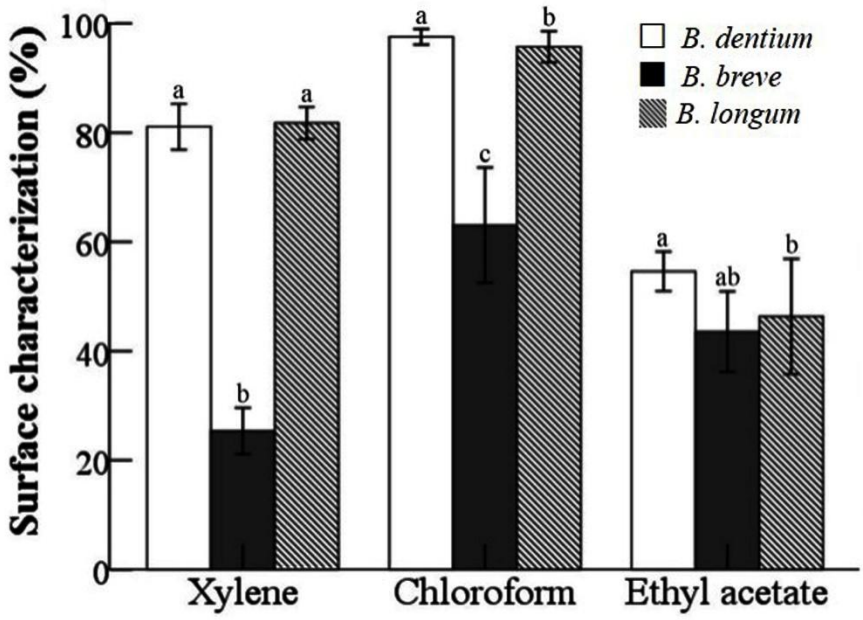


**Fig. 12.** Adhesion characteristics to different solvents of oral *Bifidobacterium* spp. of *B. dentium*, *B. breve* and *B. longum* from caries-active subjects. Shown in figure are the average ± standard deviation (% surface characterization) of hydrophobicity (affinity to xylene), surface charges (affinity to chloroform and ethyl acetate) of *Bifidobacterium* spp. Different superscript letters indicate significant differences of each parameter at $p < 0.05$.

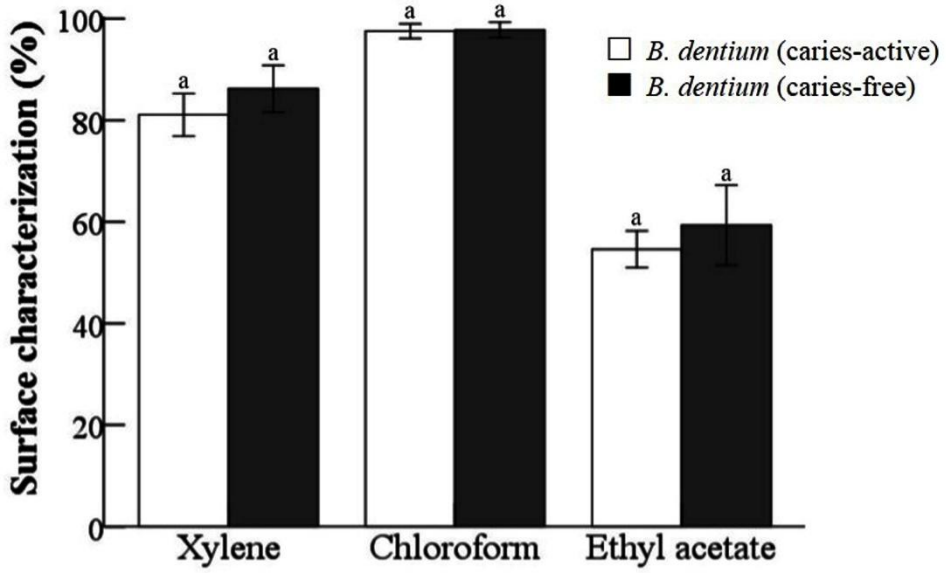


**Fig. 13.** Adhesion characteristics to different solvents of oral *B. dentium* from caries-active and caries-free groups. Shown in figure are the average ± standard deviation (% surface characterization) of hydrophobicity (affinity to xylene), surface charges (affinity to chloroform and ethyl acetate) of *B. dentium* from both groups showed no significant differences in the surface properties. Different superscript letters indicate significant differences of each parameter at $p < 0.05$.

### 6. Biofilm formation of oral *Bifidobacterium* spp.

Biofilm formation of the oral bifidobactetia accessed by biofilm assay is shown in (Fig. 14). All selected strains of oral *B. dentium* were able to form biofilm. The prominent *B. dentium* from caries-active subjects and *B. dentium* CCUG 18367 showed biofilm formation significantly higher than *B. dentium* from caries-free group, and *S. mutans* ATCC 25175.

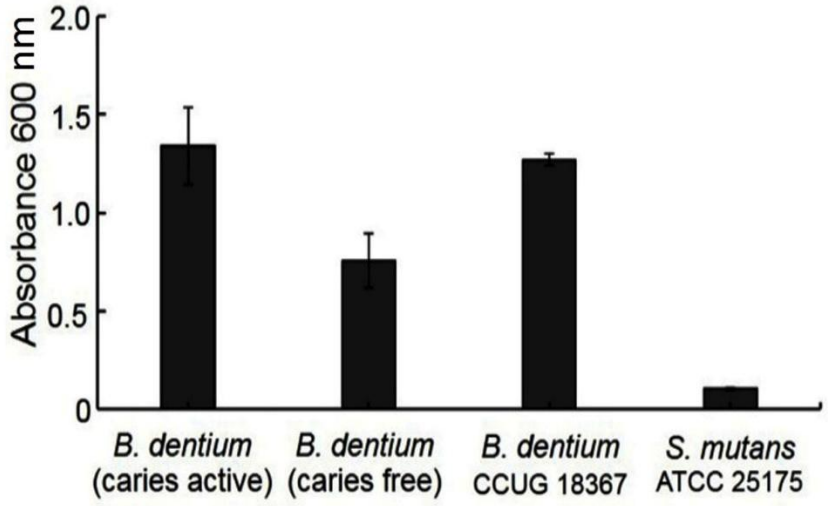


**Fig. 14.** Biofilm formation capability of oral *B. dentium* isolated from caries-active and caries-free subjects comparing with the reference strains of *B. dentium* CCUG 18367 and *S. mutans* ATCC 25175. Shown in figure are the average ± standard deviation (absorbance 600 nm) of the tested bacteria.

### 7. Cytotoxicity of oral *Bifidobacterium* spp.

The oral bifidobacteria were tested for the ability to form round cells or lyse the oral keratinocyte H357 cells *in vitro*. All selected strains of *B. dentium* from caries-active and caries-free subjects and *S. mutans* ATCC 25175 were unable to form round cells or lyse cells showing no cytotoxic activity on the cultured cells.

**Assessment of Prevalence, Adhesion and Surface Charges of *Bifidobacterium* spp. Isolated from Thai Women with Bacterial Vaginosis and Healthy Women (Paper II)**

**1. Identification of vaginal *Bifidobacterium* spp.**

A total number of 139 isolates of vaginal bifidobacteria isolated from 20 of 60 women with BV and 7 of 60 healthy women were presumptive identification and then identified into species levels by 16S rRNA genes PCR-RFLP, DGGE and DNA sequencing as previously described in Materials and Methods 1.6, 1.7 and 1.8. For vaginal bifidobacteria, there were *B. bifidum* 53 strains, *B. longum* 38 strains, *B. breve* 6 strains and *B. dentium* 4 strains from BV subjects and B. *bifidum* 21 strains, *B. longum* 11 strains, *B. breve* 6 strains from healthy group.

**2. Prevalence of vaginal *Bifidobacterium* spp. in women with BV and healthy group**

The prevalence of *Bifidobacterium* spp. in women with BV was 33.3% (20/60) which showed significantly ($p<0.05$) higher than healthy women 11.7% (7/60) as shown in (Table 6). The total count in women with BV (8.9 ± 3.4 log CFU/ml) was about 1,000 times higher than healthy subjects (5.7 ± 2.9 log CFU/ml).

**Table 6.** Prevalence of vaginal *Bifidobacterium* spp. in women with BV and healthy group

| Characteristic | No. of subjects | Prevalence, N (%) | Total count (Log CFU/ml) |
|---|---|---|---|
| Healthy | 60 | 7 (11.7) | 5.7 ± 2.9 |
| Bacterial vaginasis | 60 | 20[1](33.3) | 8.9[2]± 3.4 |

[1]Chi-square test indicates a statistical significance ($p < 0.05$) of the prevalence of bifidobacteria from women with BV and healthy subjects. [2]Mann-Whitney U Test indicates a statistical significance ($p < 0.05$) of total count from women with BV and healthy subjects.

### 3. Distribution of vaginal *Bifidobacterium* spp. in women with BV and healthy group

The distribution of vaginal *Bifidobacterium* spp. in vaginas of 60 women with BV and 60 healthy women are demonstrated in (Table 7). The frequent species of *B. bifidum*, *B. longum, B. breve* and *B. dentium* were found in women with BV, while the strains of *B. bifidum*, *B. longum* and *B. breve* were detected in healthy group. The women with BV possessed *B. bifidum* (52.5%) (53/101), *B. longum* (37.6%) (38/101), *B. breve* (5.9%) (6/101) and *B. dentium* (4.0%) (4/101), while healthy women showed *B. bifidum* (55.3%) (21/38), *B. longum* (28.9%) (11/38), and *B. breve* (15.8%) (6/38).

**Table 7.** Distribution of vaginal *Bifidobacterium* spp. in women with BV and healthy group

| Species | All subjects, N=27 | | Healthy women, N=7 | | Women with BV, N=20 | |
|---|---|---|---|---|---|---|
| | No. of subjects (%) | No. of isolates (%) | No. of subjects (%) | No. of isolates (%) | No. of subjects (%) | No. of isolates (%) |
| *B. bifidum* | 18 (66.7) | 74 (53.2) | 6 (85.7) | 21 (55.3) | 12 (60.0) | 53 (52.5) |
| *B. longum* | 14 (51.9) | 49 (35.3) | 4 (57.1) | 11 (28.9) | 10 (50.0) | 38 (37.6) |
| *B. breve* | 4 (14.8) | 12 (8.6) | 2 (28.6) | 6 (15.8) | 2 (10.0) | 6 (5.9) |
| *B. dentium* | 1(3.7) | 4(2.9) | ND | ND | 1 (5.0) | 4 (4.0) |

ND = Not detected

### 4. Adhesion ability of vaginal *Bifidobacterium* spp. to HeLa cells

The adhesion assessments of the isolated bifidobacteria to HeLa cells are shown in (Fig. 15) and (Fig. 16). All isolated bifidobacteria were able to adhere culture cells. The most adherent strains were bifidobacteria isolated from BV subjects and expressed as percentage of adhesion ability as follows: *B. dentium* (71.0%), *B. bifidum* (57.7%), *B. longum* (44.6%), and *B. breve* (49.0%). While the strains from healthy women showed lower degrees of adhesive ability

being *B. bifidum* (53.4%), *B. longum* (39.4%) and *B. breve* (49.4%) (Fig. 15). Notably, in both groups, there were no significantly differences in the adhesion property of the strains *B. bifidum*, *B. longum* and *B. breve* (Fig. 16).

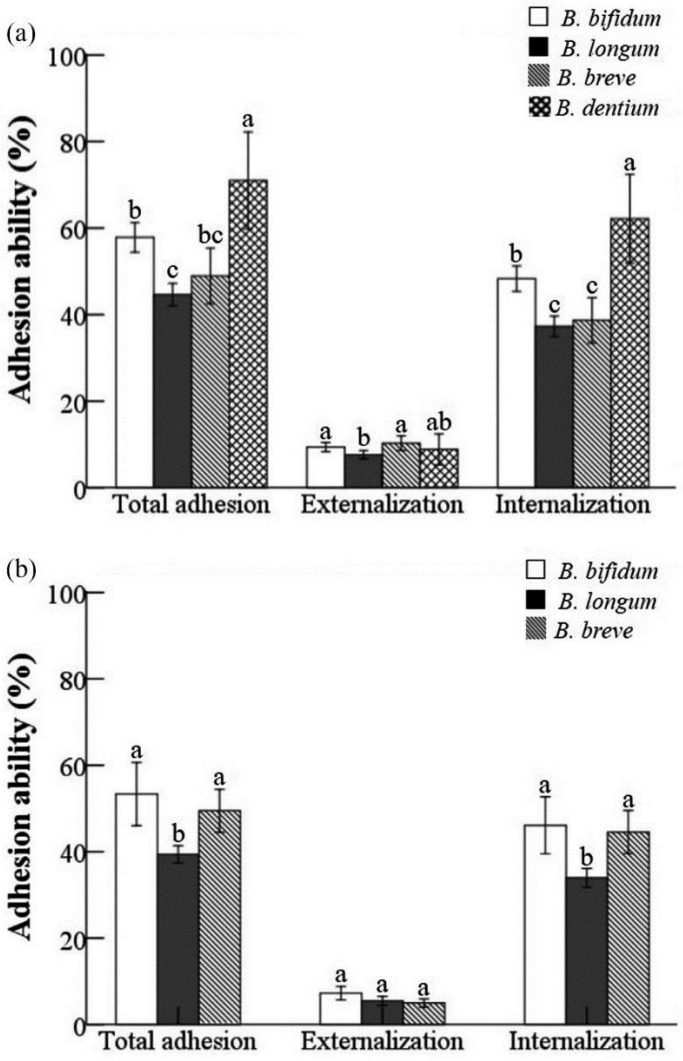


**Fig. 15.** Adhesion ability to HeLa cells of vaginal *Bifidobacterium* spp. of (a) *B. bifidum*, *B. longum*, *B. breve* and *B. dentium* from women with BV and (b) *B. bifidum*, *B. longum* and *B. breve* from healthy subjects. Shown in figure are the average ± standard deviation (% adhesion ability) of total adhesion, externalization and internalization of *Bifidobacterium* spp. Different superscript letters indicate significant differences of each parameter at $p < 0.05$.

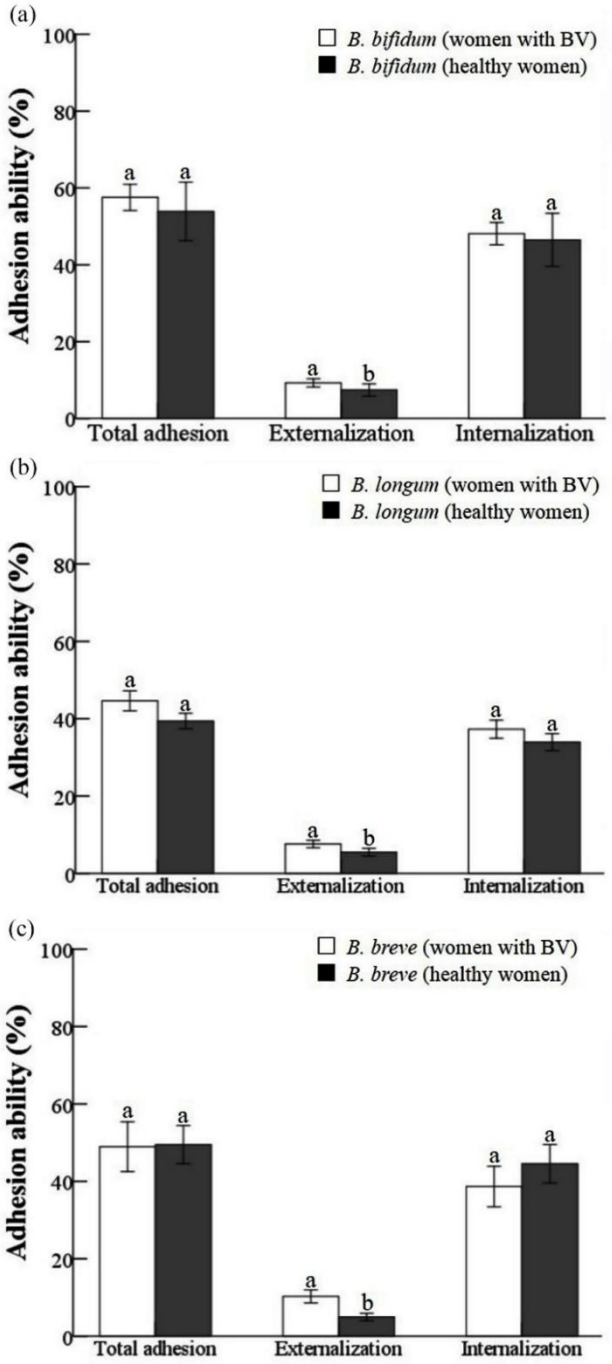


**Fig. 16.** Comparison of adhesion ability to HeLa cells of vaginal *Bifidobacterium* spp. of (a) *B. bifidum*, (b) *B. longum*, and (c) *B. breve* between women with BV and healthy subjects. Shown in figure are the average ± standard deviation (% adhesion ability) of total adhesion, externalization and internalization of *Bifidobacterium* spp. Different superscript letters indicate significant differences of each parameter at $p < 0.05$.

**5. Physicochemical cell surface properties of vaginal *Bifidobacterium* spp.**

The adhesive characteristics of bifidobacteria to xylene, chloroform and ethyl acetate are shown in (Fig. 17) and (Fig. 18). The bifidobacteria showed strain variations for the adhesive characteristics and exhibited a low to high degree of hydrophobicity and hydrophilic surface charges. The hydrophobicity (affinity to xylene) and hydrophilic surface charges (affinity to chloroform and ethyl acetate) of bifidobacteria from women with BV were *B. dentium* (94.4%, 99.5% and 88.9%) and *B. bifidum* (84.1%, 95.8% and 75.6%), followed by *B. longum* (35.9%, 52.5% and 38.7%) and *B. breve* (14.2%, 23.0% and 24.6%), while healthy women showed *B. bifidum* (44.1%, 57.7% and 39.8%), *B. longum* (30.2%, 58.1% and 34.9%), and *B. breve* (16.4%, 38.3% and 32.0%). Interestingly, the adhesion abilities ($r_s$ = 0.592, 0.570 and 0.501, $p < 0.001$) of the bifidobacteria from women with BV were in correlation with the hydrophobicity (affinity to xylene) and surface charges (affinity to chloroform and ethyl acetate) (Fig. 19), while the relationship between adhesion ability and hydrophobicity and surface charges from healthy women was not found.

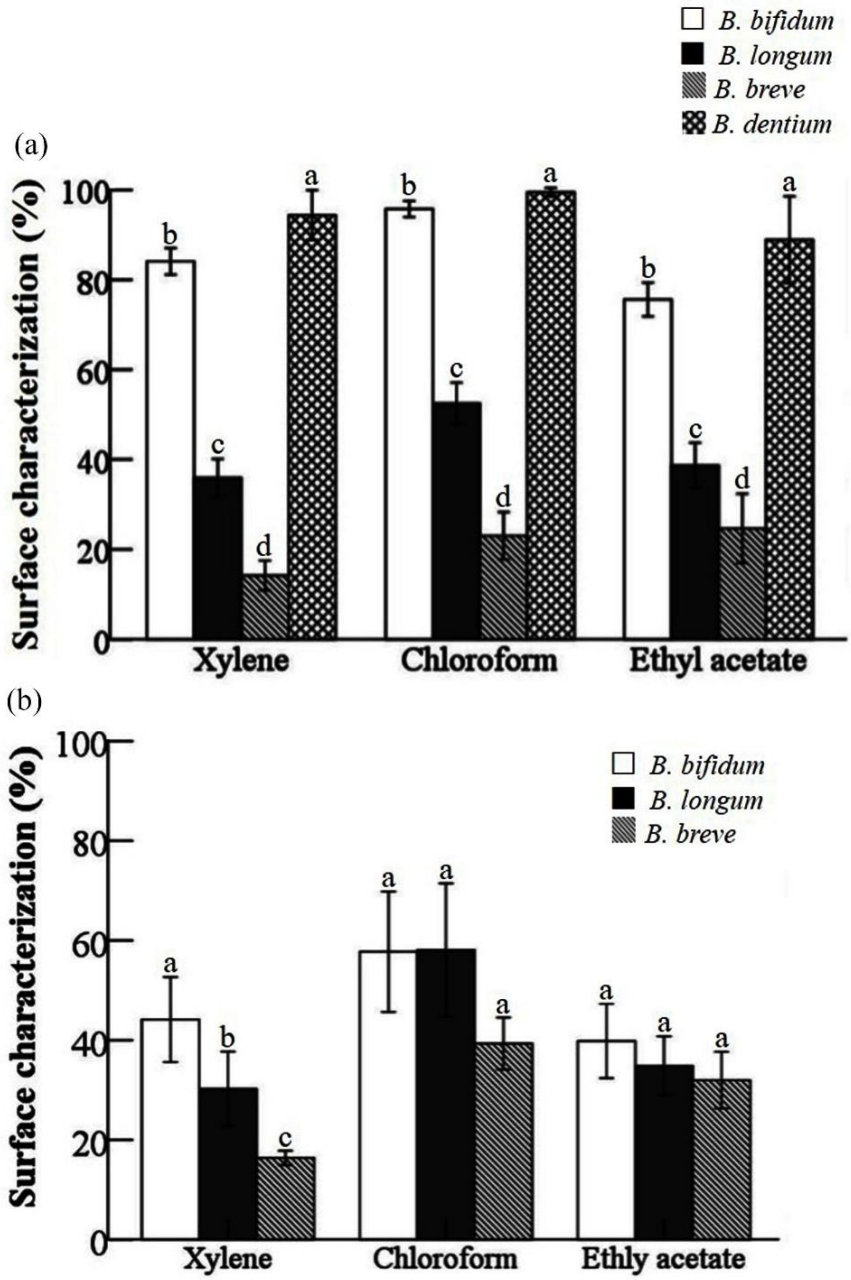


**Fig. 17.** Adhesion characteristics to different solvents of vaginal *Bifidobacterium* spp. of (a) *B. bifidum*, *B. longum*, *B. breve* and *B. dentium* from women with BV and (b) *B. bifidum*, *B. longum*, *B. breve* from healthy subjects. Shown in figure are the average ± standard deviation (% surface characterization) of hydrophobicity (affinity to xylene), surface charges (affinity to chloroform and ethyl acetate) of *Bifidobacterium* spp. Different superscript letters indicate significant differences of each parameter at $p < 0.05$.

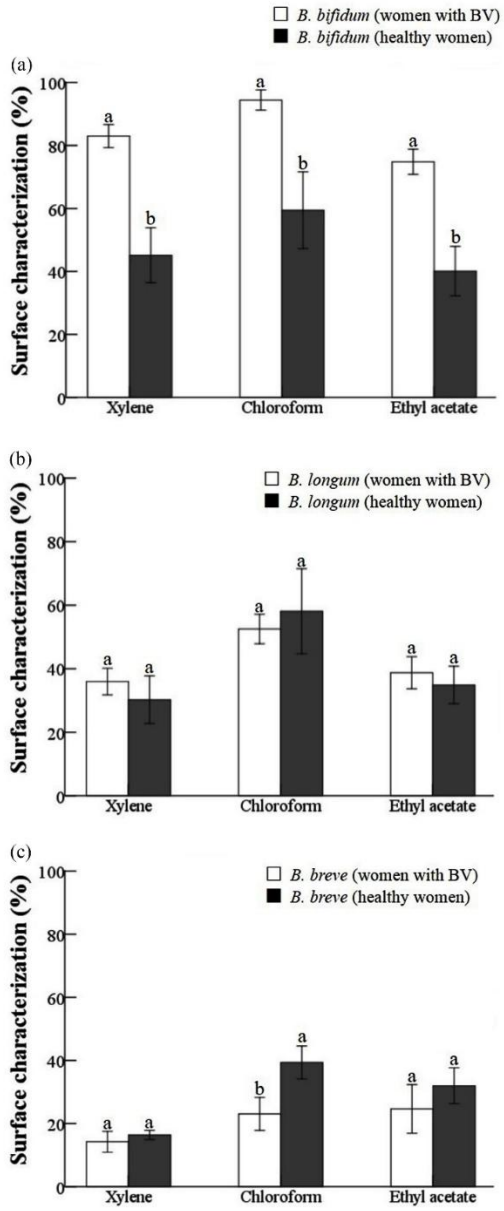


**Fig. 18.** Comparison of adhesion characteristics to different solvents of vaginal *Bifidobacterium* spp. of (a) *B. bifidum*, (b) *B. longum* and (c) *B. breve* between women with BV and healthy subjects. Shown in figure are the average ± standard deviation (% surface characterization) of hydrophobicity (affinity to xylene), surface charges (affinity to chloroform and ethyl acetate) of *Bifidobacterium* spp. from both groups. Mann-Whitney U Test indicates a statistical significance ($p \leq 0.05$) of *B. bifidum* from women with BV and healthy subjects. Different superscript letters indicate significant differences of each parameter at $p < 0.05$.

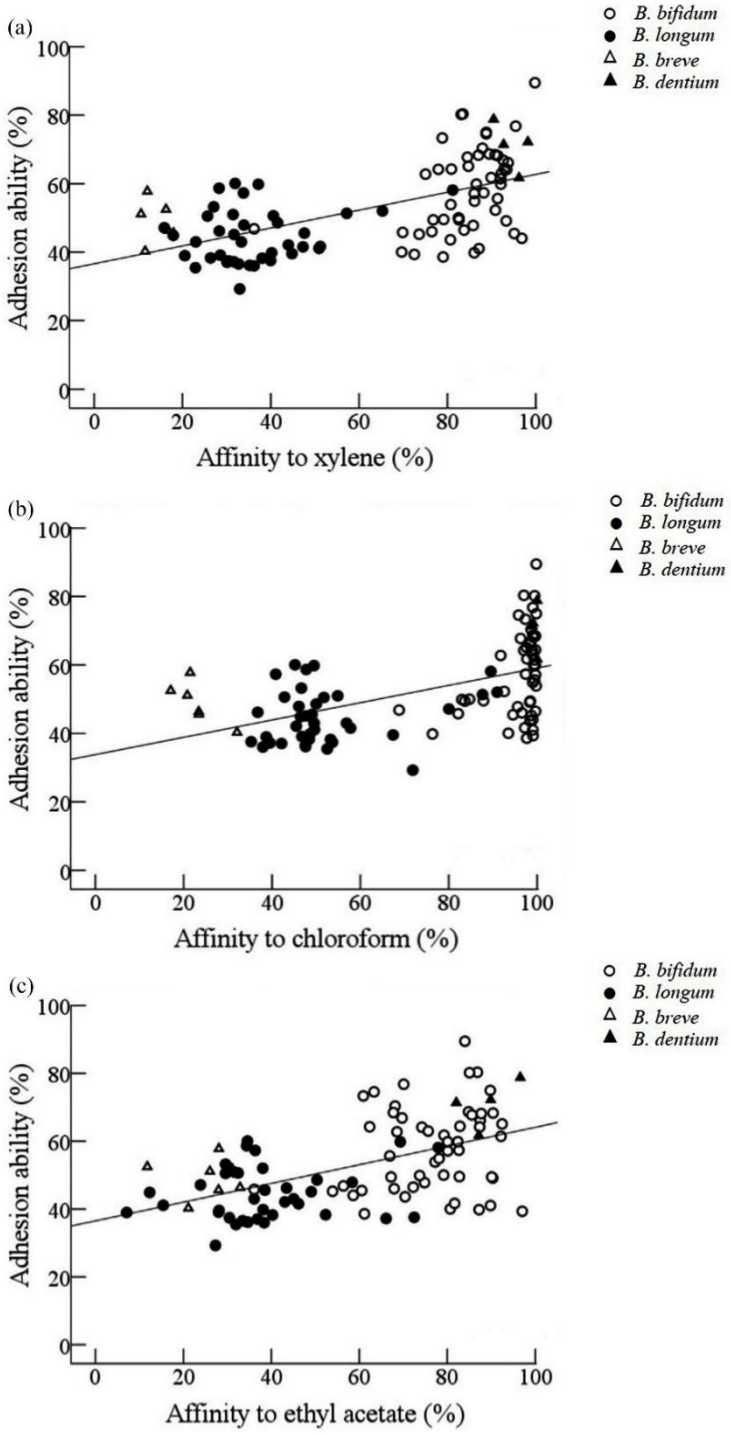


**Fig. 19.** Correlation of bifidobacterial strains isolated from women with BV between adhesion ability and surface characteristics. Spearman's rho test indicates as follows: (a) hydrophobicity: affinity to xylene, $r_s = 0.592$, $p < 0.001$, (b) surface charges: affinity to chloroform, $r_s = 0.570$, $p < 0.001$ and (c) affinity to ethyl acetate, $r_s = 0.501$, $p < 0.001$.

**6. Biofilm formation of vaginal *Bifidobacterium* spp.**

Biofilm formation of vaginal bifidobactetia were accessed by biofilm assay is shown in (Fig. 20). All selected strains of bifidobacteria were able to form biofilm. The prominent *B. dentium* from women with BV showed the highest biofilm formation than the other strains of *B. bifidum*, *B. longum, B. breve* and *G. vaginalis* CCUG 3717.

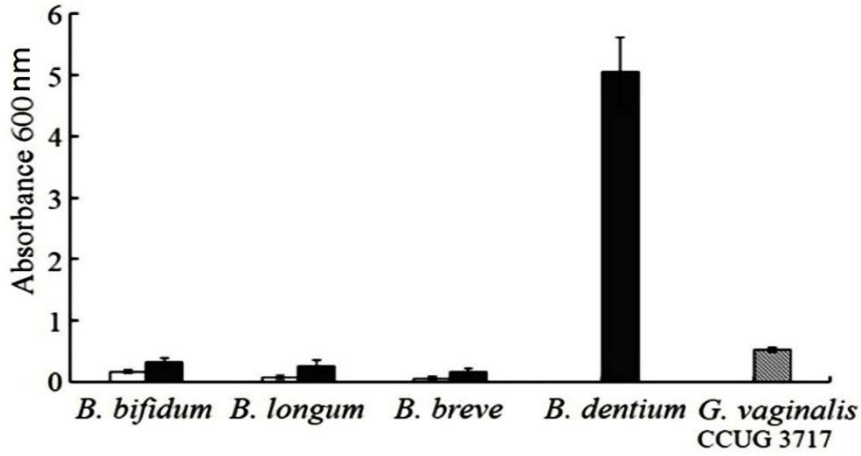


**Fig. 20.** Biofilm formation capability of vaginal *Bifidobacterium* spp. of *B. bifidum*, *B. longum, B. breve* and *B. dentium* from BV subjects and *B. bifidum*, *B. longum* and *B. breve* from healthy groups, comparing with the reference strain of *G. vaginalis* CCUG 3717. Shown in figure are the average ± standard deviation (absorbance 600 nm) of the tested bacteria.

**7. Cytotoxic activity of vaginal *Bifidobacterium* spp.**

The vaginal bifidobacteria were tested for the ability to form round cells or lyse the epithelial HeLa cells *in vitro* as shown in (Table 8). All selected bifidobacteria were able to show varying degree of cytotoxic activity. The most potential cytotoxic strain was *B. dentium* from women with BV showing score as 3 on the cytotoxic scale (51-75 % cells were rounded or lysed), while the other strains from both groups of *B. bifidum, B. longum* and *B. breve* and *G. vaginalis* CCUG 3717 showed lower score as 1 on the cytotoxic scale (< 25 % cells were rounded or lysed).

**Table 8.** Cytotoxicity score of vaginal *Bifidobacterium* spp. from healthy and BV subjects

| Strains | Cytotoxicity score* | |
|---|---|---|
| | **Healthy women** | **Women with bacterial vaginosis** |
| Medium control | 0 | 0 |
| *B. bifidum* | 1 | 1 |
| *B. longum* | 1 | 1 |
| *B. breve* | 1 | 1 |
| *B. dentium* | ND | 3 |
| *G. vaginalis* | ND | 1 |

* Cytotoxicity was scored as follows: 0, no difference between the experimental well and the control; 1, < 25 % cells were rounded; 2, 26–50 % cells were rounded; 3, 51-75 % cells were rounded; 4, >75 % cells were rounded, with partial disruption of the monolayer; 5, complete disruption/absence of the monolayer. ND = Not done

**8. Comparison of adhesion ability of oral and vaginal *B. dentium* to different cultured cell line**

Comparison of adhesion ability of oral and vaginal *B. dentium* to keratinocyte and HeLa cells between *B. dentium* isolated from caries-active subjects and women with BV is shown in (Fig. 21). By using keratinocyte cell line, oral *B. dentium* showed higher percentage of adhesion ability than vaginal *B. dentium* of total adhesion (82.6%, 57.9%), externalization (17.8%, 9.9%) and internalization (64.7%, 48.0%), respectively. On the contrary, the vaginal *B. dentium* had higher adhesion ability to HeLa cells than the oral *B. dentium* of total adhesion (71.0%, 57.6%), externalization (8.9%, 2.1%) and internalization (62.2%, 54.7%), respectively.

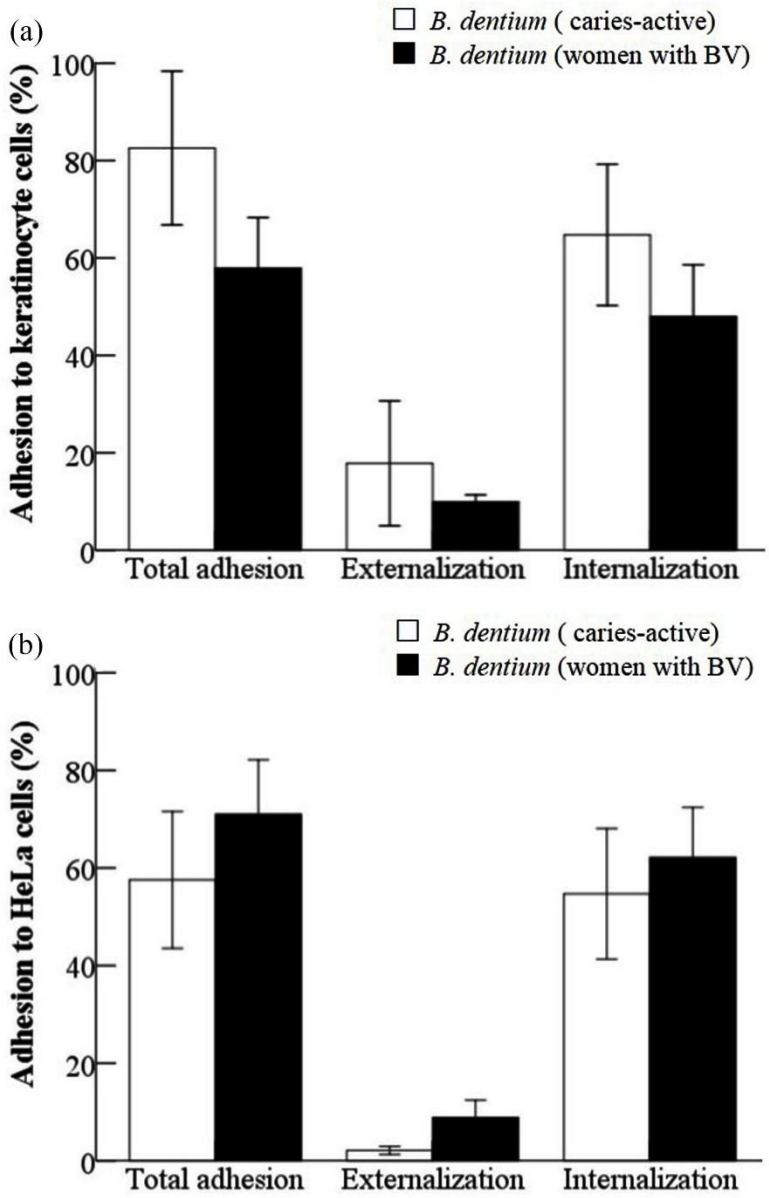


**Fig. 21.** Comparison of adhesion ability to (a) keratinocyte cells and (b) HeLa cells between *B. dentium* isolated from caries-active subjects and women with BV. Shown in figure are the average ± standard deviation (% adhesion ability) of total adhesion, externalization and internalization of *B. dentium* from caries-active subjects and women with BV.

# 4. DISCUSSION

In this study, the sample size was calculated preliminarily from pilot study. For oral sample size, the prevalence of bifidobacteria in healthy group was not found from pilot study whereas the prevalence rate was increased when all samples were tested. Presumably, the oral sample size was reasonable for further study. For the vaginal sample size from pilot study was almost similar to the test which was reliable for this study.

In this study, the prevalence of bifidobacteria in caries-active children (44%) was significantly higher than caries-free group (24%). The prevalence rate of bifidobacteria in Thai children was lower than previous reports from occlusal lesions (66%) [2] and caries saliva (95%).[4] While the bifidobacteria were isolation from only 9% of the caries-free children. Additionally, the bifidobacteria were formerly undetected in the mouths of healthy individuals [9] and in active root caries lesions.[76] Interestingly, in this study, the species of *B. dentium* was the major strain isolated from caries-active (82.9%) and caries-free (100%) groups. The results are in agreement with previous report in that the predominant *B. dentium* was found in children of caries lesions (86%), leathery lesions (100%) and sound plaques (80%).[6] While the other analyses refer the 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%).[5] 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, host habitat of particularly pH in such environment would select dominant strains of bifidobacterial species. The *B. dentium* grows well in around neutral pH and could maintain viability with mutans streptococci,[28] since saliva pH is around 7.0 for caries-free and 6.5 for caries-active group.[63] The frequently found strain of *B. dentium* associated with dental caries in children [4] and adults.[2] 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 number of pilus-like appendages around cell surface than the other bifidobacterial species [100] indicating that *B.dentium* is the most potential strain to adhere host tissue.

In dental caries, the association of bifidobacteria in caries lesions arise from crucial colonization ability to form biofilm as well as dental plaque and the production of invasive acids. The *B. dentium* isolated in this study showed dominant in the implication of adhesion ability in caries lesion and were able to form biofilm. In fact, dental biofilm comprises a coating formed with saliva and food debris on the surface of teeth. The development of caries biofilm is a complex and multiple process of various factors. Biofilm formation occurs in a few minutes up to few months mediated by adsorption of protein and carbohydrate intake, followed by immobilization of oral bacteria and consolidation of exopolysaccharide production from bacteria and finally colonization to form biofilm.[91] The accumulation of microbial consortia generate acids when sugar and/or carbohydrate supplied which results in dental disease.[101] In oral environment, bifidobacteria (*B. breve* and *B. longum*) may not attach hard tissue of teeth surface as evidenced by low affinity to hydroxyapatite.[11] However, the strain *B. dentium* as demonstrated in this study showed high adhesion ability to keratinocyte cell line. The *B. dentium* possessed high affinity to xylene and chloroform indicating the bacterial cell surface properties exhibited hydrophobic and hydrophilic of electron donor (basic) characteristics in the adherence to host tissue. Interestingly, *B. dentium* was able to adhere to keratinocyte cells representing epithelium of oral mucosa in the buccal cavity. The *B. dentium* in the free form in saliva and/or adhered cells may in turn directly attach food supply to form denture plaque which finally enhances the proliferation of *B. dentium* and other cariogenic bacteria. Importantly, the ecological factors of food consumption containing sugar and carbohydrate mediate acidogenic and aciduric bacteria to metabolize glucose or sucrose. The end products of glycolysis are mixed acids which lower environmental pH as acidogenic stage. The plaque biofilm pH of caries-free and extreme caries-active subjects are drop after sugar intake from pH 7.1 to 5.5 and pH 5.5 to 4.3, respectively.[63] It is therefore recommended that oral health care should be daily performed after each meal as best practices for particular children after consumption sugar-containing foods in order to prevent dental caries. It is interesting to note the strain of *B. dentium* and the cariogenic *S. mutans* ATCC 25175 had no cytotoxic effect on the cultured cells indicating that the bacteria had no enzyme or protein that can injure oral tissue and no virulence potential to produce oral discharge.

For bacterial vaginosis, the prevalence of bifidobacteria in women with BV (33.3%) in this study showed a significantly higher frequency than healthy women (11.7%),

which was in agreement with Rosenstein *et al*. in reported that *Bifidobacterium* spp. were found in 12% healthy women.[3] Whereas, the prevalence rate of bifidobacteria in non-pregnant women with BV in this study was considerably lower than previous reports of 83% in West African women[20] and 41-94% in Western pregnant women with BV.[3] This is not surprising since the prevalence of various etiological agents in BV varied according to age, sexual behavior, immunodeficiency diseases, pregnancy, individual vaginal flora, and regional variation. In this study, the vaginal strains of *B. bifidum, B. longum, B. breve* and *B. dentium* were found which were also in accordance with a previous report.[36, 85] An increase in 1,000 folds in number of bacteria in BV subjects indicated a strong bacterial association with BV.[102] Interestingly, all bifidobacteria from BV and healthy subjects were less acid tolerance at pH 4.0 indicating the sporadic isolation of the bifidobacterial strains as *B. dentium, B. bifidium, B. longum* and *B. breve* which supports the low prevalence rate of bifidobacteria found in healthy group. Additionally, the strain of *B. dentium* could not be isolated from healthy vagina, while only small proportions of *B. dentium* were found in BV subjects which is in agreement with a previous report.[36] It might be that different species bifidobacteria prefer to colonize in different appropriate environments of host habitat. On the other hand, the bifidobacteria may be transmitted to vaginal site by rectal-vaginal or oral-vaginal route. Since the bifidobacteria present at perianal region but are not found at vaginal site were *B. adolescentis, B. longum, B. breve, B. bifidum and B. catenulatum,*[103] while the *B. dentium* is usually found in the oral cavity.[5] Normally, the bifidobacteria are able to tolerate growth in alkaline pH. While *B. dentium* and *B. longum* are able to survive in complex media in both acidic and alkaline pH.[28] However, *B. dentium* is less able to grow in acidic condition and the growth drastically dropped within 2 h. at pH 4.0.[28] Since normal pH of vagina is 3.8-4.5 may not be optimal for growth of *B. dentium* compared to the pH that rises to a level between 5.5 and 6.0 in women with BV.

Furthermore, cell adhesion plays a crucial role for bifidobacteria to persist in the vaginal tract in order to exert their biological actions. While, there is limited information concerning the adhesion ability and cell surface charges of vaginal bifidobacteria. In this study, the adhesion property and surface charges of *Bifidobacterium* spp. were investigated using HeLa cells as an *in vitro* model. The adhesion ability to cultured cells of the bifidobacteria isolated from BV was higher than healthy groups. Particularly, *B. bifidum* and *B. dentium* showed higher

adhesion ability than *B. longum* and *B. breve* in women with BV. Recently, Foroni *et al*. demonstrated various human intestinal bifidobacteria such as *B. bifidum, B. dentium, B. longum,* and *B. adolescentis* having pilus-like appendages on the cell surface which might be involved in bacterial colonization.[38] Additionally, in this study, the morphology of *B. bifidum* and *B. dentium* isolated from BV subjects showed more branch formation than bifidobacteria isolated from healthy group (data not shown) suggesting that each bifidobacterial strain differs in the adhesion ability.[104] It is interesting to note that the *B. dentium* from BV subjects showed virulence potential of high biofilm formation and high cytotoxic activity which may indicated the association with the other polymicrobial infection in BV pathogenesis. The virulence factor of protein or enzyme produced from *B. dentium* would be interesting for further investigation.

Previous reports have demonstrated that bifidobacteria had variations in the adhesion ability and cell surface property. These properties have been assessed in an intestinal bifidobacterial strain of *B. longum* B6 and several probiotic lactobacilli using Caco-2 cell line originated from a human colonic adenocarcinoma and the bacterial cell surface had dominant hydrophobicity which was highly correlated with coaggregation ability suggesting a good relationship between *in vitro* adhesion and *in vivo* colonization.[23, 105] Notably, in this study, the vaginal *B. dentium* showed higher percentage of total adhesion ability to HeLa cells (71.0%) than keratinocyte cells (57.9%), indicating that vaginal *B. dentium* preferred to localize in the vaginal environment in the association of BV. Furthermore, in this study, *B. bifidum* and *B. dentium* isolated from women with BV exhibited higher hydrophobic and hydrophilic surface charges than *B. longum* and *B. breve*. The women with BV harboring *B. bifidum* and *B. dentium* showed significantly ($p<0.05$) higher properties of affinity to xylene, chloroform and ethyl acetate than the other strains, suggesting that surface characteristics of vaginal *B. bifidum* and *B. dentium* possessing hydrophobic and hydrophilic electron donor (basic) and electron acceptor (acidic) properties. The variety of cell surface characteristics of *B. bifidum* and *B. dentium* may be related to the adhesion property leading to colonize in the vaginal mucosa. In the fact that, hydrophobic and hydrophilic properties are depended on the density of hydrophobic amino acids and polysaccharides on the bacterial cell surfaces.[106] Therefore, it would be interesting to investigate further a more detailed of bacterial surface composition which is was mediated in mechanism of

adhesion ability of the bifidobacteria in order to understand the biological action in the BV association.

# 5. CONCLUSIONS

The present studies were performed to assess prevalence and adhesion properties of the bifidobacteria in oral cavity and vagina. For dental caries, the prevalence of oral bifidobacteria occurred significantly higher in caries-active Thai children (44%) than caries-free group (24%) with the increase in number of total bacteria in caries-active subjects. The predominant oral bifidobacterial species in both groups was *B. dentium* which showed adhesion ability and biofilm formation implying an important role of colonization to oral mucosa and in the association with caries process. It should be aware of the occurrence of oral bifidobacteria, a non-mutans streptocococi in dental caries. The potential cell surface structure of the oral bifidobacteria would be interesting to further investigation besides surface charges such as adhesin, receptor complementary to the binding site of adhesion or exopolysaccharide produced by bacteria that attached imbedded cells.

For bacterial vaginosis, the prevalence of vaginal bifidobacteria in women with BV (33.3%) was significantly higher than healthy women (11.7%) with increase the number of total count in BV subjects. All vaginal bifidobacteria from BV and healthy subjects were able to adhere cultured cells *in vitro*. The strains of *B. bifidum* and *B. dentium* showed high adhesion properties. Particularly, the *B. dentium* had high biofilm formation and cytotoxic activity implying an important role of colonization to vaginas of women with BV. The potential strain of *B. dentium* would be interesting to further investigate the production of protein, enzyme or toxin in the association with BV pathogenesis.

Interestingly, the vaginal *B. dentium* had higher adhesion ability to HeLa cells than keratinocyte cell line implying a better localization in vagina than oral environment and vice versa, the oral *B. dentium* was more in favor of colonization in the oral cavity than vaginal habitat.

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# APPENDICES

# APPENDIX A

Parada Utto, Rawee Teanpaisan and Supatcharin Piwat. Prevalence and Adhesion Properties of Oral *Bifidobacterium* species in Caries-active and Caries-free Thai Children. ***Walailak J. Sci. & Tech.*** Accepted for publication. August 11, 2016.

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

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**Running title:** Prevalence and Adhesion properties of *Bifidobacterium* spp.

**Abstract**

Several publications have reported the association of bifidobacteria with dental caries lesions but no data of prevalence and adhesion properties of oral *Bifidobacterium* spp. have been evaluated in Thai children. The objectives of this study were to compare prevalence of oral *Bifidobacterium* spp. in caries-active and caries-free Thai children and to characterize adhesion 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 adhesion ability, surface properties and biofilm formation. The prevalence of oral bifidobacteria in caries-active children (48%) (24/50) was significantly higher than caries-free group (24%) (12/50) ($p<0.05$) with total count 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 caries-active and *B. dentium* 100% (44/44) for caries-free group. All strains of bifidobacteria were able to adhere keratinocyte cell line *in vitro*. The adherent strains of *B. dentium* showed higher total adhesion ability in caries-active subjects (66%) than caries-free group (58%). The *B. dentium* showed strain variations in cell surface characteristics of hydrophobic and hydrophilic surface charges. The strains of *B. dentium* from both groups were able to form biofilm. In conclusions, the predominant strains of *B. dentium* had high adhesion ability to keratinocytes and biofilm forming capacity implying a role of colonization to 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 rod, pleomorphic branched, non-motile, non-spore-forming and possess fructose-6-phosphate phosphoketolase (F6PPK) to produce lactic acid as well as acetic acid as end products of glucose metabolism [4]. The bifidobacteria in 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- and subgingival plaques from clinical healthy teeth [8]. The other studies in England reported the isolation of the bifidobacteria from saliva of 95% of caries-active and from only 9% of caries-free children [3] and 96.8% of bifidobacteria found in saliva of caries-active older adults [1]. However, the information of the prevalence of oral bifidobacteria in Thai children has not yet been evaluated.

The caries-associated bifidobacteria may be incorporated into dental plaques where the acids are responsible for 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 adhesion ability of oral bifidobacteria and the capacity of biofilm formation on the surfaces of the teeth coating with saliva, food debris and bacterial consortia would be interesting for further investigation. The information of the adhesion property of the oral bifidobacteria and cell surface charges of hydrophobic and hydrophilic characteristics reflecting on colonization ability of the oral strains are still limited. It is postulated that the oral bifidobacteria may possess adherence to oral buccal cavity referring maintenance to colonize dental plaque. Therefore, the objectives of this study were to evaluate prevalence of bifidobacterial strains in Thai children of high risk caries-active and

caries-free groups and to investigate adhesion ability to keratinocyte cell line *in vitro*, biofilm formation as well as assessment of hydrophobic and hydrophilic surface charges of the oral bifidobacteria.

## Materials and methods

### Subjects and clinical examination

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

Total 100 children (aged 6 to 9 years old) of 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 sound teeth of 50 caries-free children and 50 samples collected from carious lesions of 50 caries 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 spreaded on Beerens agar plate. After 2 to 7 days of incubation at 37°C under an 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 the same or different colonial appearance were collected and were initially identified as bifidobacteria based on being gram-positive, pleomorphic rods, catalase negative and 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 used.

**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 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 gene 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 gene by DNA sequencing. The following reference panel strains were used for comparative identification: *Bifidobacterium longum* CCUG 28903, *Bifidobacterium breve* CCUG 30511A, *Bifidobacterium dentium* CCUG 18367, *Bifidobacterium scardovii* CCUG 13008A, *Alloscardovia omnicolens* CCUG 31649 and *Scardovia inopinata* CCUG 35729.

**Adhesion assay**

The H357 keratinocyte from 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 three 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 an anaerobic conditions (10% $H_2$, 10% $CO_2$ and 80% $N_2$). The bacterial cells were harvested and washed twice with phosphate buffered saline that contained 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 then 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 percentages from duplicates according to the formula of total adhesion or internalization as follows: (%) = ($N/N_0$) x 100, where $N_0$ and N were $\log_{10}$ number of bacterial cells (CFU/ml) before and after total adhesion or internalization. Adhesion (externalization) was calculated as total adhesion minus by 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. The 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 sec 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 three 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 two-strains of high adhesion 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 the plates were air-dried for 1 h. Biofilms were stained with 200 µl crystal violet for 30 min, then wells were washed gently to remove the crystal violet, and the plates were air-dried. After the biofilms had been visually analysed 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* species 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

### Results

#### 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 showed significantly higher than 24% (12/50) from caries-free group ($p<0.05$) as shown in **Table 1**. The quantity of bifidobacteria of caries-active subjects exhibited a bacterial count of 5.8 ± 0.9 Log CFU/ml, whereas the lower number of bacteria found in caries-free group was 2.7 ± 0.8 Log CFU/ml. The 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 caries-free group (**Table 2**).

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

The adhesion of the isolated oral bifidobacteria to keratinocytes are shown in **Figure 1**. All isolated bifidobacteria were able to adhere culture cells. There were strain variations in adhesion properties of total adhesion, externalization and internalization abilities. The adherent strains expressing as percentage of total adhesion, externalization and internalization as 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 1a**), while *B. dentium* from caries-free group showed 66%, 15% and 50% (**Figure 1b**).

**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 hydrophobicity (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%), *B. longum* (82, 98 and 46%) for caries-active subjects and *B. dentium* (86, 98 and 59%) for caries-free group respectively. Moreover, the strain *B. dentium* from both groups showed no significant differences in the surface properties.

**Biofilm formation of oral *Bifidobacterium* spp.**

Biofilm-forming bifidobactetia accessed 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 *B. dentium* from caries-free group.

**Discussion**

The prevalence rate of bifidobacteria in Thai children was lower than previous reports from occlusal lesions (67%) [7] and caries saliva (95%) [3]. While formerly the 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* were the major strains isolated from oral route in both groups. The results are in agreement with previous report in that the predominant bifidobacteria is *B. dentium* found in children of root surface plaque (80%), infected root caries lesions (100%) and soft active lesions (80%) [2]. While the other analyses referred the 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, host habitat of particularly pH in such environment would select 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 caries-free and 6.5 for caries-active group [19]. The frequently found strains of *B. dentium* are strongly in the association 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 number of pilus-like appendages around cell surface than other bifidobacterial species [20] indicating that *B. dentium* is the most potential strain to adhere host tissue. Previous reports demonstrated that bifidobacteria had variations in the adhesion ability and cell surface property [21]. These properties were assessed in an intestinal bifidobacterial strain of *B. longum* B6 and several probiotic lactobacilli using 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 crucial colonization ability to form biofilm as well as dental plaque and the production of invasive acids. The findings that *B. dentium* strains showed high ability of adhesion (both internalization and externalization) may explain high prevalence of *B. dentium* in plaque samples found in this study. Such ability may also affect the capability of biofilm formation of the *B. dentium* strains. Biofilm formation occurs in a few minutes up to few months mediated by adsorption of protein and carbohydrate intake, followed by immobilization of multispecies bacteria and 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 supplied which results in dental disease [23]. In oral environment, bifidobacteria (*B. breve* and *B. longum*) may not attach hard tissue of teeth surface as evidenced by low affinity to hydroxyapatite [10]. Whereas, the strains of *B. dentium* as demonstrated in this study showed

high adhesion ability to keratinocytes. Additionally, the *B. dentium* possessed high affinity to xylene and chloroform indicating the properties of bacterial cell surface exhibited hydrophobic and hydrophilic of electron donor (basic) characteristics in the adherence to host cells. Interestingly, *B. dentium* was able to adhere to keratinocytes representing epithelium of oral mucosa of gingival, cheek bulge and palate in oral cavity, this may facilitate the proliferation of *B. dentium.*

**Conclusions**

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

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**Figure legnds**

**Figure 1** Adhesion ability to 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 adhesion ability represents total adhesion (□), externalization (■) and internalization (▨). Data are expressed as mean ± standard deviation. Different superscript letters indicate significant differences of each parameter at $p < 0.05$.

**Figure 2** Adhesion characteristics to 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 ± standard deviation. 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 ± standard deviation.

**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 ($p < 0.05$) of the prevalence of bifidobacteria from caries-active and caries-free groups.
[2]Mann-Whitney U Test indicates a statistical significance ($p < 0.05$) of 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 ($p < 0.05$) of *B. dentium* from caries-active and caries-free groups. ND = Not detected

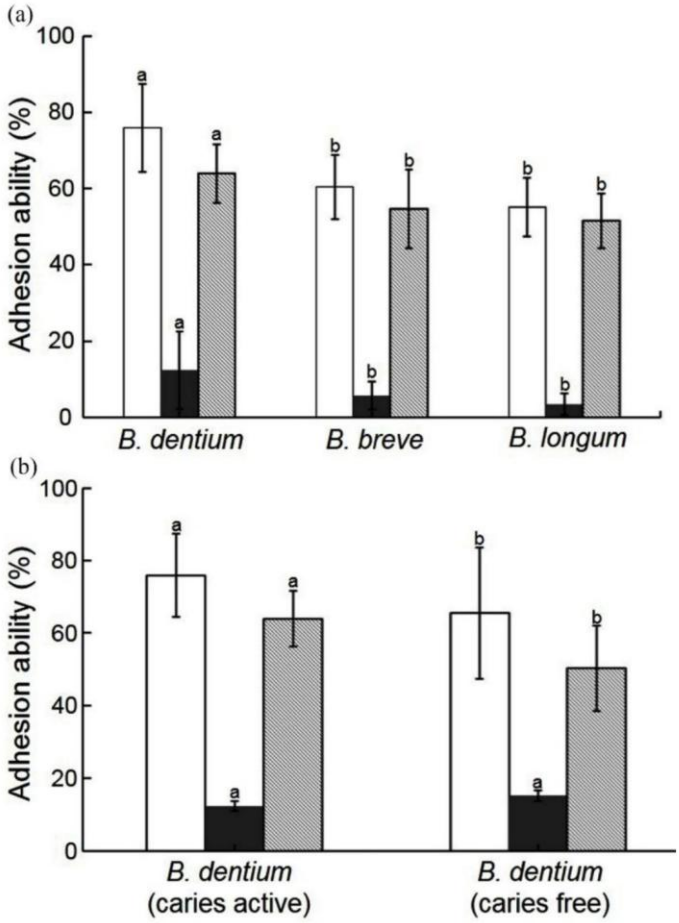


**Figure 1** Adhesion ability to keratinocyte cells of (a). *B. dentium*, *B.breve* and *B.longum* from caries-active subjects. (b). *B. dentium* from caries-active and caries-free groups. The adhesion ability represents total adhesion (□), externalization (■) and internalization (▨). Data are expressed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

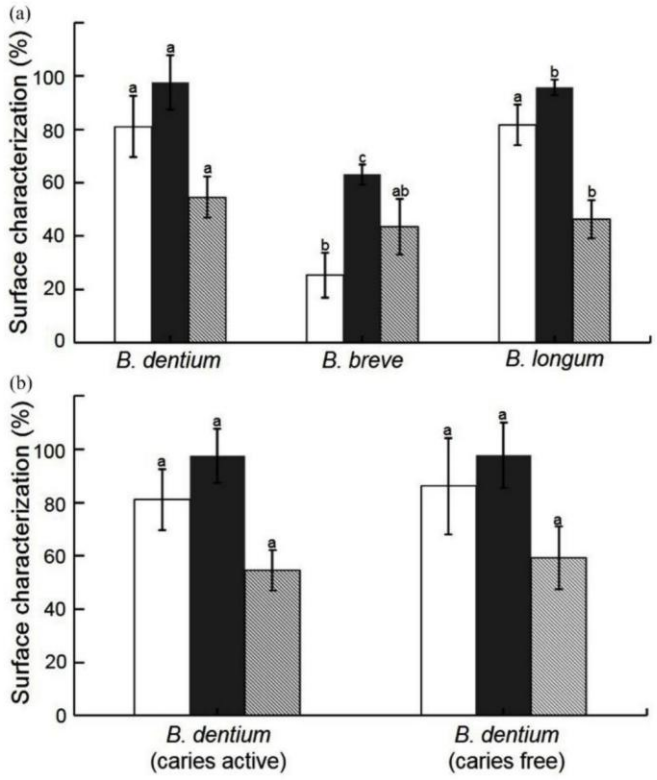


**Figure 2** Adhesion characteristics to different solvents of (a). *B. dentium*, *B.breve* and *B.longum* from caries-active subjects. (b). *B. dentium* from caries-active and caries-free groups. The solvents represent xylene (□), chloroform (■) and ethyl acetate (▨). Data are expressed as mean ± standard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

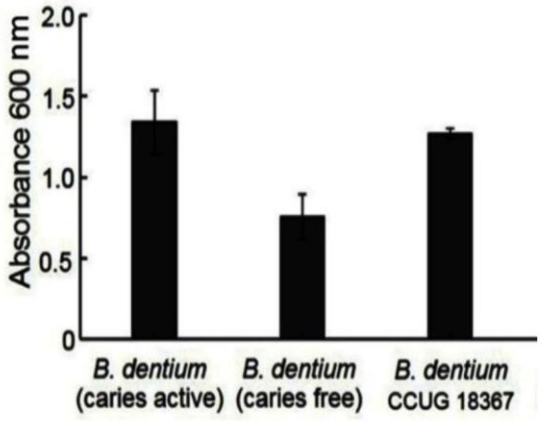


**Figure 3** Biofilm formation capacity of *B. dentium* isolated from caries-active and caries-free subjects and reference strains of *B. dentium* CCUG 18367. Data are expressed as mean ± standard deviation.

# APPENDIX B

Parada Utto, Rawee Teanpaisan, Supatcharin Piwat and Verapol Chandeying. Assessment of Prevalence, Adhesion and Surface Charges of *Bifidobacterium* spp. Isolated from Thai Women with Bacterial Vaginosis and Healthy Women. ***J Med Assoc Thai.*** Accepted for publication. June 7, 2016.

# Assessment of Prevalence, Adhesion and Surface Charges of *Bifidobacterium* spp. Isolated from Thai Women with Bacterial Vaginosis and Healthy Women

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**Abstract**

***Background****: Bifidobacterium spp. have been reported in women with BV, nevertheless data of prevalence and adhesion property of the vaginal bifidobacteria in Thai women with bacterial vaginosis (BV) is still limited.*

***Objective:*** *To determine prevalence of Bifidobacterium spp. and to evaluate adhesion ability and cell surface properties of bifidobacterial isolates from Thai women with BV compared to healthy subjects.*

***Material and Method:*** *A total number of 139 bifidobacterial isolates from 20 of 60 women with BV and 7 of 60 healthy women. The isolated strains were identified by molecular biology techniques and* were *examined for adhesion property and surface charges.*

***Results:*** *The prevalence of vaginal bifidobacteria in women with BV (33.3%) was significantly ($p<0.05$) higher than healthy women (11.7%) with total counts of 8.9 ± 3.4 Log CFU/ml and 5.7 ± 2.9 Log CFU/ml, respectively. The frequent species of B. bifidum, B. longum, B. breve and B. dentium were found in women with BV, while healthy women harbored B. bifidum, B. longum and B. breve. All vaginal bifidobacteria from BV and healthy subjects were able to adhere cultured cells in vitro. The adhesion ability of B. bifidum and B. dentium from BV subjects showed high degree of adhesion property and was in correlation with cell surface characteristics.*

***Conclusion:*** *The prevalence of vaginal bifidobacteria occurred significantly higher in women with BV than healthy group. The strains* of *B. bifidum and B. dentium showed high adhesion property which implied an important role of colonization in vaginas of women with BV.*

***Key words:*** *Prevalence, adhesion, bifidobacteria, bacterial vaginosis, surface charge*

**Introduction**

Bacterial vaginosis (BV) is one of the most common cause of abnormal vaginal discharge among women of reproductive age in the association with obstetric and gynaecologic complications[1-3]. The BV appears as a change in vaginal microbes, due to a decrease in the number of lactobacilli and overgrowth of various facultative or anaerobic bacterial species such as *Gardnerella vaginalis*, *Mobiluncus* spp., *Prevotella* spp.[4], and *Bifidobacterium* spp.[5].

The genus *Bifidobacterium* is gram-positive, anaerobic, pleomorphic branched, non-motile and non-spore-forming bacteria. The bifidobacteria were reported to be associated with BV in African[6] and Western women[7]. The prevalence of bifidobacteria varied from 12% in healthy controls, 41% to 58% of those with abnormal microflora and up to 94% in women with BV[7]. However, prevalence of bifidobacteria has not yet been reported for Thai women with BV and healthy women.

Moreover, an attention is now focused on adhesion property of bifidobacteria. Since adhesion to host tissues is the first step in bacterial colonization and also influences the subsequent phases leading to commensalism or infection. Previous studies have been reported on the adhesion properties of intestinal bifidobacterial strains [8, 9]. However, the information of the adhesion property of the vaginal bifidobacteria and cell surface charges of hydrophobic and hydrophilic characteristics reflecting on colonization ability of the vaginal strains are still limited. Therefore, the aims of this study were to determine prevalence of vaginal *Bifidobacterium* spp. and to evaluate adhesion ability and cell surface properties of vaginal bifidobacterial isolates from Thai women with BV compared to healthy subjects.

## Material and Method

### Subjects and clinical examination

A total of 120 Thai women subjects (60 of women with BV, 60 of healthy women) with age ranging from 18 to 60 years old who attended the Gynaecology clinic, Songklanagarind Hospital, Hat Yai, Songkhla were enrolled in the study. All participants gave informed consent, which the protocol was approved by the Ethics Committee of the Faculty of Medicine, Prince of Songkla University, Thailand. The diagnosis of BV was based on three of five indicators of modified Amsel's criteria: 1) homogeneous discharge, 2) vaginal pH >4.5, 3) production of fishy amine odor, 4) clue cells >20% of the total vaginal epithelial cells, and 5) absent gram-positive rods[10, 11]. Exclusion criteria were as: use of systemic antibiotics within one week prior to sampling, bilateral ovariectomized, postmenopausal, menstruating women, or having human immunodeficiency virus infection.

### Bacterial sampling and cultivation

A sterile swab was rolled over high vaginal wall and placed in sterile screw cap tubes containing 3 ml of sterile reducing transport fluid (RTF). The specimens were collected from the transport tube by centrifugation at 5,000 g for 5 min and resuspended in 1 ml phosphate buffered saline pH 7.0 (PBS, contained 0.05% L-cysteine hydrochloride). Ten-fold dilution series of each sample was made in PBS, and 0.1 ml of the diluted sample was spread on Beerens agar plate. After 2-7 days of incubation at 37°C under anaerobic condition (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 the same or different colonial appearance were collected and were initially identified as bifidobacteria based on being gram-positive rods, giving catalase negative and 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 used.

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

A total number of 139 isolates of bifidobacteria isolated from 20 of 60 women with BV and 7 of 60 healthy women 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 and Dahlen[13]. Briefly, the 16S rRNA gene sequences were amplified by PCR using the universal primers 8UA (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGGTACCTTGTTACGACTT-3'). The bacterial DNA samples were prepared using a Genomic DNA Extraction Kit (RBC Bioscience, Taipei, Taiwan) following the manufacturer's protocol for gram-positive bacteria. A 25 µl PCR reaction mixture contained 100 ng of DNA template, 1.0 µM of each primer, 2.5 µl of 10 x buffer with 2.0 mM $MgCl_2$, 1.0 U of Taq DNA polymerase, and 0.2 mM of each dNTP. Amplification condition was programmed as follows: initial heat activation at 95°C for 15 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 2 min, then a primer extension at 72°C for 1.5 min, and a final extension step at 72°C for 10 min. The PCR products were individually digested with *HpaII* (New England Biolap, Ipswich, MA) according to the manufacturer's instructions and the digested products were separated by 7.5% polyacrylamide gel electrophoresis and stained with silver staining. The discrimination of uncertain strains was confirmed by denaturing gradient gel electrophoresis (DGGE) and 16S rRNA gene by DNA sequencing. The following reference panel strains were used for comparative identification: *Alloscardovia omnicolens* CCUG 31649, *Bifidobacterium breve* CCUG 30511A, *Bifidobacterium longum* CCUG 28903, *Bifidobacterium dentium* CCUG 18367, *Bifidobacterium scardovii* CCUG 13008A and *Scardovia inopinata* CCUG 35729.

**Adhesion assay**

The HeLa cells, a continuous cell line that originated from a human cervical cancer cells, was used to assess adherence ability. The cells line were grown and maintained in Dulbecco's modified Eagle's minimal essential medium (DMEM; HyClone Laboratories Inc., Logan, UT, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; HyClone), penicillin (100 U/ml), and streptomycin (100 µg/ml), then kept in the incubator at 37°C with 95% (v/v) humidified air and 5% (v/v) $CO_2$ at 37°C. To harvest cells for experiments, the cells were lifted from a culture plate by trypsinization with 0.25% trypsin–0.05% EDTA at 37°C for 7 to 10 min and collected by centrifugation. The HeLa cells were subcultured in 24-well plates at approximately $10^5$ cells/well and were grown at 37°C in 5% $CO_2$ for 2 days to reach confluence.

The adhesion assay was performed with a modification method of Le Blay *et al*[14]. Each tested *Bifidobacterium* strain was grown anaerobically 18-24 h in 10 ml MRS broth supplement with 0.05% L-cysteine hydrochloride at 37°C. The bacterial cells were harvested and washed twice with PBS. A bacterial inoculum containing approximately $10^8$ CFU/ml suspended in DMEM was added to each well, and the plates were incubated at 37°C in a 5% $CO_2$ for 60 min. Non adherent bacteria were washed off and then the adherent bacteria plus intracellular bacteria were quantified as the adhesion. To determine the number of bacteria, the HeLa cells 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. Adhesion ability was reported as a percentage from duplicates according to the formula of adhesion as follows: (%) = $(A_0/A)$ x 100, where A and $A_0$ were $\log_{10}$ number of bacterial cells (CFU $ml^{-1}$) before and after adhesion.

**Bacterial adhesion to solvents**

The microbial adhesion to solvents (MATH) test was performed according to the modified methods of Xu *et al*[15]. The adhesion to xylene (apolar solvent) demonstrates the hydrophobic surface characteristic of bacteria. The affinities to chloroform (polar acidic solvent) and ethyl acetate (polar basic solvent) describe the electron acceptor and electron donor properties of hydrophilic surface charge, respectively. Bacterial cells were suspended in PBS to concentration of $10^8$ CFU/ml. A volume of 3 ml bacterial suspension was mixed with 1 ml of solvents; xylene, chloroform, or ethyl acetate. The mixture was vortexed for 1 min and allowed to stand for 30 min to separate into two phases. The aqueous phase was measured at room temperature, and its absorbance at 600 nm was measured. 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 three groups: those with low (0-35%), moderate (36-70%), or high (71-100%) hydrophobicity or charge surfaces.

**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 Mann-Whitney U-test was used to evaluate statistical difference between healthy women and women with BV. The Spearman's rho test was used for correlating coefficients. All analyses were performed with the Statistical Package for Social Sciences (SPSS Inc., Chicago, IL, USA) software package. The differences were considered significant when $p<0.05$.

## Results

### Prevalence of *Bifidobacterium* spp.

The distribution of *Bifidobacterium* spp. in vaginas of 60 women with BV and 60 healthy women are demonstrated in Table 1. The prevalence of *Bifidobacterium* spp. in women with BV was 33.3% (20/60 subjects), which showed significantly ($p<0.05$) higher than healthy women 11.7% (7/60 subjects). The bifidobacterial count in women with BV ($8.9 \pm 3.4$ log CFU/ml) was about 1,000 times higher than in healthy subjects ($5.7 \pm 2.9$ log CFU/ml). The frequently prevalent species of *B. bifidum*, *B. longum, B. breve* and *B. dentium* were found in both women with BV and healthy group, except *B. dentium* was not detected in healthy women. The women with BV possessed *B. bifidum* (52.5%), *B. longum* (37.6%), *B. breve* (5.9%) and *B. dentium* (4.0%), while healthy women showed *B. bifidum* (55.3%), *B. longum* (28.9%), and *B. breve* (15.8%).

### Adhesion ability of *Bifidobacterium* spp. to HeLa cells

The adhesion assessment of the isolated bifidobacteria to HeLa cells are shown in Fig.1 All isolated bifidobacteria were able to adhere culture cells. The most adherent strains were bifidobacteria isolated from BV subjects and expressed as percentage of adhesion as follows: *B. dentium* (71.0%), *B. bifidum* (57.7%), *B. longum* (44.6%), and *B. breve* (49.0%). While the strains from healthy women showed lower degrees of adhesive ability being *B. bifidum* (53.4%), *B. longum* (39.4%) and *B. breve* (49.4%).

### Physicochemical properties of bifidobacteria cell surface

The adhesive characteristics of bifidobacteria to xylene, chloroform and ethyl acetate are shown in Fig.2a-2c. The bifidobacteria showed strain variations for the adhesive characteristics and exhibited a low to high degree of hydrophobicity and hydrophilic surface

charges. The hydrophobicity (affinity to xylene) and hydrophilic surface charges (affinity to chloroform and ethyl acetate) of bifidobacteria from women with BV were *B. dentium* (94.4%, 99.5% and 88.9%) and *B. bifidum* (84.1%, 95.8% and 75.6%), followed by *B. longum* (35.9%, 52.5% and 38.7%) and *B. breve* (14.2%, 23.0% and 24.6%), while healthy women showed *B. bifidum* (44.1%, 57.7% and 39.8%), *B. longum* (30.2%, 58.1% and 34.9%), and *B. breve* (16.4%, 38.3% and 32.0%). Interestingly, the adhesion ability ($r_s$ = 0.592, 0.570 and 0.501, p = 0.000) of the bifidobacteria from women with BV were in correlation with the hydrophobicity and surface charges, while the relationship between adhesion ability and hydrophobicity and surface charges from healthy women was not found.

**Discussion**

The prevalence of bifidobacteria in women with BV (33.3%) in this study showed a significantly higher frequency than healthy women (11.7%), which was in agreement with Rosenstein *et al*. in reporting that *Bifidobacterium* spp. were found in 12% healthy women[7]. Whereas, the prevalence rate of bifidobacteria in non-pregnant women with BV in this study was considerably lower than previous reports of 83% in West African women[6] and 41-94% in pregnancy of Western women with BV[7]. This is not surprising since the prevalence of various etiological agents in BV varied according to age, sexual behavior, immunodeficiency diseases, pregnancy, individual vaginal flora, and regional variation. In this study, the vaginal strains of *B. bifidum, B. longum, B. breve* and *B. dentium* were found which were also in accordance with a previous report [5, 16]. An increase in 1,000 folds in number of bacteria in BV subjects indicated a strong bacterial association with BV[17]. Interestingly. the strain of *B. dentium* could not be isolated from healthy vagina, while only small proportions of *B. dentium* were found in BV subjects which is in agreement with a previous report[16]. It might be that different species bifidobacteria prefer to colonize in different appropriate environments of host

habitat. Normally, the bifidobacteria are able to tolerate growth in alkaline pH. While *B. dentium* and *B. longum* are able to survive in complex media in both acidic and alkaline pH [(18)]. However, *B. dentium* is less able to grow in acidic condition and the growth drastically dropped within 2 h. at pH 4.0[(18)]. Since normal pH of vagina is 3.8-4.5 may not be optimal for growth of *B. dentium* compared to the pH that rises to a level between 5.5 and 6.0 in women with BV.

Furthermore, cell adhesion plays a crucial role for bifidobacteria to persist in the vaginal tract in order to exert their biological actions. While, there is limited information concerning the adhesion ability and cell surface charges of vaginal bifidobacteria. In this study, the adhesion property and surface charges of *Bifidobacterium* spp. were investigated using HeLa cells as an *in vitro* model. The adhesion ability to cultured cells of the bifidobacteria isolated from BV was significantly higher than healthy groups. Particularly, *B. bifidum* and *B. dentium* showed higher adhesion ability than *B. longum* and *B. breve* in women with BV. Recently, Foroni *et al*. demonstrated various human intestinal bifidobacteria such as *B. bifidum, B. dentium, B. longum,* and *B. adolescentis* having pilus-like appendages on the cell surface which might be involved in bacterial colonization[(19)]. Additionally, in this study, the morphology of *B. bifidum* and *B. dentium* isolated from BV subjects showed more branch formation than bifidobacteria isolated from healthy group (data not shown) suggesting that each bifidobacterial strain differs in the adhesion ability[(20)].

Previous reports have demonstrated that bifidobacteria had variations in the surface properties [(15, 21)]. In this study, *B. bifidum* and *B. dentium* isolated from women with BV exhibited higher hydrophobic and hydrophilic surface charges than *B. longum* and *B. breve*. The women with BV harboring *B. bifidum* and *B. dentium* showed significantly ($p<0.05$) higher properties of affinity to xylene, chloroform and ethyl acetate than other strains, suggesting that surface characteristics of vaginal *B. bifidum* and *B. dentium* possessing hydrophobic, basic

electron acceptor and acidic electron donor properties. . The variety of cell surface characteristics of *B. bifidum* and *B. dentium* may be related to the adhesion property leading to colonize in the vaginal mucosa. In the fact that, hydrophobic and hydrophilic properties are depended on the density of hydrophobic amino acids and polysaccharides on the bacterial cell surfaces[22]. Therefore, it would be interesting to investigate further a more detailed of bacterial surface composition which was mediated in mechanism of adhesion ability of the bifidobacteria in order to understand the biological action in the BV association.

In conclusions, the prevalence of bifidobacteria occurred significantly higher in women with BV higher than healthy group. The strains of *B. bifidum and B. dentium* showed high adhesion and cell surface charge properties implying an important role of colonization to vaginas of women with BV.

**What is already known on this topic?**

Previous studies of Rosenstein *et al.*[7] in 1996 have reported that *Bifidobacterium* spp. were found in 12% of healthy controls, in 41% to 94% of patients with bacterial vaginosis. However, prevalence of vaginal bifidobacteria has not yet been reported for Thai women with BV and healthy women.

**What this study adds?**

Additional data was to determine the prevalence of vaginal *Bifidobacterium* spp. isolates from Thai women with BV compared to healthy subjects and to evaluate adhesion ability and cell surface properties of the vaginal bifidobacterial in order to assess a potential role in the association with BV

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**Potential conflicts of interest**

None.

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**Table 1.** Distribution of bifidobacteria among healthy women and women with BV

| *Bifidobacterium* species | All subjects, N=27 | | Healthy women, N=7 | | Women with BV, N=20 | |
|---|---|---|---|---|---|---|
| | No. of subjects (%) | No. of isolates (%) | No. of subjects (%) | No. of isolates (%) | No. of subjects (%) | No. of isolates (%) |
| *B. bifidum* | 18 (66.7) | 74 (53.2) | 6 (85.7) | 21 (55.3) | 12 (60.0) | 53 (52.5) |
| *B. longum* | 14 (51.9) | 49 (35.3) | 4 (57.1) | 11 (28.9) | 10 (50.0) | 38 (37.6) |
| *B. breve* | 4 (14.8) | 12 (8.6) | 2 (28.6) | 6 (15.8) | 2 (10.0) | 6 (5.9) |
| *B. dentium* | 1(3.7) | 4(2.9) | ND | ND | 1 (5.0) | 4 (4.0) |

ND = Not detected

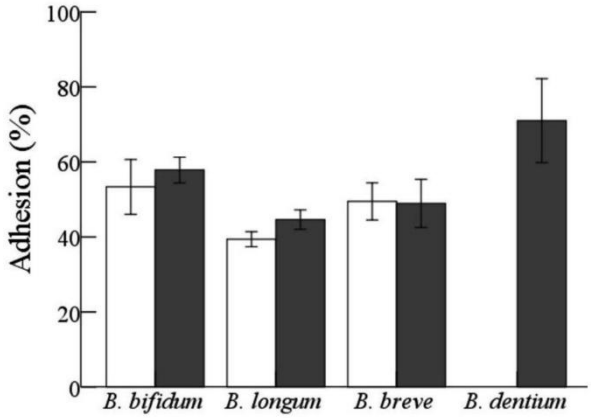


**Fig. 1** Comparison of adhesion of *Bifidobacterium* strains between healthy (□) and BV (■) subjects.

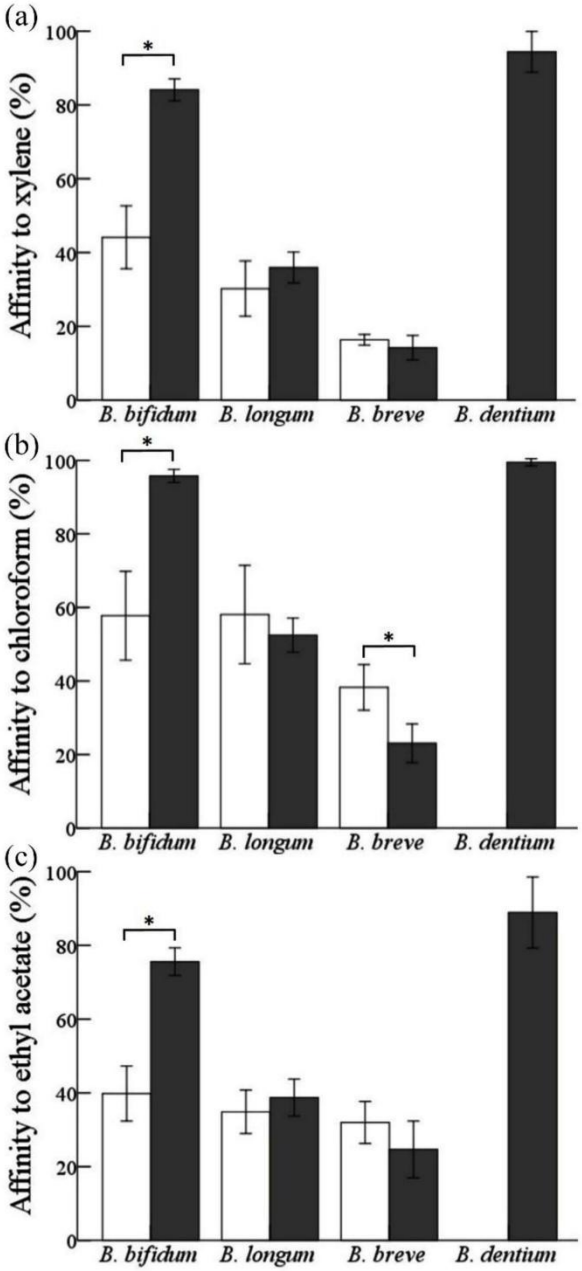


**Fig. 2** Comparison of (a) affinity to xylene, (b) affinity to chloroform and (c) affinity to ethyl acetate of *Bifidobacterium* strains between healthy (□) and BV (■) subjects. * Significance ($p < 0.05$) compared with bifidobacterial isolates between healthy women and women with BV.

***การประเมินความชุก การเกาะติดและศึกษาสมบัติผิวเซลล์ของ สกุลไบฟิโดแบคทีเรีย ที่แยกได้จากสตรีไทยผู้เป็นช่องคลอดอักเสบแบคทีเรียผสมและผู้มีสุขภาพดี***

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***ภูมิหลัง:*** *มีรายงานการตรวจพบสกุลไบฟิโดแบคทีเรียในในสตรีผู้เป็นช่องคลอดอักเสบแบคทีเรียผสม แต่อย่างไรก็ตาม ข้อมูลเกี่ยวกับความชุกและความสามารถในการเกาะติดของสกุลไบฟิโดแบคทีเรียที่แยกได้สตรีไทยผู้เป็นช่องคลอดอักเสบแบคทีเรียผสมยังมีอยู่อย่างจำกัด*

***วัตถุประสงค์:*** *เพื่อตรวจหาความชุก และประเมินค่าความสามารถในการเกาะติด รวมทั้งศึกษาสมบัติผิวเซลล์ของ สกุลไบฟิโดแบคทีเรีย ที่แยกได้จากสตรีไทยผู้เป็นช่องคลอดอักเสบแบคทีเรียผสมเปรียบเทียบกับผู้มีสุขภาพดี*

***วัสดุและวิธีการ:*** *จำนวนไบฟิโดแบคทีเรีย 139 สายพันธุ์ ที่แยกได้จากผู้เป็นช่องคลอดอักเสบแบคทีเรียผสม 20 ใน 60 ราย และผู้มีสุขภาพดี 7 ใน 60 ราย ได้นำมาจำแนกสายพันธุ์โดยเทคนิคชีววิทยาโมเลกุล และศึกษาความสามารถในการเกาะติดและสมบัติของผิวเซลล์*

***ผลการศึกษา:*** *ความชุกของเชื้อไบฟิโดแบคทีเรียในสตรีผู้เป็นช่องคลอดอักเสบแบคทีเรียผสม (33.3%) มีปริมาณสูงกว่าสตรีผู้มีสุขภาพดี (11.7%) อย่างมีนัยสำคัญทางสถิติ ($p<0.05$) โดยมีปริมาณแบคทีเรียทั้งหมด 8.9 ± 3.4 Log CFU/ml และ 5.7 ± 2.9 Log CFU/ml ตามลำดับ ไบฟิโดแบคทีเรียที่พบบ่อยในสตรีผู้เป็นช่องคลอดอักเสบแบคทีเรียผสมได้แก่ สายพันธุ์ B. bifidum, B. longum, B. breve และ B. dentium ส่วนสตรีผู้มีสุขภาพดีพบสายพันธุ์ B. bifidum, B. longum และ B. breve ไบฟิโดแบคทีเรียทุกสายพันธุ์ที่แยกได้จากสตรีผู้เป็นช่องคลอดอักเสบแบคทีเรียผสมและผู้มีสุขภาพดี มีความสามารถในเกาะติดเซลล์เพาะเลี้ยงในหลอดทดลอง โดย B. bifidum และ B. dentium ที่แยกได้จากสตรีผู้เป็นช่อง*

*คลอดอักเสบแบคทีเรียผสม มีความสามารถในเกาะติดสูง และความสามารถในการเกาะติด สัมพันธ์กับสมบัติของผิวเซลล์ของเชื้อไบฟิโดแบคทีเรีย*

***สรุป**: ความชุกของไบฟิโดแบคทีเรีย ในสตรีผู้เป็นช่องคลอดอักเสบแบคทีเรียผสมสูงกว่าผู้มีสุขภาพดีอย่างมีนัยสำคัญ B. bifidum และ B. dentium ที่แยกได้จากสตรีผู้เป็นช่องคลอดอักเสบแบคทีเรียผสม มีความสามารถในเกาะติดสูง แสดงถึงบทบาทสำคัญของการเพิ่มจำนวนไบฟิโดแบคทีเรียในช่องคลอดสตรีผู้เป็นช่องคลอดอักเสบแบคทีเรียผสม*

# APPENDIX C

Utto P. and Teanpaisan R. Screening of bifidobacteria from oral cavity to use as probiotic. The 2nd Current Drug Development International Conference; 2012 May 2-4; Phuket Graceland Resort & Spa Hotel, Phuket, Thailand. Quintessence Publishing; 2012. p. 187 -188.


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The 2nd Current Drug Development International Conference
2nd - 4th May 2012
Phuket, Thailand
Faculty of Pharmaceutical Sciences
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# Screening of bifidobacteria from oral cavity to use as probiotic

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**Abstract-** Bifidobacteria are generally found in human and animal intestines and considered one of probiotics used for promoting the intestinal health. However, bifidobacteria in oral cavity have received comparatively little research attention. Aims of this study were to investigate the prevalence of bifidobacteria in oral cavity and to explore for the strains to use as probiotics in oral cavity. Total 28 subjects gave the informed consent to participate in the study. All 50 collected plaque samples were cultured on the Columbia agar base containing propionic acid and incubated at 37°C, in anaerobic condition for 5-7 days. The tentative colonies of bifidobacteria were counted as CFU/ml, gram stained and tested for fructose-6-phosphate phosphoketolase activity. Antimicrobial production of 20 strains of bifidobacteria was tested against *Streptococcus mutans* ATCC 25175 using an agar overlay method. Results showed that the presence of bifidobacteria was found 12%; 2% of carious plaque samples and 10% of caries free plaque samples. All 20 strains (100%) of bifidobacteria could inhibit growth of *S. mutans*, however, the activities varied among the strains. In conclusion, the preliminary data demonstrated that bifidobacteria was found more frequent in caries free teeth than in carious teeth. In addition, all strains of bifidobacteria showed antagonistic activity against *S. mutans,* an oral pathogen of dental caries. The further study will include more subjects, and the role of bifidobacteria in human oral microbiota will be clarified.
**Key word**: Bifidobacteria, antagonistic interaction, *Streptococcus mutans*, Oral cavity

## Introduction

Bifidobacteria are generally found in human and animal intestines and considered one of probiotics used for promoting the intestinal health. However, bifidobacteria in oral cavity have received comparatively little research attention. Little is known of their intraoral distribution or of the environmental factors, including dietary components, which may influence their numbers or the proportions of individual species isolated from the oral cavity. Some reports have shown that bifidobacteria could be detected in high numbers in caries lesions of children (1,2), and in active root caries lesions (3). However, bifidobacteria was also detected in free caries subjects (4). Thus, the certain role of bifidobacteria regarding to its role on in oral cavity is still unclear. In addition, bifidobacteria has been used as probiotics adding in food to promote health (5). Therefore, this study aimed to investigate the prevalence and types of bifidobacteria, the relationship of bifidobacteria in health and diseased conditions and the ability of antimicrobial agents elaborated by bifidobacteria against oral pathogens.

## Materials and methods

*1)Subjects for the oral investigation:* The study comprised of 28 children with age 7-8 years old and has been informed consent by their parents. Total 50 plaque samples; 22 samples from carious lesions and 28 samples from caries free were collected.
*2)Bacterial culture and identification*: All plaque samples were cultured on the Columbia agar base containing propionic acid and incubated at 37°C, in anaerobic condition for 5-7 days. The tentative colonies of bifidobacteria were counted as CFU/ml, gram stained and tested for fructose-6-phosphate phosphoketolase activity.
*3)Antimicrobial production:* Antimicrobial ability of 20 strains of bifidobacteria was tested against *Streptococcus mutans* ATCC 25175 using an agar overlay method.

## Results and Discussions

The presence of bifidobacteria was found 12%; 2% of carious plaque samples and 10% of caries free plaque samples. All 20 strains (100%) of bifidobacteria could inhibit growth of *S. mutans*, however, the activities varied among the strains (Fig.1). This is in agreement of Lee *et al*. (6) reported that the cultured supernatant of *Bifidobacterium adolescentis* SPM1005 had the ability to inhibited growth of *S. mutans*.

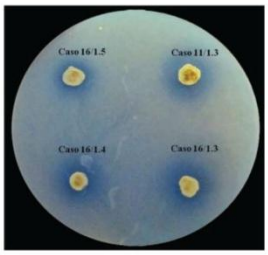


**Figure 1**. Agar overlay test of bifidobacteria against *S. mutans* ATCC 25175

**Conclusion**

The preliminary data demonstrated that bifidobacteria was found more frequent in caries free teeth than in carious teeth. In addition, all strains of bifidobacteria showed antagonistic activity against *S. mutans,* an oral pathogen of dental caries. The further study will include more subjects, and the role of bifidobacteria in human oral microbiota will be clarified.

**Acknowledgements:** This work was supported by the Thailand's Commission on Higher Education and the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission (AGR 540555M).

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# APPENDIX D

Documentary Proof of Ethical Clearance Research Ethics Committee (REC) Faculty of Dentistry, Prince of Songkla University (EC5507-26-L)

ที่ ศธ 0521.1.03/ **1176**

คณะทันตแพทยศาสตร์
มหาวิทยาลัยสงขลานครินทร์
ตู้ไปรษณีย์เลขที่ 17
ที่ทำการไปรษณีย์โทรเลขคอหงส์
อ.หาดใหญ่ จ.สงขลา 90112

**หนังสือฉบับนี้ให้ไว้เพื่อรับรองว่า**

**โครงการวิจัยเรื่อง** "การจำแนกชนิดและศึกษาคุณสมบัติของเชื้อไบฟิโดแบคทีเรีย ที่เกี่ยวข้องกับโรคฟันผุรุนแรงในเด็ก"

**รหัสโครงการ** EC5507-26-L

**หัวหน้าโครงการ** อาจารย์ ดร.ทพญ.สุพัชรินทร์ พิวัฒน์

**สังกัดหน่วยงาน** ภาควิชาทันตกรรมป้องกัน คณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์

ได้ผ่านการพิจารณาและได้รับความเห็นชอบจากคณะกรรมการจริยธรรมในการวิจัย (Research Ethics Committee) ซึ่งเป็นคณะกรรมการพิจารณาศึกษาการวิจัยในคนของคณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์ ดำเนินการให้การรับรองโครงการวิจัยตามแนวทางหลักจริยธรรมการวิจัยในคนที่เป็นสากล ได้แก่ Declaration of Helsinki, the Belmont Report, CIOMS Guidelines และ the International Conference on Harmonization in Good Clinical Practice (ICH-GCP)

**ในคราวประชุมครั้งที่** 7/2555 **เมื่อวันที่** 18 ตุลาคม 2555

**ให้ไว้ ณ วันที่** 20 พ.ย. 2555

(ผู้ช่วยศาสตราจารย์ ดร.ทพญ.ศรีสุรางค์ สุทธปรียาศรี)
ประธานคณะกรรมการจริยธรรมในการวิจัย

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| .....กรรมการ | .....กรรมการ |
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**Documentary Proof of Ethical Clearance**

**Research Ethics Committee (REC)**

**Faculty of Dentistry, Prince of Songkla University**

**The Project Entitled** Identification and cariogenic characterization of oral *Bifidobacterium* spp. associated with severe childhood caries

**REC Project No.** : EC5507-26-L

**Principal Investigator** : Dr. Supatcharin Piwat

Approved by Research Ethics Committee (REC), Faculty of Dentistry, Prince of Songkla University.

This is to certify that REC is in full Compliance with International Guidelines for Human Research Protection such as the Declaration of Helsinki, the Belmont Report, CIOMS Guidelines and the International Conference on Harmonization in Good Clinical Practice (ICH-GCP).

**Date of Approval** : 20 November 2012

(Asst. Prof. Dr. Srisurang Suttapreyasri)
Chairman of Research Ethics Committee

Surapong Vongvatchranon
(Asst. Prof.Surapong Vongvatchranon)

(Asst. Prof. Dr. Angkana Thearmontree)

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(Mr. Kamolphan Nuangsri)

(Mr. Wasin Suwannarat)

(Dr. Supatcharin Piwat)

# APPENDIX E

Documentary Proof of Ethical Clearance the Ethics Committee (EC) Faculty of Medicine, Prince of Songkla University (EC54-032-12-1-3)

EC: 54-032-12-1-3

**Documentary Proof of Ethical Clearance**

**The Ethics Committee, Faculty of Medicine, Prince of Songkla University**

| | | |
|---|---|---|
| **The Project Entitled** | : | Prevalence of Lactobacilli and Bifidobacteria in Normal Women with Bacterial Vaginosis |
| **Principal Investigator** | : | Assoc.Prof.Verapol Chandeying, M.D. |
| **Name of Department** | : | Department of Obstetrics and Gynecology, Faculty of Medicine, Prince of Songkla University |

Has been reviewed and approved by The Ethics Committee, Faculty of Medicine, Prince of Songkla University.

**Date of Approval :** December 7, 2010

(Assoc.Prof. Verapol Chandeying, M.D.)

Vice Dean in Research Affairs

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**Name** Miss Parada Utto

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**Scholarship Awards during Enrolment**

The Office of the Higher Education Commission under the CHE-PhD, 2010-2013

**List of Publication and Proceeding**

Parada Utto, Rawee Teanpaisan, Supatcharin Piwat and Verapol Chandeying. Assessment of Prevalence, Adhesion and Surface Charges of *Bifidobacterium* spp. Isolated from Thai Women with Bacterial Vaginosis and Healthy Women. ***J Med Assoc Thai.*** Accepted for publication. June 7, 2016.

Parada Utto, Rawee Teanpaisan and Supatcharin Piwat. Prevalence and Adhesion Properties of Oral *Bifidobacterium* species in Caries-active and Caries-free Thai Children. ***Walailak J. Sci. & Tech.*** Accepted for publication. August 11, 2016.

Utto P. and Teanpaisan R. Screening of bifidobacteria from oral cavity to use as probiotic. The 2nd Current Drug Development International Conference; 2012 May 2-4; Phuket Graceland Resort & Spa Hotel, Phuket, Thailand. Quintessence Publishing; 2012. p. 187 -188.