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

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Background: *Bifidobacterium spp. have been reported in women with bacterial vaginosis (BV), nevertheless data of prevalence and adhesion property of the vaginal bifidobacteria in Thai women with BV are 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 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 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 as an important role of colonization in vaginas of women with BV.*

Keywords: *Prevalence, adhesion, bifidobacteria, bacterial vaginosis, surface charge*

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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

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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 had been reported on the adhesion properties of intestinal bifidobacterial strains[8,9]. However, the information of the adhesion properties 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 the present 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 women with BV, 60 healthy women) age ranging from 18 to 60 years old attended the Gynaecology clinic, Songklanagarind Hospital, Hat Yai, Songkhla were enrolled in the present study. All participants gave informed consents, the study 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: use of systemic antibiotics within one week prior to sampling, bilateral ovariectomized, postmenopausal, menstruating women, or having human immunodeficiency virus infection.The sample size was calculated based on our pilot study by using a formula illustrated below:

$$n = [2(Z_{\alpha/2} + Z_{\beta})^2 P(1-P)]/\Delta^2=44$$

where $P = (P_{test} + P_{control})/2$, P_{test} and $P_{control}$= prevalence of bifidobacteria in BV (0.32) and in healthy subjects (0.08), respectively; $\Delta = (P_{test} - P_{control})$, $\alpha = 0.05$ ($Z_{0.025}= 1.96$), $\beta = 0.2$($Z_{0.2}= 0.84$).

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 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 supplemented 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 (MATS) 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 electron donor (basic) and electron acceptor (acidic) characteristics of hydrophilic bacterial surface charges, 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. From the testing, the data of adhesion properties and surface charges were not normal distributions, thus, the differences of adhesion properties and surface charges were analyzed using the Kruskall-Wallis test and the Mann-Whitney U-test. The Spearman's rho test was used for correlating coefficients. 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

Prevalence of *Bifidobacterium* spp.

The distribution of *Bifidobacterium* spp. in vaginas of 60 women with BV and 60 healthy women were 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± 3.4 log CFU/ml) was about 1,000 times higher than in healthy subjects (5.7±2.9 log CFU/ml). The frequent 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 were shown in Fig. 1. All isolated bifidobacteria were able to adhere to 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 *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 were 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 the present study showed significant higher frequency than healthy women (11.7%), which was in agreement with Rosenstein et al reporting that *Bifidobacterium* spp. were found in 12% healthy women[7]. Whereas, the prevalence rate of bifidobacteria in non-pregnant women with BV in the present

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

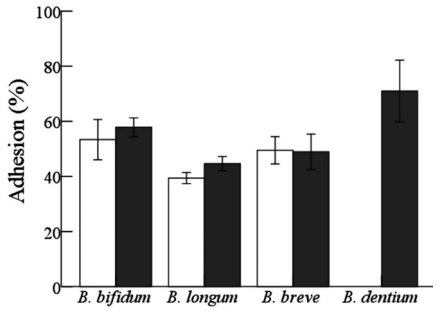

Fig. 1 Comparison of adhesion of *Bifidobacterium* spp. between healthy (□) and BV (■) subjects.

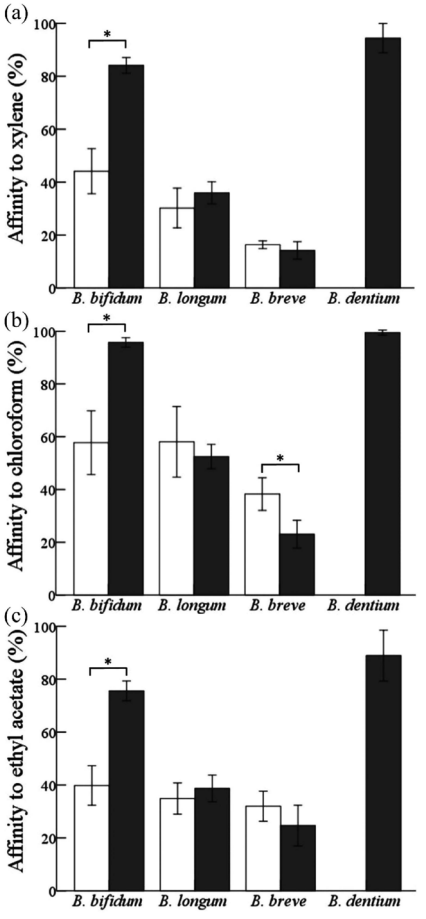

Fig. 2 Comparison of (a) affinity to xylene, (b) affinity to chloroform and (c) affinity to ethyl acetate of *Bifidobacterium* spp. between healthy (□) and BV (■) subjects.*Significance (p<0.05) compared with bifidobacterial isolates between healthy women and women with BV.

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 the present study, the vaginal strains of *B. bifidum, B. longum, B. breve* and *B. dentium* were found which were in accordance with 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 of 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 (3.8 to 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 the present 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 which supported the prevalence data of bifidobacteria in both groups. Notably, *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 the present 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 adhesion ability and cell surface property[15,21]. 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[15]. In the present 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 higher properties of affinity to xylene, chloroform and ethyl acetate than the other strains ($p<0.05$), suggesting that, surface characteristic of vaginal *B. bifidum* and *B. dentium* possessing hydrophobic and hydrophilic electron donor (basic) and electron acceptor (acidic) properties, respectively. 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 for more detailed of bacterial surface composition which was mediated the 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 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 in Thai women with BV and healthy women.

What is this study adds?

Additional data were to determine the prevalence of vaginal *Bifidobacterium* spp. isolated from Thai women with BV compared to healthy subjects and to evaluate adhesion ability and cell surface properties of the vaginal bifidobacteria 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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การประเมินความชุก การเกาะติดและศึกษาสมบัติผิวเซลล์ของแบคทีเรียสกุลไบฟิโด ที่แยกได้จากสตรีไทยผู้เป็นช่องคลอดอักเสบแบคทีเรียผสมและผู้มีสุขภาพดี

ภารดา อุทโท, รวี เถียรไพศาล, สุพัชรินทร์ พิวัฒน์, วีระพล จันทร์ดียิ่ง

ภูมิหลัง: *มีรายงานการตรวจพบแบคทีเรียสกุลไบฟิโดในในสตรีผู้เป็นช่องคลอดอักเสบแบคทีเรียผสม แต่อย่างไรก็ตาม ข้อมูลเกี่ยวกับความชุกและความสามารถในการเกาะติดของแบคทีเรียสกุลไบฟิโดที่แยกได้สตรีไทยผู้เป็นช่องคลอดอักเสบแบคทีเรียผสมยังมีอยู่อย่างจำกัด*

วัตถุประสงค์: *เพื่อตรวจหาความชุก และประเมินค่าความสามารถในการเกาะติด รวมทั้งศึกษาสมบัติผิวเซลล์ของแบคทีเรียสกุลไบฟิโด 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 มีความสามารถในการเกาะติดเซลล์เพาะเลี้ยงในหลอดทดลอง โดย B. bifidum และ B. dentium ที่แยกได้จากสตรีผู้เป็นช่องคลอดอักเสบแบคทีเรียผสมมีความสามารถในการเกาะติดสูงและความสามารถในการเกาะติดสัมพันธ์กับสมบัติของผิวเซลล์ของเชื้อไบฟิโดแบคทีเรีย*

สรุป: *ความชุกของไบฟิโดแบคทีเรีย ในสตรีผู้เป็นช่องคลอดอักเสบแบคทีเรียผสมสูงกว่าผู้มีสุขภาพดีอย่างมีนัยสำคัญ B. bifidum และ B. dentium ที่แยกได้จากสตรีผู้เป็นช่องคลอดอักเสบแบคทีเรียผสมมีความสามารถในการเกาะติดสูงแสดงถึงบทบาทสำคัญของการเพิ่มจำนวนไบฟิโดแบคทีเรียในช่องคลอดสตรีผู้เป็นช่องคลอดอักเสบแบคทีเรียผสม*