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DO LACTOBACILLI CHALLENGE *GARDENELLA VAGINALIS* BIOFILMS?

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Abstract

Bacterial vaginosis (BV) is a highly prevalent vaginal dysbiosis that has been linked to adverse pregnancy outcomes and enhanced transmission of sexually transmitted infections (STIs). Key characteristics of the disease process are thought to be depletion of vaginal *Lactobacillus* and overgrowth of anaerobes (often dominated by *G.vaginalis*) and a pH > 4.5. Currently, is consensual that BV also involves the presence of a dense, structured and polymicrobial biofilm, primarily constituted by *G.vaginalis* clusters, strongly adhered to the vaginal mucosal surface.

Biofilms are communities of microorganisms attached to a surface and encased in a selfproduced polymeric matrix. Reduction of the adhesive and biofilm forming capacity activity of *G. vaginalis* bacteria by *Lactobacillus* strains is a well-known and desired effect of strains for potential vaginal probiotic application. The objectives of the present study were to evaluate in vitro the effect of *Lactobacillus* on biofilm production by different species of *G.vaginalis* isolated from women with bacterial vaginosis (BV). A total of 36 isolates from women with BV identified as *G.vaginalis* were tested for their biofilm-forming capacity as monocultures and in bacterial coculture with confirmed non-biofilm producing strain of Lactobacillus, in a ratio of 1:1 by microtiter plate assay. *Lactobacillus* strain in our study was capable of interfering with the growth of *G. vaginalis* biofilms to different degrees. According to the criteria for biofilm-forming ability, after 24-h incubation 25%, 28% and 22% of *Gardnerella* monocultures were strong, moderate and weak biofilm producers, compared to 5.5%, 14% and 33.5% of *Gardnerella+Lactobacillus* cocultures, respectively.

Our results indicate the potential of lactobacilli as probiotics, since they effectively reduced the adheration and biofilm formation of the tested *Gardnerella* species which is a well-known and desired effect of strains for potential vaginal probiotic application.

Key words: biofilms, resistance, lactobacilli, genital.

Introduction

Bacterial vaginosis (BV) is polymicrobial syndrome characterized by the replacement of beneficial lactobacilli and the augmentation of anaerobic bacteria [1,2].

Research data indicate associations between BV and multiple male or female partners, a new sex partner, inconsistent condom use, douching and young age at sexual debut; women who have never been sexually active are rarely affected.

The precise pathogenesis of BV remains unclear by means of that the etiology of the microbial imbalance that precipitates BV is not fully understood, and there is a lack of knowledge whether this condition results from sexual transmission of a single or multiple pathogens. The vaginal microflora in a healthy women is normally residented by lactic-acid producing vaginal bacteria (mainly by Gram-positive *Lactobacillus* spp.) [3-5].

Small numbers of *Gardnerella* spp., *Prevotella bivia, Atopobium vaginae, Mobiluncus* spp. and *Bacteroides* spp are also present but in normal circumstances this does not result with a disease [6, 7]. Key characteristics of the BV pathogenessis are thought to be the dramatic shift in the normal vaginal microbiota with depletion of vaginal lactobacilli, overgrowth of anaerobes (with *G.vaginalis* being the leading pathogen) and a rise of the vaginal pH above 4.5.

BV is by far the most common infective cause of vaginal discharge in women of childbearing age; with detection rates twice higher than that of Candidiasis. The prevalence varies by population with representative 5%–25% among college students and 12%–61% among STD patients [8].

Although BV is characterized with absence of inflammation and approximately 50% of the cases are asymptomatic if clinically presented it can have a major impact on quality of life and mental resilience. Typical symptoms include a profuse vaginal discharge and a rotten fish vaginal odor which recur frequently, most commonly during the menstrual cycle [9, 10].

This highly prevalent vaginal dysbiosis may have a annihilating impact on women's reproductive health since it is linked to adverse pregnancy outcomes and enhanced transmission of sexually transmitted infections (e.g., HIV, *N. gonorrhoeae, C. trachomatis*, and HSV- 2), increased risk of complications after gynecologic surgery interventions etc. [11, 12].

Currently, is consensual that BV involves the presence of a thick, structured and polymicrobial biofilm, dominantly composed of *G.vaginalis* clusters, firmly attached to the vaginal epithelium [13, 14].

Biofilms are communities of microorganisms adhered to an organic or anorganic surface embedded in a self-produced extracellular polymeric matrix composed of polysaccharides, proteins and nucleic acids 15, 16. Due to the fact that bacteria within biofilms are not effectively eliminated by the immune system or fully destroyed by antibiotics, biofilms appear to contribute to persistence and a high rate of relapse and recurrence of BV [17, 18].

G.vaginalis biofilm mode of existence is crucial for the pathogenesis of BV hence its recalcitrance toward antibiotics is associated with further displacement of indigenous lactobacilli from the vault and consecutive elevation of vaginal pH which results with aggravation of the vaginal disbiossis [17- 20].

On the other hand, coaggregation of probiotic microorganisms to pathogens generates a hostile environment for the pathogens implying the reduction of their growth and re-establishment of indigenous microbiota. Reduction of the adhesive and biofilm forming capacity activity of *G. vaginalis* bacteria by *Lactobacillus* strains is a well-known and desired effect of strains for potential vaginal probiotic application [21- 23].

Study objectives:

The objectives of the present study were to evaluate the effect of *Lactobacillus* on biofilm production by different species of *G.vaginalis* isolated from women with bacterial vaginosis (BV).

Materials

Samples and bacterial strains

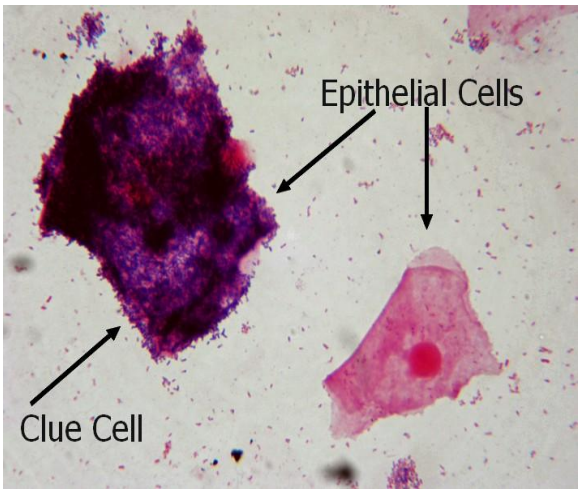
A total of 36 G. vaginalis isolates were obtained from female outpatients clinically suspected of BV referred for microbiological examination of genital swabs at the Institute of Microbiology and parasitology.

All of the identified G. vaginalis strains were tested for their ability for biofilm production:

- as monoculture and
- in bacterial co-culture with confirmed biofim non-producing strain of *Lactobacillus* (ratio 1:1) in order to evaluate the influence of lactobacilli on the growth of *G. vaginalis*.

Isolation and identification of the strains

- Direct Gram stain of vaginal swab - considered the gold standard laboratory method for diagnosing BV used to determine: the relative concentration of lactobacilli (Gram-positive rods) and Gram-variable rods (*G. vaginalis*) characteristic of BV and presence of "clue" cells (squamous epithelial cells with granular appearance due to the clusters of gram variable coccobacilli organisms attached or nonattached to their surface).



Picture 1. Clue cells on Gram-stained vaginal smears

Growth on enriched culture media (Columbia agar plates with 5% sheep blood); all plates were incubated (18–24 h; 37°C; microaerophilic conditions- 5% CO_2 atmosphere) and read following standard laboratory procedures.

Additionally, sensitivity to Bacitracin and 50 mcg metronidazole and hemolysis on human blood agar were investigated

Methods

- ***Biofilm cultivation & biomass determination***

"Tissue culture plate method (TCP)"- Chirstensen et al (1985)

The ability to form biofilm was investigated using the microtiter plate assay described by Christensen et al., a most widely used method which is considered as standard test for detection of biofilm formation by cultivation of the microorganisams in 96-well polystyrene microtitre plates [24].

For the biofilm formation assay, pre-inoculums (liquid cultures) were grown in supplemented brain heart infusion medium (sBHI, Becton Dickinson) containing 2% (w/v) gelatin (Fluka), 0,5% yeast extract (Becton Dickinson), 0.1% starch (Merck) and 1% glucose (Sigma) for sBHIG medium [25].

For liquid culture, one colony from Columbia agar mentioned above was inoculated in 5 ml of sBHIG medium and incubated for 18 h at 37 under microaerophilic conditions (5% CO_2 atmosphere). Subsequently, 10 µl of stationary (18-h) sBHIG cultures were diluted 1:100 into 1000 µl of sBHIG and bacterial density was adjusted to obtain 0.5 McFarland turbidity standard containing approximately 10^8 CFUs/ml.

For biofilm formation of *G. vaginalis monocultures* 100 µl from each bacterial suspension was inoculated into the wells of sterile, polystyrene, 96-well, flat-bottomed tissue culture plates.

For evaluation of the lactobacilli influence on *G. vaginalis* biofilms a modification of bacterial coculture technique described by Coudeyras et al. was used - in another set of wells *Lactobacillus* was added on each of the *G. vaginalis isolates*, in a ratio of 1 : 1 at 37°C in a 5% CO_2 atmosphere for 24 h.

For biofilm formation the 96-well polystyrene microtitre plates were incubated for 24 h in a normal atmosphere at at 37°C in a 5% CO_2 atmosphere, without shaking. After the incubation time the supernatant was removed using a pipette and discarded and the plates were gently washed three times with 200 µl l 85% NaCl to remove free-floating 'planktonic' bacteria.

-Semi-quantitative spectrophotometric method

In order to evaluate biofilm mass (including matrix, dead and living cells) and visualize the attachment pattern, each well of the microtiter plate was added 120 μl of 0.1% (wt/vol) crystal violet and left at room temperature for 10-15 min. Excess (unbound) stain was removed and the wells were thoroughly washed with sterile distilled water. At this point, biofilms were visible as purple rings formed on the side of each well.

The quantitative assessment of biofilm production was performed by adding 120 μl 75% ethanol per well to solubilize the bound dye. Optical density of the eluted solution was read in a microplate spectrophotometer (ELISA microplate reader) at OD_{495}. The amount of absorption was proportional with the amount of biofilm present.

Three wells containing sterile sBHIG served as a negative control during the experiment.

To compensate for the considerable variability in the assay, tests were done in triplicate on three separate occasions and the results were averaged.

Optical density (OD) of stained adherent bacteria and the negative control was calculated as an arithmetical mean of the absorbencies of the three wells. The ″cut-off″ OD value (ODc) was defined as three standard deviations (SD) above the mean OD of the negative control i.e.:

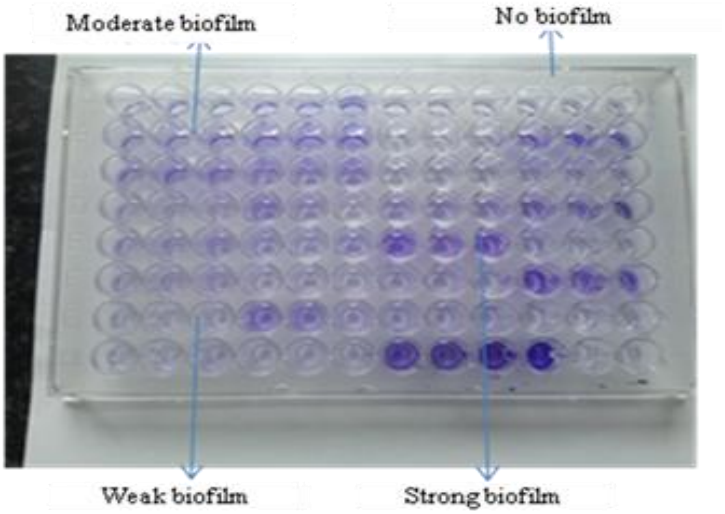
ODc=meanOD of negative control+ (3x SD of negative control).

The ODc value in this study was 0.0051.

The results for biofilm production were interpreted and the isolates were classified as presented in the table below (table 1)[26].

Table 1. Classification of **b**iofilm formation capacity according to the optical density

Strength of biofilm formation	**Average OD value**
Non-biofilm producers	OD ≤ ODc
Weak biofilm producers	ODc < OD≤ 2xODc
Moderate biofilm producers	2 × ODc < OD ≤ 4 × ODc
Strong biofilm producers	4 ×ODc < OD



Picture 2. Bacterial biofilms stained with crystal violet

Results

A total of 36 isolates of *G. vaginalis* obtained from 42 women meeting the Ames criteria for BV were examined for their biofilm production capacity as monoculture and in co-culture with Lactobacillus by TCP.

In this study, the average OD value of the negative control came to be 0.005 and the optical density cut-off value was 0.0067. The ODs of the 24-h biofilm ranged from 0.0010 to 0.0501.

Based on the biofilm formation all examined strains were were classified into the following categories: strains with $OD_{495} < 0.0067$; $0.0067 < OD_{495} \leq 0.0134$; $0.0134 < OD_{495} \leq 0.0268$ and $0.0268 < OD_{495}$ defined as none, weak, moderate and strong biofilm producers, accordingly (presented in Table 2).

Table 2. Optical density and biofilm formation of *G. vaginalis* monocultures and *G. vaginalis+Lactobacillus* co-cultures

G. vaginalis		*G. vaginalis+Lactobacillus*	
OD495	**Biofilm formation**	**OD495**	**Biofilm formation**
0.0026	absent	0.0054	absent
0.0045	absent	0.0053	absent
0.0049	absent	0.0048	absent
0.0010	absent	0.0018	absent
0.0039	absent	0.0016	absent
0.0064	absent	0.0045	absent
0.0058	absent	0.0054	absent
0.0022	absent	0.0017	absent
0.0026	absent	0.0058	absent
0.0079	weak	0.0044	absent
0.0085	weak	0.0032	absent
0.0132	weak	0.0051	absent
0.0079	weak	0.0051	absent
0.0090	weak	0.0019	absent
0.0086	weak	0.0066	absent
0.0113	weak	0.0035	absent
0.0129	weak	0.0021	absent
0.0182	moderate	0.0098	weak
0.0169	moderate	0.0096	weak
0.0255	moderate	0.0114	weak
0.0211	moderate	0.0077	weak
0.0178	moderate	0.0103	weak
0.0199	moderate	0.0086	weak
0.0213	moderate	0.0092	weak
0.0249	moderate	0.0099	weak
0.0193	moderate	0.0131	weak
0.0255	moderate	0.0117	weak
0.0297	strong	0.0162	weak
0.0454	strong	0.0125	weak
0.0317	strong	0.0155	moderate
0.0433	strong	0.0176	moderate
0.0501	strong	0.0142	moderate
0.0427	strong	0.0139	moderate

0.0501	strong	0.0213	moderate
0.0290	strong	0.0363	strong
0.0398	strong	0.0498	strong

After 24-h incubation the TCP method detected total positive biofilm production in 27 (75%) of the microtiter wells with *G. vaginalis* monocultures and in 19 (53%) wells containing mixed (*G. vaginalis+Lactobacillus*) cultures. Among these, according to the OD values, 9 (25%), 10 (28%) and 8 (22%) of *Gardnerella* monocultures were strong, moderate and weak biofilm producers, compared to 2 (5.5%), 5 (14 %) and 12 (33.5%) of *Gardnerella+Lactobacillus* cocultures, respectively (Table 3 and Figure 1).

Table 3. Comparison of biofilm formation capacities of *G. vaginalis* monocultures vs *G. vaginalis+Lactobacillus* co-cultures

N° of inoculated G.vaginalis strains		**Biofilm production**			
		Absent	**Weak**	**Moderate**	**Strong**
in **MONOCULTURE**	36	9 (25%)	8 (22 %)	10 (28%)	9 (25%)
in **CO-CULTURE** with Lactobacillus	36	17 (47%)	12 (33.5%)	5 (14%)	2 (5.5%)

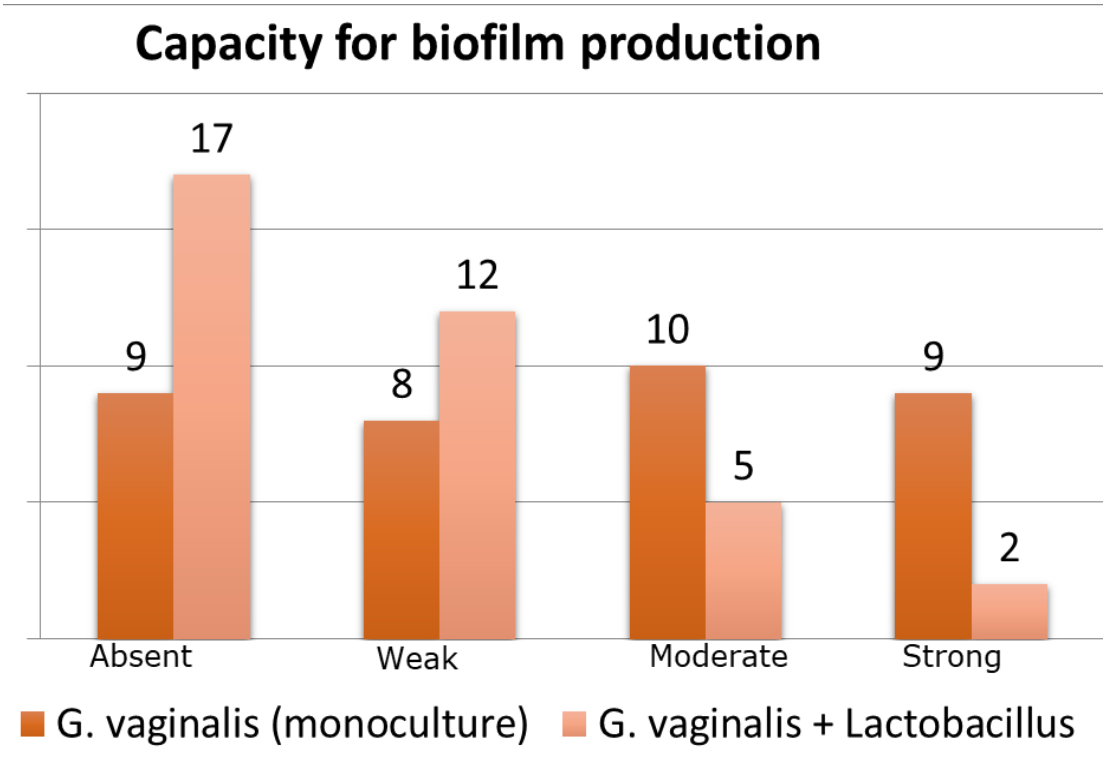


Figure 1: Biofilm formation capacities of *G. vaginalis* monocultures vs *G. vaginalis+Lactobacillus* co-cultures

Discussion

Findings of a diverse polymicrobial biofilm on the epithelial vaginal biopsy specimens from women with clinical signs of *BV* and analysis of its composition and structure position *G. vaginalis*, the major component of these microbial communities, at the center of BV pathogenesis [14, 27- 30].

Considering its potent biofilm-forming abilities, it has been implied that *G. vaginalis* commence the colonization of the vaginal epithelium and acts as a platform which other species subsequently can attach [27- 32].

Since depletion of lactic producing bacteria combined with overgrowth of various anaerobic opportunists is considered a key factor for development of BV [33] and certain strains of lactobacilli are acknowledged as crucial for (reversal of alterations in the vaginal microbiology) (restore a normal vaginal flora and eliminate bacterial vaginosis) many studies have been conducted for investigation of the effect of lactobacilli on BV biofilms and their therapeutic potentials.

In the study of Saunders et al. (2007), changes in structure and viability as well as reduced density of *Gardnerella vaginalis* biofilm pods were detected by deconvolution microscopy after a challenge with different L. strains *(L. reuteri* RC-14, *L. crispatus* 33820, *L. iners* and *L. rhamnosus* GR-1) [34]. Similarly, Hütt et al. in their study confirmed anti-adhesive activity of several investigated lactobacilli strains (*L. acidophilus*, *L. gasseri*, and *L. jensenii*s) against *C. albicans*, *E. coli*, and *G. vaginalis* [35].

In another study conducted by Attassi et al. (2006) co-culturing of *Gardnerella vaginalis* and *Prevotella bivia* on human cervix epithelial HeLa cells with *Lactobacillus acidophilus, Lactobacillus jensenii, Lactobacillus gasseri* and *Lactobacillus crispatus r*esulted with decreased viability of the pathogens and inhibition of their attachment onto HeLa cells [36].

This effect in vivo was investigated in a clinical, placebo-controlled, randomized study which included 64 women with BV. All of the participants were randomly assigned to receive tinidazole (2 g/day)+placebo or tinidazole (2 g/day)+capsules with *L. rhamnosus* GR-1 and *L. reuteri* RC-14 I a period of 28 days. According to the results, at the end of the trial, the cure rate of BV and establishment of normal vaginal microbiome were significantly higher in the probiotic group compared to placebo (87.5% vs 50.0%; $p = 0.001$ and 75.0% vs. 34.4%; $p = 0.011$, accordingly)[37].

Our results revealed strong association between lactobacilli and reduced adheration and biofilm formation of *Gardnerella* species which correlates with data from other studies in this field.

Findings in this research confirm the probiotic potentials of lactobacilli, since they effectively reduced the adheration and biofilm formation of the tested *Gardnerella* species which is a well-known and desired effect of strains with potential for eradication of vaginal biofilms. The elucidation of the antagonistic mechanisms as well as their effect on human cells may be useful in enlightening the importance of development of new products containing such microorganisms or products secreted by them.

References:

1. Machado D, Castro J, Palmeira-de-Oliveira A, Martinez-de-Oliveira J, Cerca N. Bacterial vaginosis biofilms: Challenges to current therapies and emerging solutions. Front Microbiol. 2015;6:1528.
2. Huang B, Fettweis JM, Brooks JP, Jefferson KK, Buck GA. The changing landscape of the vaginal microbiome. Clin Lab Med. 2014;34:747–61.
3. SE Hawes, SL Hillier, J Benedetti, *et al.*Hydrogen peroxide-producing lactobacilli and acquisition of vaginal infections. J Infect Dis, 174 (1996), pp. 1058-1063
4. V Redondo-Lopez, RL Cook, JD Sobel. Emerging role of lactobacilli in the control and maintenance of the vaginal bacterial microflora. Rev Infect Dis, 12 (1990), pp. 856-872
5. A Aroutcheva, D Gariti, M Simon, *et al.*Defense factors of vaginal lactobacilli. Am J Obstet Gynecol, 185 (2001), pp. 375-379

6. Hillier SL, Krohn MA, Rabe LK, Klebanoff SJ, Eschenbach DA: The normal vaginal flora, H2O2-producing lactobacilli, and bacterial vaginosis in pregnant women. Clin Infec Dis. 1993, 16: S273-81.
7. Thorsen P, Jensen IP, Jeune B, Ebbesen N, Arpi M, Bremmelgaard A, Moller BR: Few microorganisms associated with bacterial vaginosis may constitute the pathologic core: A population-based microbiologic study among 3596 pregnant women. Am J Obstet Gynecol. 1998, 178: 580-587. 10.1016/S0002-9378(98)70442-9.
8. Sobel, J. D. (2000). Bacterial vaginosis. *Annu. Rev. Med.* 51, 349–356. doi: 10.1146/annurev.med.51.1.349
9. Van Oostrum N, De Sutter P, Meys J, Verstraelen H. Risks associated with bacterial vaginosis in infertility patients: A systematic review and meta-analysis. Hum Reprod. 2013;28:1809–15.
10. Koumans, E. H., Sternberg, M., Bruce, C., McQuillan, G., Kendrick, J., Sutton, M., et al. (2007). The prevalence of bacterial vaginosis in the United States, 2001-2004; associations with symptoms, sexual behaviors, and reproductive health. *Sex. Transm. Dis.* 34, 864–869. doi: 10.1097/OLQ.0b013e318074e565
11. T. T. Suehiro, N. Malaguti, E. Damke et al., "Association of human papillomavirus and bacterial vaginosis with increased risk of high-grade squamous intraepithelial cervical lesions," *International Journal of Gynecological Cancer*, vol. 29, no. 2, pp. 242–249, 2019.
12. C. Chehoud, D. J. Stieh, A. G. Bailey et al., "Associations of the vaginal microbiota with HIV infection, bacterial vaginosis, and demographic factors," *AIDS*, vol. 31, no. 7, pp. 895–904, 2017.
13. Schwebke JR, Muzny CA, Josey WE. Role of *Gardnerella vaginalis* in the pathogenesis of bacterial vaginosis: a conceptual model. J Infect Dis. 2014;210:338–43.
14. Swidsinski, A., Mendling, W., Loening-Baucke, V., Ladhoff, A., Swidsinski, S., Hale, L. P., et al. (2005). Adherent biofilms in bacterial vaginosis. *Obstet. Gynecol.* 106, 1013–1023.
15. Heukelekian H, Heller A. Relation between food concentration and surface for bacterial growth. J Bacteriol. 1940; 40:547-58.
16. Lewandowski Z. Structure and function of biofilms. In: Evans LV, editor. Biofilms: recent advances in their study and control. Amsterdam: Harwood Academic Publishers; 2000. p. 1-17.
17. Donlan RM. Role of biofilms in antimicrobial resistance. ASAIO J 2000;46;S47-S52.
18. Thien-Fah CM, George AoO'T. Mehanismas of biofilm resistance to antimicrobial agents. Trends in microbiology 2001 January;
19. Patterson, J. L., Girerd, P. H., Karjane, N. W., and Jefferson, K. K. (2007). Effect of biofilm phenotype on resistance of *Gardnerella vaginalis* to hydrogen peroxide and lactic acid. *Am. J. Obstet. Gynecol.* 197, e1–e7.
20. Swidsinski, A., Mendling, W., Loening-Baucke, V., Swidsinski, S., Dörffel, Y., Scholze, J., et al. (2008). An adherent *Gardnerella vaginalis* biofilm persists on the vaginal epithelium after standard therapy with oral metronidazole. *Am. J. Obstet. Gynecol.* 198, 97.e1–e6.
21. Silva M, Jacobus NV, Deneke C, Gorbach SL. Antimicrobial substance from a human *Lactobacillus* strain. Antimicrobial Agents and Chemotherapy. 1987;31:1231–1233.
22. Aroutcheva AA, Simoes JA, Faro S. Defense factors of vaginal lactobacilli.Infect Dis Obstet Gynecol. 2001; 9(1):33-9.
23. Reid G, Burton J. Use of *Lactobacillu*s to prevent infection by pathogenic bacteria. Microbes Infect. 2002 Mar; 4(3):319-2*4.*
24. Christensen GD, Simpson WA, Bismo AL, Beachery EH. The adherence of the slime-producing strains of Staphylococcus epidermidis to smooth surfaces. Infect immune. 1982;37:318–26.
25. Braga PC, Dal SM, Culici M, Spallino A. Inhibitory activity of thymol on native and mature Gardnerella vaginalis biofilms: in vitro study. Arzneimittelforschung 2010;60(11):675–81.
26. Stepanovic, S.; Vukovic, D.; Hola,V.; Bonaventura, G.; Djukic, S.; Irkovic, I.C.; Ruzicka, F. Quantification of biofilm in microtiter plates: overview of testing conditions and practical

recommendations for assessment of biofilm production by staphylococci. APMIS 2007; 71 (5), 687-690.
27. Castro, J., Machado, D. & Cerca, N. Unveiling the role of *Gardnerella vaginalis* in polymicrobial Bacterial Vaginosis biofilms: the impact of other vaginal pathogens living as neighbors. *ISME J* 13, 1306–1317 (2019).
28. Hardy L, Jespers V, Dahchour N, Mwambarangwe L, Musengamana V, Vaneechoutte M, et al. Unravelling the bacterial vaginosis-associated biofilm: a multiplex *Gardnerella vaginalis* and *Atopobium vaginae* fluorescence *in situ* hybridization assay using peptide nucleic acid probes. PLoS ONE. 2015;10:e0136658.
29. Machado A, Cerca N. Influence of biofilm formation by *Gardnerella vaginalis* and other anaerobes on bacterial vaginosis. J Infect Dis. 2015;212:1856–61.
30. Hardy L, Jespers V, Van den Bulck M, Buyze J, Mwambarangwe L, Musengamana V, et al. The presence of the putative *Gardnerella vaginalis* sialidase A gene in vaginal specimens is associated with bacterial vaginosis biofilm. PLoS ONE. 2017;12:e0172522.
31. Jung HS, Ehlers MM, Lombaard H, Redelinghuys MJ, Kock MM. Etiology of bacterial vaginosis and polymicrobial biofilm formation. Crit Rev Microbiol. 2017;43:651–67.
32. Swidsinski A, Loening-Baucke V, Mendling W, Dörffel Y, Schilling J, Halwani Z, et al. Infection through structured polymicrobial *Gardnerella* biofilms (StPM-GB). Histol Histopathol. 2013;29:567–87.
33. Tamrakar, R., Yamada, T., Furuta, I. *et al.* Association between *Lactobacillus* species and bacterial vaginosis-related bacteria, and bacterial vaginosis scores in pregnant Japanese women. *BMC Infect Dis* **7,** 128 (2007).
34. Saunders S, Bocking A, Challis J, Reid G. Effect of Lactobacillus challenge on Gardnerella vaginalis biofilms. *Colloids Surf B Biointerfaces*. 2007;55(2):138-142.
35. Hütt P., Lapp E., Štšepetova J., et al. Characterisation of probiotic properties in human vaginal lactobacilli strains. *Microbial Ecology in Health & Disease*. 2016;27:1–9.
36. Atassi F, Brassart D, Grob P, Graf F, Servin AL. Lactobacillus strains isolated from the vaginal microbiota of healthy women inhibit Prevotella bivia and Gardnerella vaginalis in coculture and cell culture. *FEMS Immunol Med Microbiol*. 2006;48(3):424-432.
37. Martinez RC, Franceschini SA, Patta MC, et al. Improved cure of bacterial vaginosis with single dose of tinidazole (2 g), Lactobacillus rhamnosus GR-1, and Lactobacillus reuteri RC-14: a randomized, double-blind, placebo-controlled trial.