RESEARCH ARTICLE

Natural antimicrobials subtilosin and lauramide arginine ethyl ester synergize with conventional antibiotics clindamycin and metronidazole against biofilms of *Gardnerella vaginalis* but not against biofilms of healthy vaginal lactobacilli

Ammar Algburi\(^1,2\), Anna Volski\(^3\) and Michael L. Chikindas\(^4,\ast\)

\(^1\)Department of Biochemistry and Microbiology, Rutgers State University, New Brunswick, NJ 08901, USA, \(^2\)Department of Microbiology, Veterinary College, Diyala University, Baqubah, Iraq, \(^3\)School of Arts and Science, Rutgers State University, New Brunswick, NJ 08901, USA and \(^4\)School of Environmental and Biological Sciences, Rutgers State University, New Brunswick, NJ 08901, USA

\(*\)Corresponding author: Rutgers State University, 65 Dudley Road, New Brunswick, NJ 08901, USA. Tel: +1-848-932-5405; E-mail: tchikindas@aesop.rutgers.edu

One sentence summary: The biofilms of BV-associated pathogens can be controlled by synergistically acting combinations of conventional antibiotics and natural antimicrobials. This creates a foundation for a new strategy in the effective control of vaginal infections.

**ABSTRACT**

The purpose of this study was to evaluate the ability of clindamycin and metronidazole to synergize with natural antimicrobials against biofilms of bacterial vaginosis (BV)-associated *Gardnerella vaginalis*. Minimum bactericidal concentrations for biofilm cells (MBCs-B) were determined for each antimicrobial. The MBCs-B of lauramide arginine ethyl ester (LAE), subtilosin, clindamycin and metronidazole were 50, 69.5, 20 and 500 \(\mu\)g mL\(^{-1}\), respectively. A checkerboard assay and isobologram were used to analyze the type of interactions between these antimicrobials. The combination of metronidazole with natural antimicrobials did not inhibit planktonic lactobacilli. Clindamycin with either LAE or with subtilosin was inhibitory for planktonic but not for biofilm-associated lactobacilli. All tested antimicrobial combinations were inhibitory for BV-associated *Mobiluncus curtisi* and *Peptostreptococcus anaerobius*. LAE and subtilosin synergized with clindamycin and metronidazole against biofilms of *G. vaginalis* but not biofilm-associated vaginal lactobacilli. The biofilms of BV-associated pathogens can be controlled by synergistically acting combinations of conventional antibiotics and natural antimicrobials which will help better management of current antibiotics, especially considering robust bacterial resistance. Our findings create a foundation for a new strategy in the effective control of vaginal infections.

**Keywords:** natural antimicrobials; *Gardnerella vaginalis*; biofilm; antimicrobial synergy
INTRODUCTION

Bacterial vaginosis (BV) is a commonly spread vaginal infection which occurs in women of an adolescent (Mascarenhas et al. 2012) and child-bearing age (Sobel 2000; Forsum et al. 2005; Larsson and Forsum 2005; John, Mares and Spear 2007) due to the replacement of protective vaginal lactobacilli with anaerobic pathogens, predominantly Gardnerella vaginalis. Toxin production and the ability to form thick biofilm are the most known virulent properties of G. vaginalis (Patterson et al. 2010). In addition, there are several mechanisms contributing to biofilms-associated bacteria increased resistance to antibiotics (for review, see Stewart and Costerton 2001).

Healthy vaginal lactobacilli produce bacteriocins, hydrogen peroxide and lactic acid, which work as antimicrobial agents against G. vaginalis and its biofilms to keep the vaginal environment healthy and protected (for review, see Dover et al. 2008). In addition, Al Kassaa et al. (2014) found that some of the isolated vaginal lactobacilli inhibit vaginal pathogens such as G. vaginalis CIP7074T. The ‘antagonistic lactobacilli’ were capable to co-aggregate with these pathogens. Also, production of antimicrobial substances by biofilm-associated lactobacilli is often greater than in planktonic cells. Jones and Versalovic (2009) found that in vitro, biofilms-associated Lactobacillus reuteri modulates the production of cytokine and potentiates the antimicrobial activity of the bacteriocin reuterin. Saunders et al. (2007) referred to the possibility of eradicating the biofilm-associated G. vaginalis, the predominant bacteria associated with BV, by re-establishing the biofilms of L. reuteri, which controls the overgrowth of pathogenic and commensal microbes.

The loss of vaginal lactobacilli protection, elevation of vaginal pH above 4.5 and increasing number of anaerobic pathogens are the most common characteristic features of BV (Syed and Braverman 2001). Nugent, Krohn and Hillier (1991) proposed a scoring system for the diagnosis of BV by analyzing the bacterioscopy of a vaginal smear. On a scale from 0 to 10, the score of bacterial vaginosis is equal to 7 points or higher. While the symptoms can be mild, BV is in fact health and life threatening when it persists, causing gynecological and obstetrical complications (Scott and Smyth 1997; Graham et al. 2009).

Most of the known antibiotics are not effective in controlling biofilm-associated BV-causing pathogens. Antibiotic resistance and infection reoccurrence are the most problematic post-treatment challenges (Colli, Landoni and Parazzini 1997), Bannatyne and Smith 1998; Beigi et al. 2004). The reported devastating effects of G. vaginalis and BV-associated biofilms on human health (Mikamo et al. 1999; Cohen et al. 2012) urge researchers to put an effort into finding tools for treatment and prophylaxis of this bacterial infection. At the same time, these treatments should not affect the healthy vaginal lactobacilli which protect the vaginal environment.

Bacteriocins are ribosomally produced antimicrobial peptides of bacterial origin (for review, see Cotter, Hill and Ross 2005). Bacteriocin subtilosin A inhibits BV-associated pathogens (Sutyak et al. 2012) and their biofilms (Turovskiy et al. 2012). Similar to many bacteriocins, subtilosin targets bacterial cytoplasmic membranes. However, unlike nisin, subtilosin works selectively against vaginal pathogens without killing healthy vaginal lactobacilli (Turovskiy et al. 2012).

The natural antimicrobial lauramide arginine ethyl ester (LAE) is a cationic surfactant inhibitory against bacteria, fungi and yeast (Infante et al. 1984), and effective against biofilm-associated G. vaginalis (Turovskiy et al. 2012).

Sutyak Noll et al. (2012) reported on subtilosin’s activity against planktonic cells of G. vaginalis, alone and as a synergistically acting combination with LAE and/or polysine. Cavera, Volsky and Chikindas (2015) evaluated combinations of these antimicrobials with clindamycin and metronidazole against planktonic G. vaginalis cells.

This study’s objective emerged from the need to avoid the undesirable side effects caused by high dosage of each antimicrobial and to reduce the chance of occurrence of resistant mutants. This was done by using multicomponent, synergistically acting formulations, where stressors had different targets on microbial cells. These combinations were also evaluated against other anaerobic pathogens that are predominantly isolated from vaginal samples taken from BV-infected women. The MBCs-B of combined antimicrobials were also assessed against the predominant vaginal lactobacilli.

MATERIALS AND METHODS

Bacterial strains, culture media and growth conditions

From the frozen stock (~80 C), G. vaginalis ATCC 14018 was inoculated in three culture media and propagated at 37 C for 48 h. Brain–heart infusion (BHI) broth (Difco BD, Franklin, NJ, USA) supplemented with horse serum 3% (JRH Biosciences, KS) was used to maintain microbial growth. Human blood tissue broth (HBT) agar (Remel, Lenexa, KS, USA) supplemented with horse serum 3% (JRH Biosciences, KS) was used to confirm the purity of the frozen stock. The inoculated broth and HBT agar were incubated anaerobically (10% hydrogen, 5% carbon dioxide and 85% nitrogen) using an anaerobic gloves box (Coy Laboratory Products, Inc., Grass Lake, MI, USA). After the incubation period, the bacterial cells were transferred to BHI broth supplemented with 1% glucose (Fisher Scientific, Waltham, MA, USA) (BHIG), every 24 h twice prior to the initiation of an experiment. In order to provide suitable conditions for G. vaginalis anaerobic growth and to avoid oxidative stress, culture media were pre-incubated in the anaerobic chamber at least overnight before bacterial inoculation.

Five species of representative vaginal Lactobacillus were used in this project to evaluate the possible effect of antimicrobial combinations. Lactobacillus vaginalis ATCC 49540, L. plantarum ATCC 39268, L. acidophilus ATCC 4356, L. rhamnosus 160 (gift of Dr Aroucheva, Rush University Medical Center) and L. gasseri ATCC 33323 were taken from the frozen stock and suspended from the frozen stock (–80 C) and child-bearing age (Sobel 2000). From the frozen stock (–80 C), L. rhamnosus 160 (gift of Dr Aroucheva, Rush University Medical Center) and L. gasseri ATCC 33323 were taken from the frozen stock and suspended from the frozen stock (–80 C).

The antimicrobial interactions were also evaluated against Peptostreptococcus anaerobius ATCC 27237, Mobiluncus curtisi ATCC 35241 and Prevotella bivia ATCC 29303, the predominant anaerobes that have been identified in vaginal samples taken from BV-infected women. The anaerobes were maintained in the anaerobic chamber and transferred daily using BHI supplemented with 3% horse serum (BHIH).

Stock solutions of antibacterial agents

Four antimicrobials were evaluated for their activity against biofilm-associated G. vaginalis. LAE (Vedeqsa, Inc., L- Lamirsa, LAE-CF) was a gift from Vedeqsa, Inc. (Barcelona, Spain).
Clindamycin phosphate and metronidazole were purchased from TCI America (Portland, OR, USA). Subtilosin was produced as previously described by Sutyak et al. (2008) and stored as a stock solution containing $5.56 \pm 0.23$ mg mL$^{-1}$ of the protein. The stock solution of LAE was 10 mg mL$^{-1}$. Clindamycin phosphate and metronidazole were prepared as a stock solution of 10 mg mL$^{-1}$. The stock solutions of antimicrobials were dissolved in double deionized water (ddH$_2$O), sterilized using syringe filter 0.45 $\mu$m and kept in the refrigerator for a maximum of 3 weeks. On the day of the experiment, the stock solutions were diluted in the anaerobic chamber (to avoid oxidative stress) with pre-incubated BHIG, BHIH or MRS broth (according to the bacterial species) to avoid changing the concentrations of nutrients of growth media.

**Determination of minimum biofilm inhibitory concentrations (MICs-B)**

MIC determination was performed according to Sutyak Noll et al. (2012) with minor modifications. Briefly, the antimicrobials were diluted (a series of 2-fold dilutions) with an appropriate volume of fresh BHIG in 96-well tissue culture plate (Falcon, Corning Incorporated, Corning, NY, USA). The final volume of antimicrobial agents diluted into the broth was 100 $\mu$L in each well. The overnight cell culture at $3 \pm 2 \times 10^8$ CFU mL$^{-1}$ was diluted in BHIG to the final $5 \times 10^6$ CFU mL$^{-1}$. From the diluted bacterial cells, 100 $\mu$L was transferred in the wells containing pre-determined concentrations of antimicrobials. Plates were incubated under anaerobic conditions at 37°C for 24–28 h. Mineral oil (Sigma-Aldrich chemical, St. Louis, MO, USA) was added (75 $\mu$L) to each well to avoid evaporation. The MIC was determined by taking the endpoint reading using a plate reader (Model 550, Bio-Rad Laboratories, Hercules, CA, USA). The MIC was defined according to Clinical and Laboratory Standards Institute guidance (2010) as the lowest concentration of antimicrobial in wells with absorbance $A_{0.95}$ equal to or less than 20% of the control’s mean absorbance (bacterial growth without antimicrobial addition).

**Bacterial biofilm formation assay**

The biofilm formation assay was performed using the method described by Turovskiy et al. (2012) with minor modifications. Briefly, from an overnight culture of *G. vaginalis* in BHIIHS, 200 $\mu$L was transferred into a 50 mL test tube (Thermo Scientific, Rochester, NY, USA) containing 20 mL of BHIIH broth. The tube was incubated overnight at 37°C under anaerobic conditions using the anaerobic chamber. Following incubation, 750–1000 $\mu$L of the cell suspension was transferred into a new tube with 20 mL of fresh BHIG broth to achieve $5 \times 10^8$ CFU mL$^{-1}$. Then, 200 $\mu$L of the cell suspension was pipetted into a sterile 96-well polystyrene flat bottom tissue culture plate (Falcon, Corning Incorporated, Corning, NY, USA). An amplification tape (Nalge Nunc International, Rochester, NY, USA) was used to cover the 96-well plate to avoid medium evaporation. The culture plate was incubated anaerobically for 24–27 h at 37°C. For vaginal lactobacilli biofilm, we followed the Jones and Versalovic (2009) method with minor modifications. Briefly, frozen stock lactobacilli was taken with a disposable loop (Fisher Scientific, Pittsburgh, PA, USA) and directly inoculated into MRS broth. After overnight incubation at 37°C, 200 $\mu$L of bacterial suspension was transferred into 50 mL tube containing 20 mL MRS broth supplemented with 1% of glucose and 2% sucrose (MRS-GS) and incubated aerobically at 37°C for 24 h without agitation. To assure consistency in the number of cells used in the study, optical density (OD$_{600}$) of second overnight bacterial culture was measured (SmartSpec 3000 Spectrophotometer, Bio-Rad Laboratories, Hercules, CA, USA) and adjusted, if necessary, to OD$_{600} = 3.03 \pm 0.043$ which was $\sim 10^6$ CFU mL$^{-1}$. Then, 200 $\mu$L of cell suspension was inoculated into 20 mL MRS-GS, mixed by Vortex and incubated for 15 min at 37°C. Then, 200 $\mu$L of the last suspension ($=10^7$ CFU mL$^{-1}$) was added to the wells of a 96-well tissue culture plate. Amplification tape was used to cover the 96-well microplate and avoid medium evaporation. The microplates were incubated at 37°C under aerobic conditions for 24 h. After the incubation period, each well was gently washed twice with 200 $\mu$L of fresh medium (BHIG was used for *G. vaginalis* and MRS-GS for vaginal lactobacilli) to remove non-adhered bacteria. To disrupt biofilm, vigorous pipetting was performed with 200 $\mu$L of fresh broth. For each well, six 10-fold dilutions (10$^{-1}$–10$^{-6}$ CFU mL$^{-1}$) were made with a fresh culture media. Then, 20 $\mu$L from each dilution was plated in duplicates on agar plate (BHI was used for plating *G. vaginalis* and MRS was used for vaginal lactobacilli). The plates were incubated for 72 h at 37°C. The grown colonies were enumerated using the colony counter (Corporate Headquarters Reichert, Inc., Buffalo, NY, USA).

**Time-bactericidal activity of antimicrobials against biofilm-associated *G. vaginalis***

First, supernatant was discarded and the biofilm was gently washed twice with fresh BHIG broth to remove non-attached cells. The concentrations of antimicrobials in the wells were as following: LAE 1000 $\mu$g mL$^{-1}$, subtilosin 138.9 $\mu$g mL$^{-1}$, clindamycin and metronidazole were added at 2000 $\mu$g mL$^{-1}$. These concentrations were chosen according to our previous findings (Turovskiy et al. 2012), in which antimicrobials with almost the same concentrations were active against *G. vaginalis*’s biofilm. Viability of the cells in antimicrobials-treated biofilms was evaluated at 0, 1, 3, 8 and 18 h of incubation, in duplicates to identify the time points (s) at which antimicrobial agents show their highest activity against the well-established biofilms. At each time point, biofilms were gently washed twice with fresh BHIG broth to remove antimicrobial and free cells. Then, biofilms were disrupted by vigorous pipetting, diluted in BHIG broth and plated on BHI agar using the drop plate method (described below) to identify microbial survivability at each time point in the presence of the selected antimicrobials. MBC-B was defined as the minimum concentration of antibacterial agent that causes $\geq 3$ log reduction in the number of viable cells as compared to the positive control (Qu et al. 2010). The positive control included the biofilm grown for 24–28 h without added antimicrobial agents. Two negative controls were used, which included BHIG broth alone (control for medium sterility) and the diluted antimicrobial agents in BHIG (control for antimicrobial’s sterility). The experiment was repeated three times.

**Plate counting method**

The number of bacteria that survived was expressed in colony-forming units per milliliter (CFU mL$^{-1}$) and was enumerated using the drop plate method. The methodology is performed as described by Hamilton and Heersink (2001), with a minor modification. The washed previously described biofilm was disrupted by vigorous pipetting with 200 $\mu$L of fresh BHIG broth. Six 10-fold dilutions for each well (from $10^{-1}$–$10^{-6}$ CFU mL$^{-1}$) were made using pre-incubated fresh BHIG1% broth. Then, 20 $\mu$L of the cell suspension was transferred from each dilution and spotted in duplicate on BHI agar plates which were then incubated for
Figure 1. Checkerboard assay, an example for two antimicrobial combinations.

72 h at 37°C under anaerobic conditions. The number of colonies between 2 and 20 CFU per spot was regarded as a quantifiable number.

**Checkerboard assay**

The checkerboard assay was conducted to evaluate the activity of antimicrobial combinations against bacterial cells in biofilm using a 96-well tissue culture plate. It was performed as described by Draper et al. (2013). Following biofilm formation, the non-adherent cells were removed and the wells washed twice with a fresh broth. In a separate and sterile 96-well microplate, 2-fold dilutions were made for each antimicrobial agent with BHIG broth. From each dilution of antimicrobial B, 125 μL was added horizontally over 125 μL of antimicrobial A. The combinations of antimicrobial agents are explained (Fig. 1). From each combination, 200 μL was added to the washed biofilm in the 96 wells of the plate. The plate was incubated for 8–9 h at 37°C in the anaerobic chamber. The drop plate method was used for the viable cells enumeration. The MBCs-B of combined antimicrobials were identified, and their anti-biofilm activity (synergistic, antagonistic or additive) was assessed using the isobologram.

**Checkerboard assay, data analysis**

In our study, isobologram was used to analyze the interactions of natural antimicrobial agents with commonly used antibiotics. This method is based on the comparison of the MBC-B value of each individual antimicrobial with its MBC-B value when used in combination. Axis (X) will represent MBC-B of antimicrobial (A) with the coordinates (0, x), and axis (Y) will represent antimicrobial (B) with the coordinates (y, 0). The two points (A) and (B) are connected by a line (Turovskiy and Chikindas 2011). Each MBCs-B value of two combining antimicrobials is represented as a point on the graph. In this study, three of these points are selected and plotted. Results are expressed according to locations of MBCs-B points on the line that connects (A) and (B) as follows: when MBCs-B points are located under or above the line, the two combining antimicrobials are synergized or antagonized respectively, against the tested microorganism.

**Statistics**

Each antimicrobials combination was conducted at least three times in duplicate. The results illustrate the average of three experiments unless it is mentioned otherwise.
Evaluation of antimicrobial activity against biofilm-associated G. vaginalis and planktonic lactobacilli

MIC-B was determined using the broth microdilution method to evaluate the activity of antimicrobials against biofilm-associated G. vaginalis. Low concentrations of natural antimicrobials were effective against biofilm-associated G. vaginalis but not against vaginal lactobacilli which were tolerant to the much higher concentrations. Clindamycin at 1.56 μg mL⁻¹ inhibited the growth of G. vaginalis. However, at this concentration it was bactericidal for L. vaginalis and L. plantarum but not for other vaginal lactobacilli. While the MIC of metronidazole for lactobacilli was relatively high (>200 μg mL⁻¹), the MIC-B of metronidazole for G. vaginalis biofilm was 6.25 μg mL⁻¹. The MIC-B of LAE and subtilosin for G. vaginalis were 6.25 and 3.7 μg mL⁻¹, respectively. Vaginal lactobacilli planktonic growth was inhibited only when high concentrations of these antimicrobials were used (Table 1).

Estimation of the time required for the highest activity of the studied antimicrobials against biofilm-associated G. vaginalis

To determine the time required for the antimicrobial agents to efficiently inhibit biofilms- associated G. vaginalis at predetermined concentrations, survivability of antimicrobials-treated biofilms was evaluated at 0, 1, 3, 8 and 18 h of incubation, in duplicates (Fig. 2). The concentration of antimicrobials in this experiment were as following: LAE, 1000 μg mL⁻¹; subtilosin, 138.9 μg mL⁻¹; and clindamycin and metronidazole, 2000 μg mL⁻¹. Clindamycin alone at concentration 2000 μg mL⁻¹ produced 2.65 ± 0.17 log reduction after 8 h with no further growth inhibition of biofilm-associated cells after this time point. In order to determine the MBC-B of clindamycin, several concentrations of this antibiotic were tested (4, 6, 8, 16 and 20 mg mL⁻¹). We have noticed that only the 20 mg mL⁻¹, the MBC-B, cause 7 log reduction (killing effect ≥ 3 log reduction) in the number of viable cells (Table 2). Metronidazole 2000 μg mL⁻¹ killed biofilm-associated cells to the point of no detection (by plating) after 8 h incubation. Similarly, 500 μg mL⁻¹ of metronidazole had a bactericidal effect against biofilm-related G. vaginalis after 8 h (Table 2). LAE 1000 μg mL⁻¹ and subtilosin 138.9 μg mL⁻¹ killed 100% of biofilm cells during the first hour of treatment. We found that the MBCs-B of LAE and subtilosin were 50 and 69.5 μg mL⁻¹, respectively (Table 2).

Subtilosin synergized with clindamycin and with metronidazole against G. vaginalis biofilm

Based on the previously observed synergy in action of subtilosin and clindamycin against planktonic cells of G. vaginalis (Turovskiy et al. 2012), we proposed that combination of subtilosin with clindamycin could also decrease the high MBC-B value of clindamycin. According to our data (Fig. 3), subtilosin synergizes with clindamycin against biofilms of G. vaginalis. The MBC-B of subtilosin in combination with clindamycin decreased 8-fold from when it was used alone (8.6 μg mL⁻¹ in combination instead of 69.5 μg mL⁻¹ when it was used alone). The MBC-B of clindamycin in combination was more than 6-folds lower than when it was used alone (2.9 mg mL⁻¹ in combination instead of 20 mg mL⁻¹ alone). We noticed that metronidazole inhibited the growth of planktonic cells and biofilm formation of G. vaginalis with MIC-B 6.25 μg mL⁻¹, which was much lower than the concentration that inhibited the growth of vaginal lactobacilli, >200 μg mL⁻¹. The combination of subtilosin with metronidazole was acting synergistically against biofilm-associated G. vaginalis (Fig. 4). The MBC-B of subtilosin in combination was 16-folds lower than when it was used alone (4.3 μg mL⁻¹ in combination instead of 69.5 μg mL⁻¹ alone). The MBC-B of

**Table 1. The inhibitory concentrations of antimicrobials against biofilm-associated G. vaginalis and planktonic vaginal lactobacilli.**

<table>
<thead>
<tr>
<th>Antimicrobial agents</th>
<th>MIC-B of antimicrobials (μg mL⁻¹) / G. vaginalis</th>
<th>MIC of antimicrobials (μg mL⁻¹)/ vaginal lactobacilli</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subtilosin</td>
<td>3.7¹/9.2³ and 12³</td>
<td>&lt;500³, 725–825³</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>1.56³/16³</td>
<td>0.78–50¹, 0.78–77.5³</td>
</tr>
<tr>
<td>Metronidazole</td>
<td>6.25³/50³</td>
<td>&lt;200³, 50–100³</td>
</tr>
</tbody>
</table>

¹Data from this study, ²data from Sutyak Noll et al. (2012), ³data from Cavera, Volsky and Chikindas (2015).

**RESULTS**

**Figure 2. Bactericidal activity of antimicrobial agents against biofilm-associated G. vaginalis during 18 h incubation.** LAE, 1000 μg mL⁻¹ (reversed triangle) and subtilosin, 139 μg mL⁻¹ (open triangle) killed the biofilm cells during first hour of treatment, clindamycin, 2000 μg mL⁻¹ (closed circle); metronidazole, 2000 μg mL⁻¹ (open circle); and the control (the number cells of biofilms-associated G. vaginalis without expose to antimicrobial agents) (closed square). Error bars represent the standard deviations measured from three experiments.

**Table 2. MBCs-B of antimicrobials against biofilm-associated G. vaginalis after 8 h incubation.**

<table>
<thead>
<tr>
<th>Antimicrobial agents</th>
<th>MBC-B (μg mL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subtilosin</td>
<td>69.5</td>
</tr>
<tr>
<td>LAE</td>
<td>50</td>
</tr>
<tr>
<td>Metronidazole</td>
<td>500</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>20 000</td>
</tr>
</tbody>
</table>
metronidazole in combination was 8-folds lower than what it was used alone (62.5 μg mL\(^{-1}\) in combination instead of 500 μg mL\(^{-1}\) alone).

**LAE synergized with clindamycin and with metronidazole against biofilm-associated G. vaginalis**

LAE was combined with the two synthetic antibiotics to evaluate their interactions. Synergistic activity was found when LAE was combined with clindamycin and with metronidazole (Figs 5 and 6). The MBC-B of LAE in combination with clindamycin was eight times lower (6.25 μg mL\(^{-1}\)) than the MBC-B of the antimicrobial alone (50 μg mL\(^{-1}\)). In the same combination, MBC-B of clindamycin was almost 7-folds lower than when it was used alone (2.9 mg mL\(^{-1}\) in combination instead of 20 mg mL\(^{-1}\) alone).

Similar MBC-B value of LAE was found when it was combined with metronidazole (6.25 μg mL\(^{-1}\)) in combination instead of 500 μg mL\(^{-1}\) alone). The MBC-B of metronidazole in this combination was 8-folds lower than when it was used alone (62.5 μg mL\(^{-1}\) in combination instead of 500 μg mL\(^{-1}\) alone).

**Combinations of the studied antimicrobials do not inhibit biofilms of vaginal lactobacilli**

We noticed that when clindamycin was combined with subtilosin or with LAE, the growth of planktonic lactobacilli was inhibited. However, almost all combinations of metronidazole with subtilosin or with LAE did not inhibit normal growth of vaginal lactobacilli (Table 3). While combinations of clindamycin with subtilosin or with LAE inhibited the growth of planktonic lactobacilli, they were ineffective against biofilm-associated...


**Table 3. Antibacterial effect of synergistically acting combinations (against biofilm-associated *G. vaginalis*) on the growth ability of vaginal lactobacilli.**

<table>
<thead>
<tr>
<th>Antimicrobial combinations</th>
<th>The MBCs-B (μg mL⁻¹)</th>
<th>L.v⁺</th>
<th>L.p⁻</th>
<th>L.g⁺</th>
<th>L.a⁻</th>
<th>L.r⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subtilosin + clindamycin</td>
<td>34.7 ± 2900</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>17.3 ± 4400</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>8.6 ± 6600</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Subtilosin + metronidazole</td>
<td>4.3 ± 250</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>17.3 ± 62.5</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>LAE + clindamycin</td>
<td>6.25 ± 10 000</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>25 ± 2900</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>LAE + metronidazole</td>
<td>6.25 ± 250</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>12.5 ± 125</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>25 ± 62.5</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>±</td>
</tr>
</tbody>
</table>

(-) = Growth was inhibited, (+) = Growth ability was normal, (±) = Growth was partial inhibited. (a) *L. vaginalis*, (b) *L. plantarum*, (c) *L. gasseri*, (d) *L. acidophilus* and (e) *L. rhamnosus*.

**DISCUSSION**

Recently, a new trend emerged focused on evaluation of conventional antibiotics as formulations synergistically acting with bacteriocins (Naghmouchi et al. 2011; Draper et al. 2013). Consequently, this study focused on combinations of natural antimicrobial, LAE and subtilosin with clindamycin and metronidazole to assess synergistic activity against biofilms of *G. vaginalis*. The importance of exploring antimicrobial proteins, both alone and in combination with conventional drugs, is recognized by many investigators and as such was recently reviewed by Di Luca, Maccari and Nifosi (2014). Clindamycin and metronidazole are the commonly prescribed antibiotics for the treatment of BV (Workowski, Berman and Centers for Disease Control and Prevention 2010). Development of antimicrobial resistance (Beigi et al. 2004) and infection recurrence (Barbieri 2013) are the most dangerous side effects of abusing or overusing these antibiotics. The ability to use alternative medications such as bacteriocins, alone or in combination with synthetic antibiotics, is highly desirable for avoiding the disadvantages that come from using antibiotics alone. In our study, we evaluated the anti-biofilm activity of natural antimicrobials alone and in combination with the most commonly used antibiotics against biofilm-associated *G. vaginalis*. To evaluate the activity of antimicrobials against these biofilms, the labor-intensive direct plate count was used instead of more advanced methods, which appear to be less reliable according to our thorough investigation (Turovskiy et al. 2012). The bactericidal activity of the selected antimicrobial agents against *G. vaginalis* biofilm was evaluated for 18 h and confirms 8 h as a preferential time of exposure for the most efficient inhibition of the targeted microorganism by the studied antimicrobials and their formulations. When clindamycin 2000 μg mL⁻¹ was used, about 2.65 ± 0.17 log reductions were achieved after 8 h and the viable cells numbers remained unchanged after the next 10 h of incubation. When LAE 50 μg mL⁻¹, subtilosin 69.5 μg mL⁻¹ and metronidazole 500 μg mL⁻¹ were used, again the bactericidal activity reached its peak after 8–9 h. This finding is in accord with what we see as a convenient application time of antimicrobial agents in the vaginal environment during the night resting period (about 8–9 h). In our previous work, we reported that MBC-B of both subtilosin and LAE against biofilm-associated *G. vaginalis* which was 100 μg mL⁻¹ produce 3 and 5 log reduction, respectively, while clindamycin at concentration 1600 μg mL⁻¹ caused only 2 log reduction (Turovskiy et al. 2012). A discrepancy had been detected when MBC-Bs were measured between our data and what Turovskiy et al. (2012) found. That was due to the differences in brand names of the used antimicrobials, the exposure time to antimicrobials and experimental method that followed to determine the MBC-Bs. To avoid the undesirable side effects of using the conventional antibiotics alone for BV treatment (Bradshaw et al. 2006; Oduyebo, Anorlu and Ogunsola 2009), innovative solutions are urgently required. Synergistically acting combinations of natural antimicrobials and synthetic antibiotics are promising solutions for the future pharmaceutical formulations (Baker et al. 2007; Yang et al. 2014). In our study, four combinations were evaluated. The natural antimicrobials subtilosin and LAE were combined with antibiotics, clindamycin or metronidazole, using a checkerboard assay. After identifying the MBC-B value of each compound, a checkerboard assay was performed to evaluate the nature of combination between two antimicrobial agents. The linear logistical isobologram was used to analyze the nature of antimicrobial combinations if they are synergistic or antagonistic. Isobolograms are commonly used to strengthen the graphical and statistical design and support the data mathematically and biologically (Chen and Pounds 1998). In this study, synergistic activity was found when antimicrobials subtilosin and LAE were combined with clindamycin and metronidazole against biofilm-associated *G. vaginalis*. Subtilosin forms temporary pores leading to cell death (Sutyak Noll et al. 2012). A solid-state NMR experiment using model phospholipid bilayers showed that when subtilosin is used at high concentration, it binds to the lipid head group region and then partially embeds in the lipid bilayer membrane causing a lipid perturbation and permeabilization defect (Thennarasua et al. 2005). Many studies reported dangerous side effects of metronidazole, such as carcinogenicity in animals, genotoxicity in humans and in vitro mutagenicity (Stranz and Bradley 1981; Benderly, Menéndez and Ostrosky-Wegman 2002; Koss et al. 2012). Importantly, the synergy between natural antimicrobials and metronidazole may help to avoid the undesirable side effects which may be caused by high dosage of the antibiotic alone, and decrease the opportunity of bacterial mutation that leads (singular) to antibiotic resistance. LAE, the cationic surfactant, altered the permeability of cytoplasmic membrane to ions, such lactobacilli at concentrations inhibitory for *G. vaginalis* biofilms (data not shown).
as potassium ions, in both Gram-positive and -negative bacteria (Rodriguez et al. 2004). The antimicrobial activity of LAE against bacterial biofilm is not fully understood yet. Bonnau et al. (2010) noticed a strong interaction between LAE and anionic polymers such as alginate. Cotton, Graham, and Lee (2009) found that the anionic polysaccharide alginate is required for biofilm’s tolerance to antimicrobials. Therefore, it is possible that LAE interacts with alginate at the surface of biofilm, modifying its biopolymer structure and enhancing the biofilm susceptibility to clindamycin and metronidazole. Our findings are in accord with Cavaer, Volsky and Chikindas (2015), who analyzed the combination of natural antimicrobials with each other and with antibiotics using a fractional inhibition concentration index. Cavaer, Volsky and Chikindas (2015) noticed that subtilosin and LAE synergized with clindamycin as well as metronidazole to inhibit the growth of planktonic cells of G. vaginalis. Synergistic activity was detected when subtilosin was combined with the natural antimicrobials: LAE, glycerol monolaurate and PL against BV-associated pathogen G. vaginalis (Sutjak Noll et al. 2012). These data and ours confirm synergistic activity when natural antimicrobials combined with each other and/or with the synthetic antibiotics against G. vaginalis and its biofilm. It is imperative for any study focused on inhibition or killing of BV-associated pathogens to identify if these treatments influence non-pathogenic vaginal lactobacilli due to the importance of this microbiota in maintaining vaginal health. While there were many studies focused on the control of BV-associated pathogens, all of them neglected elucidation of the activity of antimicrobial compounds on vaginal lactobacilli (Hubrechts et al. 1984; Braga et al. 2010; Schwebke and Desmond 2011; Henriquez, Martinez-de-Oliveira and Cerca 2012; Brocklehurst et al. 2013; Hymes et al. 2013; Kandimalla et al. 2013; Pathak et al. 2014). On the contrary, we tested the selected combinations of antimicrobials (Table 4) against five species of vaginal lactobacilli using the broth microdilution method. Our study showed that only planktonic cells of vaginal lactobacilli were inhibited by the combination of clindamycin with either LAE or subtilosin. However, these combinations were harmless for biofilm-associated lactobacilli. At the same time, combinations of metronidazole with subtilosin or with LAE did not reduce the normal growth ability of either planktonic or biofilm-associated lactobacilli. Lack of metronidazole’s activity at concentration >200 μg mL⁻¹ against lactobacilli as observed in this study is in agreement with previously published reports (Simeon et al. 2001; Austin, Meyn and Hillier 2006). Moreover, Anukam and Reid (2008) found that metronidazole at 1 mg mL⁻¹ does not inhibit normal growth of L. rhamnosus GR-1 and L. plantarum KCA which was in agreement with the study by Ocan’a et al. (2004). Finally, according to Martin et al. (2008), healthy vaginal isolates of L. gasseri and L. plantarum were more resistant to metronidazole than to clindamycin, gentamicin, ciprofloxacin, trimethoprim and sulfametoxazole. The mechanism (s) of resistance to metronidazole is not fully understood. Church et al. (1996) explained that lactobacilli as facultative aerobic bacteria lack ferredoxin-linked hydrogenase, which is an essential enzyme for metronidazole-intracellular activity. According to previous studies and our data, the importance of using lower concentrations of metronidazole combined with the natural antimicrobials such as LAE and subtilosin may lead to enhance the antibiotic susceptibility of BV-associated pathogens, while keeping the probiotic vaginal lactobacilli alive. Clindamycin is a protein synthesis blocker (Chambers 2003). Our data illustrated that vaginal lactobacilli were sensitive to clindamycin 0.78 μg mL⁻¹; however, higher concentrations of subtilosin (>500) μg mL⁻¹ and LAE (16–62.5) μg mL⁻¹ were required for bacterial growth inhibition. Also, our findings are in agreement with the studies showing sensitivity of planktonic but not biofilm-associated lactobacilli to clindamycin (Coppola et al. 2005; Klare et al. 2007). In regard to vaginal lactobacilli biofilm, we found that the antimicrobial combinations (which were bactericidal to G. vaginalis-associated biofilm) had no effect on the normal growth of lactobacilli-formed biofilm. Biofilm-associated cells are often reported as being 100 to 1000 times more tolerant to various stresses than planktonic cells due to different mechanisms used to withstand these factors. Kubota et al. (2009) found that antimicrobial tolerance of biofilm-associated lactobacilli with different growth phases was higher than resistance in planktonic cells. Biofilm formation by lactobacilli (Ocana and Nader-Macias 2004) confers positive health effects for the vaginal environment. These advantages include a replacement of biofilm formed by pathogenic bacteria (Woojin, Jae-Sook and Jaesook 2011), improvement of the production of anti-pathogenic agents and increasing tolerance of biofilm-associated cells to antimicrobial factors. In our study, L. acidophilus and L. vaginalis formed rather thin and patchy biofilms when grown in a 96-well tissue culture plate. However, L. rhamnosus, L. plantarum and L. gasseri were capable of biofilm formation with a bacterial count of 5 × 10⁸–10⁹ CFU mL⁻¹. The ability of some lactobacilli species to attach on surfaces and establish their biofilms may depend on the cell hydrophobicity and the charge of the vaginal epithelial surface (Millsap et al. 1997). It is also believed that the establishment of biofilm by lactobacilli is genetically encoded. Sturme et al. (2005) found that production of cyclic thiolactone autoinducing peptide, which is encoded by luxS, the plantarum regulator, is associated with bacterial adherence. A luxS knockout in L. rhamnosus GG showed a defect in biofilm formation and metabolic activity (Lebeer et al. 2007). The genome sequences of L. plantarum WCF51 (GenBank accession no. NP_784522) and L. gasseri (GenBank accession no. ZP_00046310) confirmed the presence of luxS homologs. Also, it has been found that pili and cell surface proteins play an important role in lactobacilli adhesion and biofilm formation. Pili-coding genes were identified in genome sequences of some lactobacilli species (Forde et al. 2011), enhancing bacterial adhesion and biofilm formation (Danne and Dramsi 2012; Lebeer et al. 2012). The S-layer cell surface proteins of lactobacilli and their role in biofilm formation have been studied. Lorita et al. (1992) and Golowczycz et al. (2007) referred to the role of S-layer in lactobacilli aggregation and biofilm development such as enhancing bacterial cell adhesion. Biofilm formation may be influenced by the culture media that are used to grow lactobacilli. The MRS-GS broth was used as growth medium in our experiment in order to obtain a developed biofilm of lactobacilli in the 96-well tissue culture plate. The number of bacterial cells in biofilms was very low when lactobacilli were grown using MRS alone or supplemented with either sucrose or glucose. In addition to sucrose, glucose is considered as a main carbon source for lactobacilli (Kandler and Weiss 1986). Tenuta

<table>
<thead>
<tr>
<th>Antimicrobials combinations</th>
<th>MBC-B (μg mL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subtilosin + clindamycin</td>
<td>34.7 + 2900, 17.3 + 4400, 8.6 + 6600</td>
</tr>
<tr>
<td>Subtilosin + metronidazole</td>
<td>4.3 + 250, 17.3 + 62.5</td>
</tr>
<tr>
<td>LAE + clindamycin</td>
<td>25 + 2900, 6.26 + 10000</td>
</tr>
<tr>
<td>LAE + metronidazole</td>
<td>6.25 + 250, 12.5 + 125, 25 + 62.5</td>
</tr>
</tbody>
</table>

### Table 4. The MBCs-B of antimicrobial combinations which synergized against biofilm- associated G. vaginalis.
et al. (2006) found that when growth medium was supplemented with glucose, fructose and sucrose, the number of lactobacilli in biofilm became higher than when compared with the results obtained on the medium without these sugars. Lactobacillus rhamnosus was unable to form biofilm when MRS broth or MRS broth without glucose was used as the growth medium (Lebeer et al. 2007). Ismail, Razak and AbdulRahim (2006) reported that using medium supplemented with glucose and sucrose could promote lactobacilli biofilm formation and development. While glucose is considered as an essential part of exopolysaccharides (de Vuyst et al. 2001), addition of sucrose to TY medium increased the adhesion and biofilm formation abilities of the cells (Shemesh, Tam and Steinberg 2007). BV is associated with multispecies biofilms (Swidsinski et al. 2008). However, isolation of G. vaginalis from 99% of women who have the BV Infection (Hillier et al. 1990) does not neglect the role of other anaerobic pathogens, such as M. curtisi and P. anaerobius, in establishment of vaginal biofilm and its antimicrobial tolerance. Both M. curtisi and P. anaerobius were incapable of forming a developed biofilm on their own when grown in BHIG broth. Our observation was in agreement with Swidsinski et al. (2008) who found that a thick and adherent biofilm was formed only by G. vaginalis. Machado, Jefferson and Cerca (2013) reported that G. vaginalis biofilm ‘encouraged’ other anaerobes to ‘participate’ in a formation of a multispecies biofilm. The combinations of antimicrobials synergistic against biofilm-associated G. vaginalis were evaluated against planktonic cells of M. curtisi, and P. anaerobius, the predominant BV-associated anaerobes (Hillier et al. 1990). In this study, all combinations (Table 4) caused partial inhibition of M. curtisi growth and were bactericidal for P. anaerobius. Recurrence and antibiotic tolerance of BV-associated microorganisms may be connected with the prevalence of other BV-causing anaerobes such as Mobiluncus species. Schwebke and Lawing (2001) found Mobiluncus in 84.5% of samples, in which 77.3% were M. curtisi. Unlike bacterial sensitivity to clindamycin, M. curtisi were resistant to metronidazole and its hydroxyl metabolite (Spiegel 1987). According to Joesoef, Schmid and Hillier (1999), after antibiotics treatment the percentage of BV-associated infection reoccurrence was generally about 50%. Michelle et al. (2008) suggested that the reason behind 67.9% of BV reoccurrence (with high Nugent scores) is in the presence of M. curtisi. A relationship has been found between high Nugent scores and M. curtisi resistance to initial treatment. Sensitivity of P. anaerobius to clindamycin and metronidazole has been reported by Könönen et al. (2007). It is known that metronidazole works by selectively targeting anaerobic microbes, including Peptostreptococcus species. The presence of nitroimidazole-resistance encoded nir genes was considered the cause of Peptostreptococcus resistance to metronidazole (Theron, Janse van Rensburg and Chalkley 2004). Könönen et al. (2007) reported the antimicrobial resistance of P. anaerobius to some of the used β-lactam antibiotics, although bacterial cells were unable to produce β-lactamase. The slow growth of Peptostreptococcus may explain their resistance to antimicrobials which target the growth factors (Higaki et al. 2000). Synergistic activity was reported when natural antimicrobials combined with conventional used antibiotics against planktonic and biofilm-associated pathogens (Choi and Lee 2012a,b; Minahk, Dupuy and Morero 2004; Kaur and Sharma 2013). Minahk, Dupuy and Morero (2004) found that the cationic peptide enterocin CRL35, which is produced by Enterococcus munditii, was synergized with tetracycline, erythromycin and chloramphenicol against Listeria innocua. Using the checkerboard assay, Choi and Lee (2012b) reported synergistic activity when pleurocidin was combined with ampicillin, chloramphenicol and erythromycin against six of the tested bacterial species. Pleurocidin is a positively charged and amphipathic antimicrobial peptide extracted from mucus secretions of Pleurocetes americanus, a winter flounder (Choi and Lee 2012a). In a separate publication, Choi and Lee (2012b) found that arenicin-1, the positively charged antimicrobial peptide isolated from Arenicola marina, strongly synergized with antibiotics against studied pathogenic bacteria. Choi and Lee (2012b) noticed that arenicin-1 enhanced the penetration of erythromycin and chloramphenicol by perturbing the permeability of the cytoplasmic membrane. Pleurocidin and arenicin-1 induced the formation of hydroxyl radicals when they combined with antibiotic and exerting anti-biofilm activity (Choi and Lee 2012a,b). Kaur and Sharma (2013) evaluated the antimicrobial combinations of cell-free supernatants (CFS) of vaginal lactobacilli with ciprofloxacin, streptomycin, oxolinic acid and rifampicin against Salmonella typhimurium and Pseudomonas aeruginosa. They found that CFS synergized with antibiotics and increased sensitivity of P. aeruginosa to antibiotic treatment (Kaur and Sharma 2013). Since most natural antimicrobials target the cytoplasmic membrane or bacterial cell envelope, they are paving the way for antibiotics to finalize their mechanism of action and avoid the problematic issue of antibiotics resistance. In the future, an in vivo study needs to be conducted to ensure the safe use of these combinations without any harmful side effects. Our future studies will follow the suggested strategy in experimental design and in the format of the data report (Lourenço et al. 2014).

**FUNDING**

We are thankful to Jiangsu Sinoyoung Biopharmaceutical Co., Ltd for financial support.

**Conflict of interest.** None declared.

**REFERENCES**


Chen DG, Pounds JC. A nonlinear isologobeam model with boxcox transformation to both sides for chemical mixtures. Environ Health Persp 1998;106:1367–71.


Könönen E, Bryk A, Niemi P, et al. Antimicrobial susceptibilities of Peptostreptococcus anaerobius and the newly described


