The Natural Antimicrobial Subtilosin A Synergizes with Lauramide Arginine Ethyl Ester (LAE), ε-Poly-lysine, Clindamycin Phosphate and Metronidazole, Against the Vaginal Pathogen *Gardnerella vaginalis*

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**Abstract** Bacterial vaginosis (BV) is a common, recurrent vaginal infection linked to increased chances of preterm delivery, incidence of sexually transmitted infections and fertility problems. BV is caused by a shift of the vaginal ecosystem from predominately *Lactobacillus* to a multi-species *Actinomyces* biofilm with the most common representatives identified as *Gardnerella vaginalis* and *Prevotella* spp. Current treatments have been associated with increased resistance as well as negative effects on healthy microbiota. The objective of this study was to evaluate the synergistic potential of ten two-antimicrobial combinations against *G. vaginalis* and four representative lactobacilli. The four tested antimicrobials were lauramide arginine ethyl ester, ε-poly-lysine, clindamycin phosphate, metronidazole, and the bacteriocin subtilosin A. The use of bacteriocins as either synergist or alternative treatment positions bacteriocins as an excellent alternative to current antibiotics. The microdilution method was used to determine the minimum inhibitory concentration (MIC) of each of the antimicrobials individually, and the checkerboard assay was used to evaluate these MICs in combination. Clindamycin and subtilosin (CS), and metronidazole and subtilosin were synergistic against *G. vaginalis* in terms of fractional inhibitory concentration index (FICI). All tested combinations were found to have Bliss synergy. The combination of clindamycin and polylysine (CP) was identified as antagonistic against *L. acidophilus* in terms of both FICI and Bliss synergy. The combination of clindamycin and metronidazole (CM) was antagonistic against *L. vaginalis* for both FICI and Bliss synergy. The combinations of CP, clindamycin and LAE, CS, and LAE and polylysine were identified as Bliss antagonistic against *L. vaginalis* but did not indicate FICI antagonism.

**Keywords** Bacteriocin · Antimicrobial · Synergy · Bacterial vaginosis

**Introduction**

*Gardnerella vaginalis* is one of the most prevalent bacteria identified in the biofilm of bacterial vaginosis (BV). BV is characterized by the shift of a predominantly lactobacilli environment to one that consists of pathogenic anaerobic bacteria including those from the *Actinomycete* genera [1]. BV may cause vaginal discomfort and discharge as well as far-reaching consequences such as preterm delivery and an increased rate of sexually transmitted infection (STI). It is considered the most common vaginal infection in women of childbearing age [1–3]. Given its frequency, a number of antimicrobials have been introduced in order to treat this infection. In particular, clindamycin phosphate and metronidazole have been shown to inhibit the growth of the BV-associated pathogenic bacteria [1, 4]. Clindamycin phosphate (hereafter, referred to as clindamycin) is a lincosamide, which prevents bacterial replication by interfering with protein synthesis by binding to the 23s portion of the 50S subunit in bacterial ribosomes...
causing premature dissociation of the peptidyl-tRNA from the ribosome. This interference does not occur in eukaryotic cells due to structural differences [5]. Metronidazole is a nitroimidazole derivative with activity against anaerobic bacteria and parasites [6, 7]. Reduced metronidazole molecules bind nonspecifically to bacterial DNA, which inactivates DNA molecules leading to an increased rate of DNA breakage. A decrease in thioredoxin reductase activity occurs resulting in the impaired removal of hydrogen peroxide removal by peroxidases [6–8]. Nitroimidazoles are effective against cells with electron-transport proteins with a sufficiently negative redox potential, therefore positioning it as active against organisms with anaerobic metabolisms [6–8].

Use of such antimicrobials is associated with inhibition of normal flora and increased resistance in strains of G. vaginalis [9]. Given these concerns regarding antimicrobial resistance (AMR), novel or alternative antimicrobial compounds are of particular interest. Bacteriocins are ribosomally synthesized antimicrobial peptides (AMPs) of bacterial origin, which kill closely related microorganisms and are characterized by heterogeneity in mode of action, molecular size and biochemical properties [9–11]. Subtilosin A (hereafter, referred to as subtilosin) (Table 1) is a bacteriocin produced by the Gram-positive spore forming bacteria Bacillus subtilus, B. atrophaeus and B. amyloliquifaciens [10–13]. Subtilosin consists of 35 amino acids with three cross-links, formed between the sulfurs of Cys13, Cys7 and Cys4 and the α-positions of Phe22, Thr28 and Phe31, respectively. It has been shown to inhibit the growth of G. vaginalis by creating transient pores in the cytoplasmic membrane, which leads to an efflux of intracellular ions and ATP and eventual cellular death [12]. This information, and the unprecedented posttranslational linkage of a thiol to the α-carbon position subtilosin as an effective alternative to conventional antibiotics [10]. Our group has previously demonstrated the inhibitory properties of subtilosin against G. vaginalis alone and in combination with other antimicrobials against planktonic cultures and against biofilms [4, 12, 14]. Also, we reported on subtilosin’s formulation by passive entrapped in polyethylene glycol-based hydrogels in order to inhibit G. vaginalis while having no effect on normal vaginal flora [15]. These data position subtilosin as a promising synergist in assisting in the improvement and overall health maintenance of the vaginal ecosystem.

In addition, we reported on the inhibitory properties of glycerol monolaurate (GML), LAE and polylysine alone against G. vaginalis [14]. Lauramide arginine ethyl ester (hereafter, referred to as LAE) was given GRAS (generally recognized as safe) status in the USA in 2005 and is currently allowed as a food preservative at quantities up to 225 mg/kg bw/day for individuals over the age of two [16, 17]. LAE rapidly hydrolyzes to form N-ε-lauroyl-L-arginine (LAS) and then hydrolyzes to arginine, which is converted into urea and ornithine. These are catabolized via the urea and citric acid cycles to form carbon dioxide and urea to be excreted through respiration or urination, respectively. The mechanisms of action of LAE is through the disruption of the plasma membrane bilayer without causing cellular lysis leading to reduced cellular growth [16–18]. Similarly, ε-poly-L-lysine (hereafter, referred to as polylysine) received GRAS status in 2010 and has been identified as safe up to 250 mg/kg bw/day [19]. Polylysine is a homopolymer of the amino acid L-lysine produced through fermentation of the bacterium Streptomyces albus subsp. lysinopolymerus [18]. The mechanism of action is through one of two physical ionic interactions within microbial cell membranes; one is through the induction of pore formations while the second is through the disintegration of the cellular membrane. Both mechanisms increase permeability of other antimicrobials, positioning polylysine as a potent synergist [4, 19].

The study presented here builds off of combination work previously performed by our group as well as that performed by Draper et al. [20] in which it was indicated that

Table 1 MIC of clindamycin, polylysine, LAE, metronidazole and subtilosin against G. vaginalis and four clinical lactobacilli spp.

<table>
<thead>
<tr>
<th>Species</th>
<th>Antimicrobial compound</th>
<th>Clindamycin</th>
<th>Polylysine</th>
<th>LAE</th>
<th>Metronidazole</th>
<th>Subtilosin</th>
</tr>
</thead>
<tbody>
<tr>
<td>G. vaginalis</td>
<td>Clindamycin</td>
<td>16.67</td>
<td>25</td>
<td>10</td>
<td>50</td>
<td>12</td>
</tr>
<tr>
<td>L. acidophilus</td>
<td>Clindamycin</td>
<td>9</td>
<td>133.33</td>
<td>50</td>
<td>100</td>
<td>1,000</td>
</tr>
<tr>
<td>L. gasseri</td>
<td>Clindamycin</td>
<td>77.5</td>
<td>111.6</td>
<td>31.25</td>
<td>75</td>
<td>825</td>
</tr>
<tr>
<td>L. plantarum</td>
<td>Clindamycin</td>
<td>25</td>
<td>1,786</td>
<td>62.5</td>
<td>100</td>
<td>785</td>
</tr>
<tr>
<td>L. vaginalis</td>
<td>Clindamycin</td>
<td>0.78</td>
<td>55.8</td>
<td>15.63</td>
<td>50</td>
<td>725</td>
</tr>
</tbody>
</table>

MIC of the four tested antimicrobials and one bacteriocin against the BV-associated pathogen G. vaginalis and four clinical isolates of lactobacilli. Some of these data are confirmatory while other data expand upon original data (4, 13). All MIC provided in μg/mL. All data are the average of four separate experiments in duplicates. All assays conducted resulted in identical results for all substances (no standard deviation).
the two peptide lantibiotic lacticin 3147 and polymyxins acted synergistically against Gram-negative microorganisms. This group has looked further into the action of lacticin 3147, having recently identified it as capable of preventing Streptococcus mutans biofilm formation [21]. In addition, Naghmouchi et al. [22–24] validated synergy between several bacteriocins and antibiotics against Gram-positive and Gram-negative microorganisms.

Commercially available nature-derived antimicrobials (LAE and polysyne), currently approved drugs (clindamycin phosphate and metronidazole) and a bacteriocin (subtilisin A) were tested in combinations against clinical isolates of healthy vaginal lactobacilli (Lactobacillus acidophilus, L. gasseri, L. plantarum, and L. vaginalis) and a clinical isolate of Gardnerella vaginalis. These antimicrobials and bacteriocin were first tested individually and then combined using a checkerboard assay in order to assess the minimum inhibitory concentration (MIC) of each possible combination against planktonic cultures. These combinations were evaluated for synergy against tested microorganisms using both FICI and Bliss independence. Data collected in these studies will be used in evaluation of synergistic combinations against biofilm-associated cells.

Materials and Methods

Preparation of Antimicrobial Solutions

MIRENAT-CF (LAE) was a gift from Vedeqsa Corp (Vedeqsa, Barcelona, Spain) and contained 1 mg/mL stock solution of polylysine was a gift from the Chisso America, Inc, Corporation (lot 2090501; Rye, NY). A 98 % stock of clindamycin phosphate was obtained from Tokyo Chemical Industry (TCI, Tokyo, Japan). A 99 % (M.W. 171.16) stock of metronidazole was purchased from Acros Organics (Acros Organics, New Jersey, USA). All antimicrobial solutions were filter-sterilized using a 0.45-μm filter (Nalgene, Rochester, NY, USA) prior to use.

Subtilisin was obtained in house through fermentation of Bacillus amyloliquefaciens KATMIRA1933, as previously described [4]. The stock solution was stored at 4 °C until needed for experiments.

Bacterial Growth Conditions

Gardnerella vaginalis ATCC 14018 was maintained as previously described [4]. Briefly, bacterial cultures were stored at −80 °C in brain heart infusion (BHI) medium (Difco, Sparks, MD, USA) supplemented with 3 % horse serum (JRH Biosciences, KS, USA) and 15 % (v/v) glycerol. Frozen stocks were cultured on human blood bilayer-Tween (hereafter, referred to as HBT) agar (Remel, Lenexa, KS, USA) and grown in anaerobic conditions in a Type A Coy Laboratory Vinyl Anaerobic Chamber (Coy Laboratory Products, Grass Lake, MI, USA) at 37 °C. An anaerobic environment was established by 2.5 % hydrogen gas and 5 % CO₂. Isolated colonies were inoculated in brain heart infusion supplemented with 3 % horse serum (hereafter, referred to as BHI + 3 %HS) media for 48 h. Initial cultures were subcultured twice before use. Cultures used for checkerboard assays were grown to 10⁸ CFU/mL and then diluted 100-fold in growth medium for a working concentration of 10⁶ CFU/mL. All media and agar were preincubated in the aforementioned anaerobic conditions overnight to remove oxygen-related stress.

The four reference species of lactobacilli were selected as they represent clinical isolates that have been found in healthy women (Lactobacillus gasseri ATCC 33323 and L. plantarum ATCC 39268) and those with recurrent vaginal infections (L. acidophilus ATCC 4356 and L. vaginalis ATCC 49540)[25, 26]. All bacteria were stored at −80 °C in DeMan, Rogosa and Sharpe (MRS) broth (Difco, Sparks, MD, USA) containing 15 % glycerol (v/v) until use. The cells were cultured on MRS agar and grown anaerobically in the same anaerobic chamber used for G. vaginalis experiments. Single colonies were inoculated in MRS broth and grown anaerobically for 24 h without agitation. Cells were subcultured twice before use.

Determination of Minimal Inhibitory Concentration (MICs)

Stock solutions of all antimicrobials were prepared in 100 mM phosphate-buffered saline (PBS) solution. Antimicrobials were filter-sterilized using 0.45 μm syringe filters (Fisher, Pittsburgh, PA, USA) and diluted to 2 x final working concentration. This was done in order to prevent further dilution upon addition of media. MICs were determined as follows. Briefly, 24 h cultures of G. vaginalis in BHI + 3 %HS were transferred to fresh media to obtain an optical density at 595 nm (OD₅95) of 0.2. From the stock solutions, tenfold serial dilutions of each antimicrobial were made, following the microdilution method as described by Amrouche et al. [27] and adapted further by Noll et al. [14]. A sterile, 96-well microplate (Corning, Inc, Corning, NY, USA) was prepared by adding the serial dilutions of antimicrobials horizontally from the highest to lowest concentration tested. Antimicrobials were tested in 20 μL increments in duplicate. The volume of the well was increased to a total volume of 100 μL with the addition of PBS buffer, with the contents mixed by an Eppendorf Xplorer automatic pipette (Eppendorf Hauppauge, NY, USA). Equal volumes (100 μL) of bacteria and serial
dilutions of each antimicrobial were mixed into the wells. Control wells were those with neither bacteria nor any of the dilutions of antimicrobials. Heavy mineral oil was placed on top of each well to prevent evaporation during optical density readings. The use of mineral oil also assists in the prevention of evaporation-induced spikes in data. This procedure was identical for the four tested lactobacilli species except for the use of MRS broth instead of BHI + 3% HS broth. Experiments were performed four times in duplicates. Plates were prepared in the anaerobic chamber to prevent oxygen-related stress from interfering with the experiment.

Checkerboard Assays

The interaction between all the antimicrobials was tested via the checkerboard assay as described by Badaoui Najjar et al. [28] with some modifications. For each combinatorial experiment, antimicrobial A was placed in horizontal rows while antimicrobial B was placed into the vertical columns. Using the stock solution that was tenfold higher than the respective MIC, each compound was aliquoted to test concentrations above, equal to or below the individual MIC of each tested antimicrobial (Table 1). The checkerboard assay was carried out in a manner identical to the MIC experiments (Table 2). Experiments were performed four times in duplicate.

Data were collected in a Maxline Series 1 microplate reader (Molecular Devices, Sunnyvale, California) using SOFTmax Pro (Molecular Devices, Sunnyvale, CA, USA). The growth kinetics of all bacteria was recorded as turbidometric measurements of absorbance (OD$_{595}$) every hour for 24 h.

Synergy Interpretation Using FICI

There are numerous models and approaches, which assess in vitro drug interactions. Currently, the two prevailing theories are the Loewe additivity (LA) and Bliss independence (BI) models because they fulfill the no-interaction theory in which it is stated that only synergy or antagonism, respectively, can be claimed. In LA, it is posited that an agent should not have synergistic interactions with itself or similar agents. It is further stated that if two similar drugs are given in equal concentrations, the effect of the drug will be doubled [29, 30].

The MICs of each single antimicrobial agent and all combinations were determined in a checkerboard assay. For each antimicrobial combination, the fractional inhibitory concentration index (FICI) was determined by computing the ratio of the MIC of the combination divided by the MIC of the antimicrobial alone and then adding these two ratios together. Equation 1 defines the LA model:

$$\text{FICI} = \frac{\text{MIC}_A}{\text{MIC}_A} + \frac{\text{MIC}_B}{\text{MIC}_B}$$

FICI data were interpreted using the following criteria: A FICI $\leq$ 0.5 is defined as synergistic while a value that is $>0.5$ and $\leq 4.0$ is identified as having no effect (no interaction). A FICI of $>4.0$ is identified as antagonistic [31].

<table>
<thead>
<tr>
<th>Combination $^b$</th>
<th>Tested bacterium</th>
<th>$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$L.\text{acidophilus}$</td>
<td>$L.\text{gasseri}$</td>
</tr>
<tr>
<td>CM</td>
<td>30/2.56</td>
<td>64/4.8</td>
</tr>
<tr>
<td>CP</td>
<td>30/160</td>
<td>12/160</td>
</tr>
<tr>
<td>CL</td>
<td>4.8/50</td>
<td>4.8/20</td>
</tr>
<tr>
<td>CS</td>
<td>12/400</td>
<td>12/400</td>
</tr>
<tr>
<td>MP</td>
<td>2.56/25.6</td>
<td>2.56/25.6</td>
</tr>
<tr>
<td>ML</td>
<td>2.56/1.28</td>
<td>2.56/1.28</td>
</tr>
<tr>
<td>MS</td>
<td>6.4/160</td>
<td>16/64</td>
</tr>
<tr>
<td>LP</td>
<td>1.28/160</td>
<td>8/160</td>
</tr>
<tr>
<td>LS</td>
<td>5/500</td>
<td>3.125/275</td>
</tr>
<tr>
<td>PS</td>
<td>66.66/500</td>
<td>55.8/82.5</td>
</tr>
</tbody>
</table>

The MIC$^a$ of clindamycin, polylsine, LAE, metronidazole and subtilosin in combination against four clinical isolates of lactobacilli and the BV-associated pathogen $G.\text{vaginalis}$. MIC$^a$ provided in $\mu g$/ml.

$^b$ To save on space, antimicrobial combinations are listed by their first letters. Combinations are as follows: CM clindamycin/metronidazole, CP clindamycin/polylsine, CL clindamycin/LAE, CS clindamycin/subtilosin, MP metronidazole/polylsine, ML metronidazole/LAE, MS metronidazole/subtilosin, LP LAE/polylsine, LS LAE/subtilosin, PS polylsine/subtilosin
Synergy Using Bliss Independence-Based Drug Interaction Analysis

Bliss independence (BI) considers two drugs that have reached their maximal effect (growth inhibition) and, once doing so, do not contribute to the overall effect of the other drug. In BI, the two agents are considered as independent, i.e., as having different targets. BI is described by the following equations:

\[ E_{\text{IND}} = E_A + E_B - E_A \times E_B. \]  

(2)

\( E_A \) is defined as the percentage of bacterial growth inhibition from of \( x \) mg/L of drug A. \( E_B \) is defined as the percentage of bacterial growth inhibition at \( y \) mg/L alone. \( E_{\text{IND}} \) is the expected percentage of bacterial growth, which results from a noninteractive, thus independent, theoretical combination of drugs A and B. The difference between the observed (\( E_{\text{OBS}} \)) and expected (\( E_{\text{IND}} \)) percentage of growth inhibition from these drug combinations is described by Eq. 3:

\[ \Delta E = E_{\text{OBS}} - E_{\text{IND}} \]  

(3)

If \( \Delta E \) and its 95 % confidence level (CI) were >0 (i.e., \( E_{\text{OBS}} > E_{\text{IND}} \), more growth inhibition was observed than if the two drugs were acting independently), Bliss synergy was concluded for the drug combination. If \( \Delta E \) and its 95 % CI were <0 (i.e., \( E_{\text{OBS}} < E_{\text{IND}} \), less growth inhibition was observed than if the two drugs were acting independently), Bliss antagonism was concluded for the particular drug combination. In cases in which the 95 % CI of \( \Delta E \) = 0, the conclusion was deemed Bliss independence [32].

Graphical Representation, Statistical Analysis and Presentation of Data

All statistical analyses were performed in Sigma Plot 11.0 except for LA and BI data analysis, which was performed using MS Excel 2007. All statements regarding statistical significance are at the 95 % level [33]. Chart representation of data was created in MS Word 2007. All figures were drawn using Instant JChem 6.2.1 (MathWorks).

Results

Antimicrobial Susceptibility of Tested Lactobacilli

The MICs of clindamycin, LAE, polysine, metronidazole and subtilosin against the four tested lactobacilli were determined by the microdilution method. As seen in Table 1, much of the data are confirmatory from our previously published results [4]. It expands upon these data by indicating that \( L. \) gasseri is inhibited by 77.5 \( \mu \)g/mL of clindamycin, and identifying that the MICs of subtilosin appeared to be greater than originally suspected. The MICs of metronidazole did not vary much between the four tested lactobacilli species, ranging between 50 and 100 \( \mu \)g/mL.

The MIC of clindamycin against \( L. \) acidophilus was 9 \( \mu \)g/mL and differed greatly from the other observed clindamycin MIC values. The MIC of polysine (133.33 \( \mu \)g/mL) was similar to that of \( L. \) gasseri (111.6 \( \mu \)g/mL) but varied from that of \( L. \) plantarum (1,768 \( \mu \)g/mL) and \( L. \) vaginalis (55.8 \( \mu \)g/mL).

Antimicrobial Susceptibility of \( G. \) vaginalis

The MICs of clindamycin, LAE, polysine, metronidazole and subtilosin against the BV-associated pathogen \( G. \) vaginalis were determined via the microdilution method. As seen in Table 1, all tested antimicrobials inhibited \( G. \) vaginalis at concentrations generally lower as compared to the four tested lactobacilli. Clindamycin inhibited \( G. \) vaginalis at 16.67 \( \mu \)g/mL, which is lower than \( L. \) gasseri and \( L. \) plantarum (77.5 and 25 \( \mu \)g/mL, respectively) while \( L. \) acidophilus and \( L. \) vaginalis were inhibited at significantly lower concentrations (9 and 0.78 \( \mu \)g/mL, respectively). All other tested antimicrobials were found to inhibit \( G. \) vaginalis at concentrations lower as compared to tested lactobacilli (Table 1).

Determination of Synergy Between Two Antimicrobial Substances

Having identified all individual MICs against the five tested bacteria, a checkerboard assay was performed with all possible combinations of antimicrobials. Assays were designed to test a wide range of concentrations, starting with above each individual antimicrobial’s MIC and decreasing in a serial dilution to 0 \( \mu \)g/mL (negative control). Wells that resulted in complete inhibition of the bacterium had the concentrations of each antimicrobial recorded (Table 2). These values were then analyzed using FICI and BI so as to determine their interaction.

Identification of Interaction Against Tested Lactobacilli

The combinations of metronidazole and LAE (ML), and metronidazole and subtilosin (MS) were synergistic against all tested lactobacilli in terms of both FICI and BI. The combination of LAE and subtilosin (LS) was synergistic against all tested lactobacilli except \( L. \) plantarum, for which there was no interaction. Similarly, the combination of polysine and subtilosin (PS) indicated synergy against all tested lactobacilli except for \( L. \) acidophilus, which also indicated no interaction (Table 3).
The combination of clindamycin and metronidazole (CM) was found to be synergistic in terms of FICI and indicated Bliss synergy against L. gasseri while having no interaction against L. acidophilus or L. plantarum and being antagonistic against L. vaginalis. Bliss antagonism was also noted for the combination of CM against L. vaginalis. The combination of clindamycin and polylysine (CP) was found to have no interaction against all tested lactobacilli except L. acidophilus for which it was antagonistic. All other tested combinations indicated combinations of synergism and no interaction in terms of FICI. Bliss antagonism was also found for the combinations of clindamycin and LAE (CL), clindamycin and subtilosin (CS) and LAE and polylysine (LP) against L. vaginalis (Table 4).

Identification of Interaction Against G. vaginalis

None of the tested combinations antagonized in their action against G. vaginalis in terms of FICI values or BI (Tables 3, 4). Four of the tested combinations were synergistic, clindamycin and subtilosin (CS), metronidazole and polylysine (MP), metronidazole and subtilosin (MS) and polylysine and subtilosin (PS) in terms of FICI. All combinations tested indicated Bliss synergy.

Discussion

Gardnerella vaginalis is one of the primary causes of BV, the most common vaginal infection identified in women of childbearing age. Its prevalence and the associated ramifications of this infection indicate the necessity to effectively inhibit the growth of the causative bacteria and assist in the recovery of the healthy microbiota. Currently used antimicrobials lead to AMR and inhibition of normal microbiota. The inhibition of healthy microorganisms lengthens the overall recovery time of the natural vaginal ecosystem [34]. The aim of this study was twofold. First, it determined whether combinations of commercial antibiotics acted synergistically against the BV-associated pathogen G. vaginalis while also evaluating the interaction of these same combinations against clinical isolates of lactobacilli. Second, the interaction of the bacteriocin subtilosin in combination with four antimicrobials was observed against all five bacteria. It was found that subtilosin is a good synergist, particularly the combination of CP, which was found to be antagonistic against L. acidophilus with both FICI and BI. The combinations of CM, CP, CL, CS and LP were found to have Bliss antagonism against L. vaginalis, but these combinations were not found to be antagonistic in terms of FICI except for CM. The combination of ML was found to be synergistic against all tested lactobacilli while having no interaction again G. vaginalis.

This study utilized FICI and BI, two of the most common parametric models concurrently in order to determine possible synergy in action of ten antimicrobial combinations against five bacteria in an in vitro system. In the described array design, these approaches are considered to be valid and utilized by many investigators [31]. The use of both of these tools allows for an analysis of the

Table 3 FICI values for combinatorial data

<table>
<thead>
<tr>
<th>Combination</th>
<th>Tested bacterium</th>
<th>L. acidophilus</th>
<th>L. gasseri</th>
<th>L. plantarum</th>
<th>L. vaginalis</th>
<th>G. vaginalis</th>
</tr>
</thead>
<tbody>
<tr>
<td>CM</td>
<td></td>
<td>3.36(\alpha)</td>
<td>0.89(\alpha)</td>
<td>0.10(\alpha)</td>
<td>4.01(\sigma)</td>
<td>0.62(\sigma)</td>
</tr>
<tr>
<td>CP</td>
<td></td>
<td>4.53(\alpha)</td>
<td>1.59(\alpha)</td>
<td>1.29(\alpha)</td>
<td>1.48(\sigma)</td>
<td>0.70(\sigma)</td>
</tr>
<tr>
<td>CL</td>
<td></td>
<td>1.53(\alpha)</td>
<td>0.70(\alpha)</td>
<td>0.70(\alpha)</td>
<td>2.25(\sigma)</td>
<td>0.73(\sigma)</td>
</tr>
<tr>
<td>CS</td>
<td></td>
<td>1.73(\alpha)</td>
<td>0.64(\alpha)</td>
<td>1.71(\alpha)</td>
<td>2.55(\sigma)</td>
<td>0.23(\sigma)</td>
</tr>
<tr>
<td>MP</td>
<td></td>
<td>0.51(\alpha)</td>
<td>0.26(\kappa)</td>
<td>0.04(\kappa)</td>
<td>0.51(\sigma)</td>
<td>0.36(\sigma)</td>
</tr>
<tr>
<td>ML</td>
<td></td>
<td>0.05(\kappa)</td>
<td>0.08(\kappa)</td>
<td>0.11(\kappa)</td>
<td>0.33(\kappa)</td>
<td>1.36(\kappa)</td>
</tr>
<tr>
<td>MS</td>
<td></td>
<td>0.22(\kappa)</td>
<td>0.29(\kappa)</td>
<td>0.36(\kappa)</td>
<td>0.35(\kappa)</td>
<td>0.33(\kappa)</td>
</tr>
<tr>
<td>LP</td>
<td></td>
<td>1.23(\alpha)</td>
<td>1.69(\alpha)</td>
<td>0.41(\kappa)</td>
<td>3.38(\sigma)</td>
<td>0.87(\sigma)</td>
</tr>
<tr>
<td>LS</td>
<td></td>
<td>0.60(\sigma)</td>
<td>0.43(\kappa)</td>
<td>1.10(\kappa)</td>
<td>0.20(\kappa)</td>
<td>2.88(\kappa)</td>
</tr>
<tr>
<td>PS</td>
<td></td>
<td>1.00(\sigma)</td>
<td>0.60(\sigma)</td>
<td>0.50(\sigma)</td>
<td>0.70(\sigma)</td>
<td>0.48(\sigma)</td>
</tr>
</tbody>
</table>

The FICI numbers generated from the data included in Table 3

\(\alpha\) FICI numbers are rounded to two decimal places. Combination efficacy is denoted as such: (\(\kappa\)) denotes synergy (\(\sigma\)) no effect, (\(\rho\)) denotes antagonism

To save on space antimicrobial combinations are listed by their first letters. Combinations are as follows: CM clindamycin/metronidazole, CP clindamycin/polylysine, CL clindamycin/LAE, CS clindamycin/subtilosin, MP metronidazole/polylysine, ML metronidazole/LAE, MS metronidazole/subtilosin, LP LAE/polylysine, LS LAE/subtilosin, PS polylysine/subtilosin
The outcome of this study is twofold. It provides a foundation into the effects of the studied antimicrobials in particular which combinations are synergistic against the tested pathogen and which should not be considered because of their activity against the healthy microbiota.

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Conflict of interest The authors declare that they have no conflict of interests.

References


Table 4 BI Data from combinatorial data from Table 3

<table>
<thead>
<tr>
<th>Combination</th>
<th>Tested bacterium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L. acidophilus</td>
</tr>
<tr>
<td>CM</td>
<td>10.13³</td>
</tr>
<tr>
<td>CP</td>
<td>−38.19⁰</td>
</tr>
<tr>
<td>CL</td>
<td>2.06³</td>
</tr>
<tr>
<td>CS</td>
<td>36.03³</td>
</tr>
<tr>
<td>MP</td>
<td>130.62³</td>
</tr>
<tr>
<td>ML</td>
<td>64.84³</td>
</tr>
<tr>
<td>MS</td>
<td>1313.39³</td>
</tr>
<tr>
<td>LP</td>
<td>48.06³</td>
</tr>
<tr>
<td>LS</td>
<td>469.55³</td>
</tr>
<tr>
<td>PS</td>
<td>661.37³</td>
</tr>
</tbody>
</table>

BI data generated from Table 3. Combination responses are denoted as either Bliss synergy (⁺) or Bliss antagonism (⁻). BI responses are rounded to two decimal places.

concentration effect of two antimicrobials in combination and an evaluation of the effectiveness against these bacteria. Previously, our group used isobolograms to determine such pharmacodynamic interactions but since the tested combinations included a maximum of two antimicrobials, it was not appropriate. The data represented here indicate both the reproducibility of using subtilosin in combination while also expanding upon its synergy using two different analysis methods. This includes the synergy of subtilosin with polylysine [14], which is confirmed here as a control. However, in the same study, the synergism of LAE and subtilosin (LS) was also indicated, which was not observed in this study. The two pharmacodynamic interaction methods used indicated that overall most of the tested combinations were synergistic while the combination of CP was antagonistic against L. acidophilus and CM was antagonistic against L. vaginalis. The synergy of subtilosin with three of the four tested antimicrobials through FICI and its Bliss synergy with all of the antimicrobials indicate its potential as a potent synergist.

BV is a polymicrobial infection; however, the cytotoxicity and biofilm-forming potential of G. vaginalis largely position it as the causative agent of the disorder, which is why it was the only BV-associated pathogenic bacterium tested in this study. Future synergy studies will incorporate other essential microorganisms associated with BV as well as biofilm studies. While the study is a continuation of our group’s investigation of synergistically acting antimicrobials, it is also influenced by the growing interest in improving conventional antibiotic by combining them with nature-derived and synergistically acting antimicrobials.

Doing so may act as a novel and more effective treatment method [20]. Further, some synergistic combinations against G. vaginalis were found to be highly effective against the tested vaginal lactobacilli, thus indicating that these combinations would, in practice, be rather detrimental to the individual and impede recovery of the vaginal ecosystem [35, 36].

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ferredoxin oxidoreductase and thioredoxin reductase are involved in 5-nitroimidazole activation while flavin metabolism is linked to 5-nitroimidazole resistance in *Giardia lamblia*. J Antimicrob Chemother 66:1756–1765. doi:10.1093/jac/dkr192


