AAC
Journals.ASM.org

The Natural Antimicrobial Peptide Subtilosin Acts Synergistically with Glycerol Monolaurate, Lauric Arginate, and ε-Poly-L-Lysine against Bacterial Vaginosis-Associated Pathogens but Not Human Lactobacilli

Katia Sutyak Noll,[a]* Mark N. Prichard,[b] Arkady Khaykin,[a] Patrick J. Sinko,[a] and Michael L. Chikindas[a]

Rutgers, The State University of New Jersey, New Brunswick, New Jersey, USA,[a] and University of Alabama at Birmingham, Birmingham, Alabama, USA[b]

Subtilosin is a cyclical antimicrobial peptide produced by *Bacillus amyloliquefaciens* that has antimicrobial activity against the bacterial vaginosis-associated human pathogen *Gardnerella vaginalis*. The ability of subtilosin to inhibit *G. vaginalis* alone and in combination with the natural antimicrobial agents glycerol monolaurate (Lauricidin), lauric arginate, and ε-poly-L-lysine was tested using a checkerboard approach. Subtilosin was found to act synergistically with all of the chosen antimicrobials. These promising results indicate that lower concentrations of subtilosin in combination with other compounds could effectively be used to inhibit growth of the pathogen, thereby decreasing the risk of developed antimicrobial resistance. This is the first report on the effects of subtilosin combined with other natural antimicrobials against *G. vaginalis*.

In recent years, the advent of multidrug-resistant pathogens has been a subject of great concern among both the scientific community and the general public. The urgent need for new therapy options has led to a growing interest in bacteriocins. Bacteriocins are bacterially produced, ribosomally synthesized peptides that have antimicrobial activity against other organisms, typically those closely related to the producer species (19). Although they have traditionally been used for food preservation purposes, bacteriocins have shown promise as safe, natural alternatives to conventional antibiotics. One such bacteriocin, subtilosin, has great potential for treating the condition known as bacterial vaginosis (BV), and is the subject of our current study.

Subtilosin A (referred to hereafter as subtilosin) is a cyclical peptide of 35 amino acids characterized by its complex, cross-linked structure (18). First isolated from a *Bacillus subtilis* culture by Babasaki et al. (4), it was recently shown to be produced by the dairy product-derived *Bacillus amyloliquefaciens* KATMIRA1933 (35). Subtilosin can inhibit the growth of several human pathogens, including the human pathogen *Gardnerella vaginalis*, the primary causative agent of BV (35). Most importantly, it is completely safe for human vaginal epithelial cells and healthy vaginal lactobacilli (35, 36), which indicates that its inclusion in personal care products would not adversely affect human health.

Bacterial vaginosis (BV) is a serious yet common infection characterized by the replacement of healthy vaginal lactobacilli with facultative and anaerobic microorganisms, especially *G. vaginalis* and *Prevotella*, *Peptostreptococcus*, *Porphyromonas*, and *Mobiluncus* spp. (11, 12, 21, 33). Estimates predict that 10 to 30% of North American women are affected by BV, although many of these cases remain asymptomatic (2). This poses a significant risk for women of reproductive age, as the uncontrolled proliferation of organisms linked to BV has been associated with the development of pelvic inflammatory disease (14) and a variety of pregnancy-related complications, including intra-amniotic infections leading to fetal brain damage (13, 24), preterm births with an elevated risk of infant death (26), low infant birth weight (17), and spontaneous abortion (10, 23). BV, and particularly its causative agent *G. vaginalis*, is also associated with an elevated probability of contracting HIV and increased proliferation of the virus in multiple cell lines (15–17, 31, 38; for a recent review, see 41).

The common antibiotics metronidazole and clindamycin are typically prescribed for oral and/or intravaginal BV treatment. While effective, these broad-spectrum drugs do not effectively inhibit BV-associated pathogens. Subsequently, there is a high BV recurrence rate of ~20% (43), which is often characterized by newly developed antibiotic resistances (7, 20, 22). Furthermore, the concentrations of drugs required to inhibit the healthy vaginal microbiota are up to hundreds-fold than the concentrations used to control BV-associated pathogens, often resulting in eradication of the healthy vaginal microbiota (3). In turn, this creates a challenge for treatment recovery. Therefore, it has become critically important to develop new treatments specifically targeted at BV-associated pathogens that carry a low risk of developed resistance and are safe for human use.

One way to circumvent the development of bacterial drug resistance is through the use of combinations of antimicrobial agents. In this approach, low concentrations of antimicrobials with different molecular mechanisms of action and targets are combined to create "multiple hurdles" against undesired microorganisms. Elucidation of the antimicrobial activity delivered by combinations of these compounds may reveal compositions with synergistic or additive effects that allow for the use of each compound in amounts lower than their individual effective concentrations. There have been several recent reports on the synergy of bacteriocins with other natural antimicrobials (25). Badaoui Najjar et al. (5) showed that nisin, a bacteriocin generally recognized as safe (GRAS) for several food preservation purposes, acts syner-

Received 5 October 2011 **Returned for modification** 11 November 2011
Accepted 3 January 2012
Published ahead of print 17 January 2012
Address correspondence to Michael L. Chikindas, tchikindas@aesop.rutgers.edu.
* Present address: Kraft Foods, Tarrytown, New York, USA.

doi:10.1128/AAC.05861-11

gistically with ε-poly-L-lysine (hereafter referred to as polylysine) against the food-borne pathogens *Listeria monocytogenes* and *Bacillus cereus*. On a related note, Amrouche et al. (1) demonstrated that subtilosin has synergy with nano-encapsulated curcumin, a plant phytochemical with antimicrobial activity, zinc lactate, and polylysine against *L. monocytogenes*.

In light of these results, we investigated the combinatorial relationship of subtilosin with three natural antimicrobials: glycerol monolaurate (GML), lauric arginate (LAE), and polylysine. Glycerol monolaurate, a common ingredient in food and cosmetic industry preparations, is a monoglyceride that the Food and Drug Administration (FDA) has given GRAS status for oral use (9a). Low concentrations of GML have been shown to inhibit the growth of *G. vaginalis* (34), likely through inhibition of signal transduction at microbial plasma membranes (27, 30, 42). Lauric arginate, a derivative of lauric acid, L-arginine, and ethanol, is a GRAS compound with antimicrobial activity against a broad spectrum of microorganisms. LAE is known to cause disruptions and instability in the plasma membrane lipid bilayer without causing cell lysis, leading to inhibition of bacterial growth (6). Polylysine is a microbially produced short polypeptide comprised of repeating lysine subunits that adsorbs to cell surfaces and interferes with cellular membranes (32). The different mechanisms of action of these antimicrobials make them strong candidates for synergistic activity with subtilosin, the discovery of which could lead to more effective formulations of personal care products targeted at BV prophylaxis and/or treatment. This is the first report investigating the synergy of subtilosin combined with various natural antimicrobials against *G. vaginalis*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Clinical isolates of the BV-associated pathogens *Gardnerella vaginalis* ATCC 14018, *Peptostreptococcus anaerobius* ATCC 27337, and *Mobiluncus curtisii* ATCC 35241 were grown anaerobically in brain heart infusion (BHI) broth (Difco, Sparks, MD) containing 3% horse serum (JRH Biosciences, Lenexa, KS) at 37°C without agitation. *B. amyloliquefaciens* KATMIRA1933 cultures were grown overnight in MRS broth (Difco) at 37°C without agitation. Human clinical isolates of lactobacilli were propagated as follows. *Lactobacillus acidophilus* ATCC 4356, *L. vaginalis* ATCC 49540, *L. crispatus* ATCC 33820, and *L. jensenii* ATCC 25258 were grown in MRS broth at 37°C with a modified (5% CO_2) environment. *L. plantarum* ATCC 39268 was grown aerobically in MRS broth at 35°C, and *L. gasseri* ATCC 33323 was grown aerobically in MRS broth at 37°C. Initial cultures of all organisms were subcultured multiple times before use. For experimental purposes, all organisms were grown overnight to an approximate cell concentration of 10^8 CFU/ml and then diluted 100-fold in growth medium for a working concentration of 10^6 CFU/ml. Stock cultures of all organisms were kept at −80°C in their appropriate growth medium supplemented with 15% (vol/vol) glycerol.

Preparation of antimicrobial solutions. The partially purified preparation of subtilosin was prepared as previously described (35). Sterile Lauricidin (glycerol monolaurate) was a gift from A. A. Aroutcheva of Rush Medical Center, Chicago, IL. A 2-mg/ml stock solution of glycerol monolaurate was prepared in BHI broth plus 3% horse serum broth prewarmed to 37°C. MIRENAT-CF was a gift from Vedeqsa Corp. (Barcelona, Spain) and contained 1 mg/ml lauric arginate (N^{α}-lauroyl-L-arginine ethyl ester monohydrochloride [LAE]). A stock solution containing 25% ε-poly-L-lysine (250 mg/ml) was a gift from Chisso America, Inc. (lot 2090501; Rye, NY). All antimicrobial solutions were filter sterilized using a 0.45-μm filter (Nalgene, Rochester, NY) prior to use.

Determination of MICs. The ability of each antimicrobial agent to individually inhibit *G. vaginalis* growth was determined using the broth microdilution method by the method of Amrouche et al. (1) with slight modifications. From the stock solutions, 10-fold serial dilutions of each antimicrobial were made (230 to 0.023 μg/ml for subtilosin, 200 to 0.02 μg/ml for glycerol monolaurate, 10,000 to 10 μg/ml for lauric arginate, and 25,000 to 25 μg/ml for polylysine) in the proper diluent. *G. vaginalis* cells were grown overnight and prepared as previously described. A sterile, 96-well microplate (Corning, Inc., Corning, NY) was prepared by adding the serial dilutions of antimicrobials in horizontal rows from the highest concentration to the lowest concentration tested. The antimicrobials were tested in 20-μl increments (0 to 100 μl), with each volume tested in duplicate. The volume of each well was increased to a total volume of 100 μl with the addition of sterile double-distilled water (ddH_2O), and the contents of each well were mixed by gentle pipetting. One hundred microliters of *G. vaginalis* cells was added to each well; wells containing cells alone, antimicrobial alone, water alone, and growth medium alone were used as controls. Fifty microliters of sterile mineral oil was pipetted onto the top of each well to form an airtight seal that would allow for anaerobic growth of the *G. vaginalis* cells. Each plate was then transferred into a Coy type C anaerobic chamber (Coy Laboratory Products, Inc., Grass Lake, MI) (80% N_2–10% H_2–5% CO_2 atmosphere) and placed in a Bio-Rad model 550 microplate reader (Bio-Rad Life Sciences, Hercules, CA). The turbidity of each well was recorded at 595 nm every 60 min for 48 h at 37°C. In order to prevent mixing of the mineral oil seal with the contents of each well, the plate was not shaken prior to each measurement. Data were gathered and analyzed using Microplate Manager (version 5.1.2) software (Bio-Rad) and Microsoft Excel 2007 (Microsoft, Redmond, WA). The lowest concentration of each antimicrobial that showed no increase in optical density (no bacterial growth) was designated the MIC. Each assay was performed at least twice in duplicate.

TABLE 1 MICs of subtilosin, glycerol monolaurate, lauric arginate, and polylysine against the BV-associated pathogen *G. vaginalis*[a]

Antimicrobial compound	Starting concn	MIC (μg/ml) for *G. vaginalis*
Subtilosin	229.5 μg/ml	9.2
Glycerol monolaurate (GML)	2 mg/ml	20
Lauric arginate (LAE)	1 g/ml	100
Polylysine	250 mg/ml	25

[a] Each MIC assay tested a wide range of concentrations for each compound and was conducted at least twice in duplicate. All assays conducted resulted in identical results for all substances (no standard deviation).

Checkerboard assays. The interaction between subtilosin and the chosen antimicrobials was tested via a "checkerboard" assay that allowed for testing of two antimicrobials at various concentrations at the same time. The checkerboard assays were performed by the method of Badaoui Najjar et al. (5) with the following modifications. In each experiment, a sterile 96-well microplate (Corning) was prepared so that subtilosin (horizontal rows) would be combined with one of the chosen antimicrobials (vertical columns). Using a stock solution of a 10-fold-higher concentration than its respective MIC, each compound was aliquoted into the appropriate row or column. Each plate was designed to test concentrations directly above, equal to, and, particularly, below the individual MIC of each antimicrobial (Table 1). The volume of each well was raised to 100 μl using sterile ddH_2O. *G. vaginalis* cells were grown overnight and prepared as previously described; 100 μl of this preparation was added to each well. The first row and column of the microplate served as controls (no antimicrobials), as did a row of water alone and growth medium alone. Fifty microliters of sterile mineral oil was pipetted onto the top of each well to ensure anaerobic conditions. Each plate was run using the same equipment and under the same conditions as described in the previous section. Each assay was performed at least twice in duplicate.

The ability of subtilosin to synergize with two antimicrobials at one time was tested according to the guidelines given by Prichard et al. (28, 29). Briefly, the interaction of three antimicrobial compounds was tested

by using two compounds at different dilutions (0.25, 0.5, 0.75, 1.0, and 1.25) of their MIC while keeping the concentration of the third static (0.25-, 0.5-, and 0.75-fold MIC). For a control, each antimicrobial was also tested alone. The multiple-drug synergy assays were conducted in 96-well plates; as previously described, the volume of each well was raised to 100 μl using sterile ddH_2O, and 100 μl of *G. vaginalis* preparation was added to each well. Wells containing *G. vaginalis* cells alone and uninoculated broth medium served as positive and negative controls. Finally, 50 μl of sterile mineral oil was pipetted onto the top of each well to ensure anaerobic conditions. Each plate was run using the same equipment and under the same conditions as described in the previous section. Each assay was performed at least three times in duplicate.

The ability of the optimally synergistic combinations of subtilosin and the selected antimicrobials to inhibit the growth of healthy vaginal *Lactobacillus* isolates and two BV-associated pathogens (*M. curtisii* and *Peptostreptococcus anaerobius*) were tested via 96-well microplate assays. Briefly, each plate tested the effect of the three most effective triple-drug combinations, along with each drug individually and all combinations of two compounds. As stated above, the volume of each well was raised to 100 μl using sterile ddH_2O, and 100 μl of the appropriate cell culture preparation was added to each well. Wells containing the tested organism alone and uninoculated broth medium served as positive and negative controls. Initial absorbance readings were measured at 595 nm in a ThermoMax microplate reader (Molecular Devices, Sunnyvale, CA). Each plate was incubated in the appropriate growth conditions for the tested organism for 24 h, after which a final endpoint reading was taken at the same settings as the initial reading. Each experiment was performed at least three times in duplicate. Data were gathered and analyzed using SOFTMax Pro (version 4.0.1) software (Molecular Devices) and Microsoft Excel 2007.

Graphical presentation of the data. The kinetic growth curve data from all assays were analyzed using Microsoft Excel 2007. The results from microplate assays combining subtilosin with two additional antimicrobials were analyzed using the MacSynergy II (version 1.0) software program (31). The results from assays performed using three antimicrobials were analyzed using the MacSynergy III software program, a variation on the MacSynergy II program that is modified for use with combinations of three drugs.

RESULTS

Determination of MICs. The MICs of subtilosin, glycerol monolaurate (GML), lauric arginate (LAE), and polylysine against *G. vaginalis* were determined by the broth microdilution method in BHI broth supplemented with 3% horse serum. As seen in Table 1, all of the tested substances were able to completely inhibit the growth of the selected vaginal pathogen. Subtilosin proved to be quite effective with an MIC of only 9.2 μg/ml, while GML and polylysine had MICs of 20 μg/ml and 25 μg/ml, respectively. The MIC of GML is supported by the findings of Strandberg et al., who demonstrated that GML had an MIC of 10 μg/ml against a clinical isolate of *G. vaginalis* (34). As previously stated, all MIC assays were run at least two times in duplicate. The results for each compound did not deviate between assays, despite the extensive range of tested concentrations; thus, there was no standard deviation recorded for these results (Table 1).

Determination of synergy between two antimicrobial substances. Once the individual MICs of all the chosen compounds were calculated, a checkerboard assay was performed using subtilosin in combination with one other substance. Each assay was designed to test a wide range of concentrations, beginning with one slightly above each compound's individual MIC and decreasing in a serial manner to a zero concentration (negative control). Combinations of concentrations below each of the MIC levels that caused complete inhibition of microbial growth were analyzed with the MacSynergy II software program to determine the presence of synergy, additive effect, or antagonism.

TABLE 2 MICs of antimicrobial compounds tested in a checkerboard assay against *G. vaginalis*[a]

Antimicrobial compound	Combinatorial synergy MIC (μg/ml)	Subtilosin synergy MIC (μg/ml)
Glycerol monolaurate (GML)	2	4.6
Lauric arginate (LAE)	25	4.6
Polylysine	2.5	4.6

[a] Subtilosin was combined with one other antimicrobial agent per assay; the data in this table represent the minimum concentration of each compound required to inhibit *G. vaginalis* growth in a combinatorial manner. When combined with subtilosin, GML and LAE had a 4-fold reduction in their MIC, while polylysine had a dramatic 10-fold reduction. The MIC of subtilosin was reduced 2-fold when combined with GML, LAE, and polylysine. Each checkerboard assay tested a wide range of concentrations for each compound and was conducted at least twice in duplicate. All assays conducted resulted in identical results for all substances (no standard deviation).

(i) Interaction between subtilosin and glycerol monolaurate. Since GML has demonstrated antimicrobial activity against the BV-associated pathogen *G. vaginalis*, it was the first substance tested for synergy with our target peptide, subtilosin. From the checkerboard assay, the lowest combined concentrations of subtilosin and GML that caused total growth inhibition of *G. vaginalis* were 4.6 and 2 μg/ml, respectively (Table 2). When used in combination, there was a 2-fold reduction in subtilosin's MIC and a 4-fold reduction in GML's MIC. The MacSynergy II calculations also showed synergy between the two compounds (data not shown).

(ii) Interaction between subtilosin and lauric arginate. The second natural antimicrobial, lauric arginate, was previously studied for synergy with the *Lactobacillus rhamnosus*-produced bacteriocin lactocin 160 against *G. vaginalis* (40). As described for GML, its potential synergy with subtilosin was assessed and an isobologram was constructed using the individual MICs of subtilosin and LAE (Table 1). The checkerboard assay showed the lowest concentration combination of subtilosin and LAE that caused complete inhibition of *G. vaginalis* growth to be 4.6 μg/ml and 25 μg/ml, respectively (Table 2). This combination caused a 2-fold decrease in subtilosin's individual MIC and a 4-fold reduction in LAE's MIC. When analyzed using the MacSynergy II software, there was synergy between subtilosin and lauric arginate (data not shown), although the strength of this interaction was less than that of subtilosin and glycerol monolaurate.

(iii) Interaction between subtilosin and ε-poly-L-lysine. The third antimicrobial compound, polylysine, was previously demonstrated to synergize with both nisin and subtilosin against the food-borne pathogen *Listeria monocytogenes* (1, 5), supporting the possibility of synergy with subtilosin against *G. vaginalis*. As previously described, an isobologram was constructed using the individual MICs of subtilosin and polylysine (Table 1). The checkerboard assay exhibited the lowest concentration combination of subtilosin and polylysine to completely inhibit *G. vaginalis* growth as 4.6 μg/ml and 2.5 μg/ml, respectively (Table 2). This combination caused a 2-fold decrease in subtilosin's individual MIC and a significant 10-fold reduction in polylysine's MIC. When analyzed using the MacSynergy II software, there was synergy between subtilosin and polylysine (data not shown), although it was the weakest synergistic interaction of the three tested combinations of two antimicrobials.

TABLE 3 Synergistic and antagonistic interactions between subtilosin, lauric arginate, glycerol monolaurate, and polylysine when combined against *G. vaginalis*[a]

Antimicrobial combination[b]	MacSynergy II/III synergy value	MacSynergy II/III antagonism value
Subt + LAE	176	0
Subt + GML	1,204	0
Subt + PL	70.4	−4.37
Subt + LAE + 0.25 GML	142	0
Subt + LAE + 0.5 GML	279	0
Subt + LAE + 0.75 GML	243	0
Subt + GML + 0.25 PL	80.3	0
Subt + GML + 0.5 PL	131	0
Subt+ GML + 0.75 PL	720	0
Subt + LAE + 0.25 PL	100	0
Subt + LAE + 0.5 PL	161	0
Subt + LAE + 0.75 PL	103	0

[a] Subtilosin was combined with one or two additional antimicrobials. The effect of each combination was tested against the BV-related pathogen in a 96-well plate assay. The kinetic growth data were analyzed using either the MacSynergy II (two-drug assay) or MacSynergy III (three-drug assay) programs and are given with 99% confidence.
[b] Subt, subtilosin; LAE, lauric arginate; GML, glycerol monolaurate; PL, polylysine. The number before the last antimicrobial agent shows the fraction of MIC for the drug (e.g., 0.25 GML is 0.25 MIC of GML).

TABLE 4 Average growth inhibition of BV-associated organisms when exposed to subtilosin, lauric arginate, glycerol monolaurate, and polylysine alone and in combination[a]

Antimicrobial treatment[b]	Growth inhibition (%)	
	M. curtisii	*P. anaerobius*
Subt	100	98.1
Subt + LAE	100	75
Subt + GML	100	98.1
Subt + PL	90	77.6
Subt + LAE + 0.75 PL	92.2	100
Subt + LAE + 0.5 GML	100	100
Subt + GML + 0.5 PL	92.2	83.3

[a] The effect of each combination was tested against the BV-related pathogen in a 96-well plate assay. The average final growth rate for each set of antimicrobials tested was compared to the average growth rate for control cells to calculate the percent inhibition. Each experiment was performed at least three times in duplicate.
[b] Subt, subtilosin; LAE, lauric arginate; GML, glycerol monolaurate; PL, polylysine. The number before the last antimicrobial agent shows the fraction of MIC for the drug (e.g., 0.75 PL is 0.75 MIC of PL).

Determination of synergy between three antimicrobial substances. Once the interactions between the combinations of subtilosin and one additional antimicrobial were calculated, a checkerboard assay was performed combining subtilosin with two other substances. Each assay was designed to test subtilosin and one antimicrobial at different concentrations (0.25-, 0.-5, 0.75-, 1.0-, and 1.25-fold MIC), while the concentration of the third antimicrobial (0.25-, 0.5-, or 0.75-fold MIC) was constant in each assay. The interactions between the three drugs were analyzed using the MacSynergy III software program.

(i) Interaction between subtilosin, lauric arginate, and glycerol monolaurate. All three of the tested concentration combinations of subtilosin, lauric arginate, and glycerol monolaurate were synergistic against *G. vaginalis*. According to the MacSynergy III analyses (Table 3), the highest overall level of synergy occurs when subtilosin and lauric arginate are combined with glycerol monolaurate at its 0.5 MIC level. However, when glycerol monolaurate was used at a 0.25 MIC level, there was still a significant level of synergy between the antimicrobials. Although the overall synergy value was lower for this three-drug combination than for any of the two-drug combinations, the results indicate that these antimicrobials may be used in a synergistic multiple-hurdle approach against *G. vaginalis*.

(ii) Interaction between subtilosin, lauric arginate, and polylysine. All three of the tested concentration combinations of subtilosin, lauric arginate, and polylysine were synergistic against *G. vaginalis*. According to the MacSynergy III analyses (Table 3), the highest overall level of synergy occurs when subtilosin and lauric arginate are combined with polylysine at its 0.75 MIC level. The synergy between the three compounds at this combination was nearly 5.5-fold higher than when polylysine was used at its 0.5 MIC level. However, since all three tested combinations of these compounds were synergistic, they are all viable options for use in a synergistic multiple-hurdle approach against *G. vaginalis*.

(iii) Interaction between subtilosin, glycerol monolaurate, and polylysine. All three of the tested concentration combinations of subtilosin, glycerol monolaurate, and polylysine were synergistic against *G. vaginalis*. According to the MacSynergy III analyses (Table 3), the highest level of overall synergy occurs when subtilosin and glycerol monolaurate were combined with polylysine at its 0.5 MIC level. This combination was only slightly more synergistic than the other two combinations tested, indicating that polylysine could be used at the lowest tested concentration without any negative effect on the ability of this combination to inhibit *G. vaginalis*. Thus, it is clear that all three tested combinations of these compounds could be used in a synergistic multiple-hurdle approach against *G. vaginalis*.

Effect of synergistic antimicrobials on healthy vaginal lactobacilli and BV-associated pathogens. The most synergistic triple combinations of the selected antimicrobials (subtilosin [Subt] plus LAE plus 0.75 MIC polylysine [PL], Subt plus LAE plus 0.5 MIC GML, and Subt plus GML plus 0.5 MIC PL) were tested for their effect on a variety of human *Lactobacillus* clinical isolates and two BV-associated microorganisms. None of the antimicrobials caused growth inhibition of the lactobacilli (data not shown), indicating that they would be safe for healthy vaginal microflora. Conversely, there was significant if not total inhibition of *M. curtisii* and *Peptostreptococcus anaerobius* (Table 4).

DISCUSSION

The antimicrobial activity of subtilosin and three natural antimicrobials were investigated alone and in combination against the BV-associated pathogen *G. vaginalis*. A checkerboard assay was utilized to study multiple concentrations of subtilosin and another antimicrobial compound for the presence of synergy, additive effect, or antagonism against the target microorganism. Individually, subtilosin had the lowest MIC against subtilosin at 9.2 μg/ml, although GML, LAE, and polylysine also had MICs in the μg/ml range. However, when each of the three compounds was tested in combination with subtilosin, there was a dramatic reduction in their MIC. The MICs of both GML and LAE were reduced 4-fold, while subtilosin's MIC decreased by half. The 10-fold drop in polylysine's MIC was the most significant change (Table 2).

While the ability to use considerably smaller amounts of each compound to inhibit *G. vaginalis* growth was a promising result, our main interest lay in whether these interactions were the result of synergy between the two compounds. The result of pairing subtilosin with one antimicrobial were confirmed in a more specific manner through the use of the MacSynergy II program, which analyzed the kinetic growth data for all the combinations tested. Based on these results, the interactions between subtilosin and two additional antimicrobials were further investigated via checkerboard 96-well plate assays. Again, the MacSynergy III software data analysis confirmed that there was synergy for all tested combinations of three antimicrobials. More specifically, the analyses revealed the three most optimal concentration combinations: subtilosin plus lauric arginate plus (0.75 MIC) polylysine, subtilosin plus lauric arginate plus (0.5 MIC) glycerol monolaurate, and subtilosin plus glycerol monolaurate plus (0.5 MIC) polylysine. Taken together, the various methods of determining synergy between antimicrobial compounds clearly confirm that subtilosin synergizes with all three of the tested natural antimicrobials.

These optimal synergistic combinations were then tested against clinical isolates of human lactobacilli, as well as two major BV-contributing microorganisms. While the antimicrobials did not inhibit any of the lactobacilli, they did cause partial or full inhibition of both *M. curtisii* and *Peptostreptococcus anaerobius*. These results confirm our assumptions that a formulation combining subtilosin with selected natural antimicrobials would be a novel, effective way of controlling the pathogens involved in the etiology of BV without harming the healthy microflora of the vagina. Interestingly, when the synergistic drug combinations were tested against three strains of *Candida albicans*, a fungal vaginal pathogen, there was no inhibition of growth (data not shown) despite the known ability of GML to inhibit *Candida* (34). However, this difference can be explained by the fact that the MIC of GML is within the range of 100 to 500 μg/ml, which is well above the concentrations tested in this study. Thus, future research should investigate the effect of increasing drug concentrations on *Candida* in the hopes of further expanding the potential applicability of the final antimicrobial formulation.

While there are many accepted methods for analyzing the interactions between antimicrobial compounds, one of the most commonly used forms of analysis is isobologram analysis. Although isobolograms do provide a clear, visual representation of the interactions occurring for specific combinations of drugs at different concentrations, they do not provide an overall analysis of the collective behavior of the drugs. For our purposes, we found the data analysis provided by the MacSynergy II and III suites of software to provide a far more comprehensive indication of how the antimicrobials were working with or against each other. Furthermore, the MacSynergy programs take into account all of the tested concentration combinations, which are not typically shown on an isobologram. In the frames of our study, we also attempted to utilize the CompuSyn software program (version 3.0.1, ComboSyn, Paramus, NJ) for analysis of drug interactions. However, while this program has been designed specifically for pharmacological research (8, 9), it was not suited to microbiological kinetics and antimicrobial assay data. Indeed, the CompuSyn analyses indicated strong antagonism between all of the two-drug and three-drug combinations tested. This was in direct contrast to the biological effects seen in the kinetic growth assays, which clearly supported the fact that sub-MIC concentration combinations of drugs were fully inhibiting growth of *G. vaginalis*. Therefore, we respectfully suggest that fellow researchers conducting similar assays choose the form of data analysis that is most appropriately designed for their forms of experimentation.

The presence of synergy between subtilosin and these substances is a promising result that creates a wide range of possibilities for future formulations of personal care products targeted at the prophylaxis and treatment of BV. There are many documented instances of drug-resistant cases of BV developing after treatment with the regularly prescribed antibiotics (7, 20, 22), indicating the need for new treatment options. Considering the multitude of health risks associated with BV, ideally, there would be a low possibility of BV-associated organisms developing resistance to these new alternatives. The multiple-hurdle approach is therefore ideal, since it uses combinations of synergistic substances in concentrations lower than their individual effective doses (1). The use of more than one antimicrobial, especially those with differing mechanisms of action, makes it very difficult for the pathogen to overcome each "hurdle" and lowers the chances of significant numbers of cells surviving (5). Due to its cyclic structure, subtilosin has a unique mechanism of action. Kawulka et al. (18) first posited that subtilosin may bind to a surface receptor rather than solely interacting with the cell membrane. Later, Thennarasu et al. (39) suggested that subtilosin may in fact attach to the target cell's lipid bilayer, causing the leakage of unilamellar vesicles. However, our own research has shown that, at least for *G. vaginalis*, subtilosin forms transient pores in the cell membrane that disrupt components of the cell's proton motive force and allow for efflux of ATP (37). As detailed in the introduction, the different mechanisms of action of GML, LAE, and polylysine would all be suitable counterparts to that of subtilosin, and would indeed provide the multiple "hurdles" required to effectively control the growth of BV-associated pathogens like *G. vaginalis*. This is the first report on the synergy of subtilosin with natural antimicrobials against *G. vaginalis*, and it provides information crucial to the development and formulation of novel treatment and prophylaxis approaches for bacterial vaginosis.

ACKNOWLEDGMENTS

This research was sponsored by the NIH NIAID grant 1R01AI084137 "Multiplex Nanocarrier-Based Hydrogels for Prevention of Vaginal HIV Transmission, Highly Innovative Tactics to Interrupt Transmission of HIV (HIT-IT)," the Bill and Melinda Gates Foundation Grand Challenges Exploration Phase 5 grant OPP1025200 "The natural spermicidal antimicrobial subtilosin controls vaginal infections," and the Rutgers University Life Science Commercialization Fund "Formulation and production of an antimicrobial peptide for control of bacterial vaginosis" (2010 to 2011).

REFERENCES

1. **Amrouche T, Sutyak Noll K, Wang Y, Huang Q, Chikindas ML.** 2010. Antibacterial activity of subtilosin alone and combined with curcumin, poly-lysine, and zinc lactate against *Listeria monocytogenes* strains. Probiotics Antimicrob. Proteins **2**:250–257.
2. **Amsel R, et al.** 1983. Nonspecific vaginitis. Diagnostic criteria and microbial and epidemiologic associations. Am. J. Med. **74**:14–22.
3. **Aroutcheva AA, Simoes JA, Behbakht K, Faro S.** 2001. *Gardnerella vaginalis* isolated from patients with bacterial vaginosis and from patients with healthy vaginal ecosystems. Clin. Infect. Dis. **33**:1022–1027.
4. **Babasaki K, Takao T, Shimonishi Y, Kurahashi K.** 1985. Subtilosin A, a new antibiotic peptide produced by *Bacillus subtilis* 168: isolation, structural analysis, and biogenesis. J. Biochem. **98**:585–603.
5. **Badaoui Najjar M, Kashtanov D, Chikindas ML.** 2007. Epsilon-poly-L-lysine and nisin A act synergistically against Gram-positive food-borne

pathogens *Bacillus cereus* and *Listeria monocytogenes*. Lett. Appl. Microbiol. **45**:13–18.
6. **Bakal G, Diaz A.** 2005. The lowdown on lauric arginate. Food Qual. **12**:54–61.
7. **Bannatyne RM, Smith AM.** 1998. Recurrent bacterial vaginosis and metronidazole resistance in *Gardnerella vaginalis*. Sex. Transm. Infect. **74**: 455–456.
8. **Chou T-C.** 2006. Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. Pharmacol. Rev. **58**:621–681.
9. **Chou T-C.** 2008. Preclinical versus clinical drug combination studies. Leuk. Lymphoma **49**:2059–2080.
9a. **Code of Federal Regulations.** 2010. Title 21. Food and drugs. Chapter I. Food and Drug Administration. Subchapter B. Food for human consumption. Part 184. Direct food substances affirmed as generally recognized as safe. Subpart B. Listing of specific substances affirmed as GRAS. 21 CFR 184.1505–mono- and diglycerides. http://cfr.vlex.com/vid/184-1505-mono-and-diglycerides-19707498.
10. **Eckert LO, Moore DE, Patton DL, Agnew KJ, Eschenbach DA.** 2003. Relationship of vaginal bacteria and inflammation with conception and early pregnancy loss following in-vitro fertilization. Infect. Dis. Obstet. Gynecol. **11**:11–17.
11. **Falagas ME, Betsi GI, Athanasiou S.** 2007. Probiotics for the treatment of women with bacterial vaginosis. Clin. Microbiol. Infect. **13**:657–664.
12. **Gibbs RS.** 2007. Asymptomatic bacterial vaginosis: is it time to treat? Am. J. Obstet. Gynecol. **196**:495–496.
13. **Goldenberg RL, Culhane JF, Johnson DC.** 2005. Maternal infection and adverse fetal and neonatal outcomes. Clin. Perinatol. **32**:523–529.
14. **Haggerty CL, Hillier SL, Bass DC, Ness RB, PID Evaluation and Clinical Health Study Investigators.** 2004. Bacterial vaginosis and anaerobic bacteria are associated with endometritis. Clin. Infect. Dis. **39**:990–995.
15. **Hashemi FB, Ghassemi M, Roebuck KA, Spear GT.** 1999. Activation of human immunodeficiency virus type 1 expression by *Gardnerella vaginalis*. J. Infect. Dis. **179**:924–930.
16. **Hashemi FB, Ghassemi M, Faro S, Aroutcheva A, Spear GT.** 2000. Induction of human immunodeficiency virus type 1 expression by anaerobes associated with bacterial vaginosis. J. Infect. Dis. **181**:1574–1580.
17. **Hillier SL, et al.** 1995. Association between bacterial vaginosis and preterm delivery of a low-birth-weight infant. N. Engl. J. Med. **333**:1737–1742.
18. **Kawulka KE, et al.** 2004. Structure of subtilosin A, a cyclic antimicrobial peptide from Bacillus subtilis with unusual sulfur to alpha-carbon cross-links: formation and reduction of alpha-thio-alpha-amino acid derivatives. Biochemistry **43**:3385–3395.
19. **Klaenhammer TR, Fremaux C, Ahn C, Milton K.** 1993. Molecular biology of bacteriocins produced by *Lactobacillus*, p 151–180. *In* Hoover DG, Steenson LR (ed), Bacteriocins of lactic acid bacteria. Academic Press, Inc, New York, NY.
20. **Liebetrau A, Rodloff AC, Behra-Miellet J, Dubreuil L.** 2003. *In vitro* activities of a new des-fluoro(6) quinolone, garenoxacin, against clinical anaerobic bacteria. Antimicrob. Agents Chemother. **47**:3667–3671.
21. **Lin L, et al.** 1999. The role of bacterial vaginosis in infection after major gynecologic surgery. Infect. Dis. Obstet. Gynecol. **7**:169–174.
22. **Lubbe MM, Botha PL, Chalkley LJ.** 1999. Comparative activity of eighteen antimicrobial agents against anaerobic bacteria isolated in South Africa. Eur. J. Clin. Microbiol. Infect. Dis. **18**:46–54.
23. **Nelson DB, et al.** 2007. First trimester bacterial vaginosis, individual microorganism levels, and risk of second trimester pregnancy loss among urban women. Fertil. Steril. **88**:1396–1403.
24. **Newton ER, Piper J, Peairs W.** 1997. Bacterial vaginosis and intraamniotic infection. Am. J. Obstet. Gynecol. **176**:672–677.
25. **Nykanen A, Weckman K, Lapvetelainen A.** 2000. Synergistic inhibition of *Listeria monocytogenes* on cold-smoked rainbow trout by nisin and sodium lactate. Int. J. Food Microbiol. **61**:63–72.
26. **Oakeshott P, Kerry S, Hay S, Hay P.** 2004. Bacterial vaginosis and preterm birth: a prospective community-based cohort study. Br. J. Gen. Pract. **54**:119–122.
27. **Pechous R, Ledala N, Wilkinson BJ, Jayaswal RK.** 2004. Regulation of the expression of cell wall stress stimulon member gene *msrA1* in methicillin-susceptible or -resistant *Staphylococcus aureus*. Antimicrob. Agents Chemother. **48**:3057–3063.
28. **Prichard MN, Shipman C, Jr.** 1990. A three-dimensional model to analyze drug-drug interactions. Antiviral Res. **14**:181–205.
29. **Prichard MN, Prichard LE, Shipman C, Jr.** 1993. Strategic design and three-dimensional analysis of antiviral drug combinations. Antimicrob. Agents Chemother. **37**:540–545.
30. **Projan SJ, Brown-Skrobot S, Schlievert PM, Vandenesch F, Novick RP.** 1994. Glycerol monolaurate inhibits the production of beta-lactamase, toxic shock toxin-1, and other staphylococcal exoproteins by interfering with signal transduction. J. Bacteriol. **176**:4204–4209.
31. **Sewankambo N, et al.** 1997. HIV-1 infection associated with abnormal vaginal flora morphology and bacterial vaginosis. Lancet **350**:546–550.
32. **Shima S, Matsuoka H, Iwamoto T, Sakai H.** 1984. Antimicrobial action of episolon-poly-L-lysine. J. Antibiot. (Tokyo) **37**:1449–1455.
33. **Srinivasan S, Fredericks DN.** 2008. The human vaginal bacterial biota and bacterial vaginosis. Interdiscip. Perspect. Infect. Dis. **2008**:750479.
34. **Strandberg KL, et al.** 2010. Glycerol monolaurate inhibits *Candida* and *Gardnerella vaginalis in vitro* and *in vivo* but not *Lactobacillus*. Antimicrob. Agents Chemother. **54**:597–601.
35. **Sutyak KE, Wirawan RE, Aroutcheva AA, Chikindas ML.** 2008. Isolation of the *Bacillus subtilis* antimicrobial peptide from the dairy product-derived *Bacillus amyloliquefaciens*. J. Appl. Microbiol. **104**:1067–1074.
36. **Sutyak KE, et al.** 2008. Spermicidal activity of the safe natural antimicrobial peptide subtilosin. Infect. Dis. Obstet. Gynecol. **2008**:540758.
37. **Sutyak Noll K, Sinko PJ, Chikindas ML.** 2011. Elucidation of the molecular mechanism of action of the natural antimicrobial peptide subtilosin against the bacterial vaginosis-associated pathogen *Gardnerella vaginalis*. Probiotics Antimicrob. Proteins **3**:41–47.
38. **Taha TE, et al.** 1999. HIV infection and disturbances of vaginal flora during pregnancy. J. Acquir. Immune Defic. Syndr. Hum. Retrovirol. **20**: 52–59.
39. **Thennarasu S, et al.** 2005. Membrane permeabilization, orientation, and antimicrobial mechanism of subtilosin A. Chem. Phys. Lipids **137**:38–51.
40. **Turovskiy Y, Chikindas ML.** 2011. Zinc lactate and sapindin act synergistically with Lactocin 160 against *Gardnerella vaginalis*. Probiotics Antimicrob. Proteins **3**:144–149.
41. **Turovskiy Y, Sutyak Noll K, Chikindas ML.** 2011. The aetiology of bacterial vaginosis. J. Appl. Microbiol. **110**:1105–1128.
42. **Vetter SM, Schlievert PM.** 2005. Glycerol monolaurate inhibits virulence factor production in *Bacillus anthracis*. Antimicrob. Agents Chemother. **49**:1302–1305.
43. **Weir E.** 2004. Bacterial vaginosis: more questions than answers. Can. Med. Assoc. J. **171**:448.