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Zinc Lactate and Sapindin Act Synergistically with Lactocin 160 Against *Gardnerella vaginalis*

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Abstract

Lactocin 160 is a vaginal probiotic-derived bacteriocin shown to selectively inhibit the growth of *Gardenerella vaginalis* and some other pathogens commonly associated with bacterial vaginosis. The natural origin of this peptide, its safety, and selective antimicrobial properties make it a promising candidate for successful treatment and prophylaxis of bacterial vaginosis (BV). This study evaluated interactions between lactocin 160 and four other natural antimicrobials in the ability to inhibit *G. vaginalis*. We report that zinc lactate and soapnut extract act synergistically with lactocin 160 against this pathogen and therefore have a potential to be successfully used as the components of the multiple-hurdle antimicrobial formulation for the treatment of BV.

Keywords

Bacteriocin; Natural antimicrobial; Antimicrobial synergy

Introduction

Bacterial vaginosis is a complex multispecies infection of the lower genital tract, which affects millions of women each year [1]. Aside from having a dramatic negative impact on the quality of a woman's life, this condition is notorious for causing serious gynecological and obstetric complications. Less than satisfactory results produced by the conventional antibiotic treatments for BV have prompted researchers to look for natural alternatives to antibiotics, particularly among the antimicrobial products of healthy vaginal lactobacilli. The topical application of these lactobacillus-derived antimicrobials, especially if followed by probiotic treatment, can potentially be used to restore a healthy microbial balance in BV-affected individuals [2]. Lactocin 160, a bacteriocin-like compound produced by a vaginal isolate of *Lactobacillus rhamnosus* 160, is a promising alternative treatment for BV. This antimicrobial peptide selectively inhibits some BV-associated pathogens, including *Gardnerella vaginalis*, without affecting the commensal vaginal lactobacilli [3]. Moreover, the safety of lactocin 160 for topical applications has been demonstrated using both in vivo and in vitro vaginal models [4].

The emergence of bacterial resistant strains to current antibiotics is a rapidly advancing problem in clinical microbiology [5]. The widespread resistance of *G. vaginalis* to metronidazole and clindamycin, commonly used for treatment of BV, has already been reported [6, 7]. Moreover, the ability of *G. vaginalis* to uptake DNA from other vaginal

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microorganisms drastically increases its chances of developing antimicrobial resistance [8]. One of the most effective ways to minimize the chance of developing resistance is by using multiple antimicrobial hurdles, with each having a different mode of action [9].

The multiple-hurdle approach relies on the use of multiple stress factors that simultaneously deplete various resources of a target cell, making the microbial adaptation processes more challenging. Generally, stressors with different molecular targets are used as hurdles because they tend to act synergistically when used in combination [9]. Multiple-hurdle technology has been utilized for many years to control microorganisms in clinical settings and in food preservation [9]. A secondary advantage of this practice is its cost-effectiveness; synergistically acting components of the antimicrobial formulation can be used in lower concentrations. In addition, the activity of an antimicrobial preparation can be modulated to a desired specificity by using a multicomponent formulation [9, 10]. In this study, we evaluated interactions between lactocin 160 and other natural antimicrobials in an effort to control the growth of *G. vaginalis* so the data can ultimately be used for the design of an effective multiple-hurdle treatment for BV.

Turovskiy et al. [11] demonstrated that lactocin 160 targets the cytoplasmic membranes of *G. vaginalis* cells, ultimately dissipating both components of the proton motive force and causing depletion of the cellular ATP content. The exact molecular mechanism of action is yet to be determined, but there is evidence that lactocin 160 facilitates formation of transient pores across the cytoplasmic membranes of *G. vaginalis*, thereby triggering transmembrane traffic of ions and molecules [11, unpublished data].

The four natural antimicrobials selected for the synergy study were zinc lactate, soapnut extract, poly-L-lysine, and lauric arginate. These substances were chosen because their antimicrobial mode of action is likely to differ from the mode of action of bacteriocins and because they have previously been approved for human use.

Zinc lactate is a salt of lactic acid, which is a major fermentative product of lactic acid bacteria (LAB), including vaginal lactobacilli. As a result, lactates are prevalent in the lower genital tract of healthy women [12]. Lactic acid is a crucial defense factor of healthy vaginal microbiota; thus, a number of feminine hygiene products, including those designed for treatment of BV, contain this compound [13]. Additionally, lactates are used as food preservatives. Several mechanisms are responsible for the antimicrobial properties of lactic acid and its salts. In an acidic environment, these antimicrobials act as ionophores, which drop the intracellular pH in bacteria [14, 15]. Lactates also create a hostile environment for the proliferation of microorganisms by decreasing the water activity [15]. Zinc salt of lactic acid was selected for this study over other lactate species because (1) zinc ions were shown to enhance the activity of the bacteriocin nisin against *Listeria monocytogenes* [15], and (2) there are reports of ionic zinc having both antiviral and spermicidal properties [16, 17]—two effects desirable in a feminine hygiene product.

Epsilon-poly-L-lysine (poly-L-lysine) is a secondary metabolite secreted by various *Streptomycetaceae* bacteria. This antimicrobial is a cationic polypeptide that consists of 25–35 L-lysine residues connected by amide bonds between ε-amino and α-carboxyl groups [18]. Commercially, poly-L-lysine is produced through a fermentation process involving *Streptomyces albulus* [18]. Numerous in vivo studies demonstrated that poly-L-lysine is safe for consumption [18, 19]. This antimicrobial is currently on the commercial market in Japan as a food preservative [20]. The antimicrobial activity of poly-L-lysine is related to its electrostatic adsorption to a cell's surface. The exact mechanism of its action is largely unknown, although it has been proposed that this ionic adsorption strips the outer membrane in Gram-negative cells [20, 21].

Saponins are steroid or triterpenoid glycosides produced by a variety of plants and by some marine organisms. This group of natural detergents is very common in both human and animal diets [22]. Plant extracts containing saponins are commonly used as food additives. For example, *Quillaja saponaria* extract is widely used in both the food and beverage industries without any reported toxicity [23]. Saponins derived from the fruit pericarp of *Sapindus mukorossi* (soapnut) are of particular interest for this study because they have only an insignificant effect on the proliferation of vaginal lactobacilli. Additionally, *Sapindus* saponins have spermicidal properties and are used as active ingredients in the contraceptive cream CONSAP [24]. Finally, there are some reports of saponins inhibiting the replication of the HIV-1 virus [22, 25]. Although it is clear that the antimicrobial activity of saponins is related to their detergent-like properties, the exact mechanism of action is still unknown. Studies involving liposomes, however, suggest that saponins permanently damage cytoplasmic membranes, making them permeable to macromolecules [22].

Lauramide arginine ethyl ester (LAE) is a derivative of lauric acid, L-arginine, and ethanol. This antimicrobial is generally recognized as safe (GRAS) status for use in meat, poultry, and other food products (GRAS Notice No. GRN 000164). The antimicrobial activity of LAE is thought to be related to the compound's surfactant properties [26]. Studies conducted by Rodriguez et al. [26] using transmission electron microscopy (TEM) indicated that LAE induced swelling of the outer membrane in a Gram-negative bacterium, *Salmonella typhimurium*. In contrast, LAE induced the formation of white spots and clear zones in the cytoplasmic membranes of *Staphylococcus aureus*, a Gram-positive bacterium. In both strains, these alterations induced the flux of potassium ions across the cytoplasmic membrane [26].

In this manuscript, we demonstrate that zinc lactate and soapnut extract act synergistically with lactocin 160 against *G. vaginalis*. This synergistic interaction indicates a possibility of these natural antimicrobials being successfully used as the components of a multiple-hurdle approach for control of BV-related pathogens.

Materials and Methods

Bacterial Strains and Growth Conditions

Frozen stocks of *G. vaginalis* ATCC 14018 were maintained at −70 °C in Brain Heart Infusion (BHI) broth (Difco, Sparks, MD) containing 3% horse serum (JRH Biosciences, KS), mixed with 15% glycerol. BHI containing 3% horse serum was also used to propagate the culture, which was passed through the medium overnight at least twice prior to being used in experiments. The cells were always inoculated into fresh medium contained in 50-mL centrifuge tubes (1% v/v) that were pre-incubated with a loosened cap, under anaerobic conditions, to minimize the stress effect; this way, the microorganism was directly transferred into a warm, anaerobic environment. The culture was then incubated under anaerobic conditions at 37 °C.

Preparation of Antimicrobial Solutions

The partially purified preparation of lactocin 160 was produced at the Cell Production and Recovery Facility (Waksman Institute, Rutgers University, NJ) using the method previously described by Aroutcheva et al. [3, 11]. The 10 AU mL^{-1} stock solution was prepared by dissolving 300 mg of this preparation in 1 mL of double distilled water. The total protein concentration in the stock solution was quantified by using Micro BCA™ Protein Assay Kit (Thermo Scientific, Rockford, IL), and its specific activity was determined to be 10.4 AU mg^{-1} protein.

The other four antimicrobials were generous gifts provided to us by their manufacturers. The sample of zinc lactate (PURAMEX ZN) was given to us by Purac America (Lincolnshire, IL). We received soapnut extract (SAPINDIN) from Sabinsa Corp. (Piscataway, NJ). Poly-L-lysine (250 mg mL^{-1}) was sent to us by Chisso America, Inc. (Rye, NY), and lauramide arginine ethyl ester (100 mg mL^{-1}, MIRENAT-CF) was a gift from Vedeqsa Corp. (Barcelona, Spain). Prior to being used in the experiments, the aqueous solutions of all the antimicrobials were filter-sterilized through 0.2-μm syringe filters (NALGENE, Rochester, NY).

Determination of the Minimal Inhibitory Concentrations (MICs) of the Antimicrobials

The minimal inhibitory concentrations (MICs) of all the antimicrobials were determined using the method reported by Badaoui Najjar et al. [27] with the some modifications. A separate microplate assay was conducted for each antimicrobial. Aqueous solutions covering a wide range of antimicrobial concentrations were prepared using sterile double distilled water. One hundred microliters of each dilution was then placed into a 96-well plate (Corning, Inc., Corning, NY), in duplicate, immediately followed by 100 μL of the newly inoculated *G. vaginalis* culture. Sterile, double distilled water was used as a negative control to reveal the growth patterns of *G. vaginalis* without any antimicrobial restraint. Subsequently, the surface of the wells was covered with sterilized mineral oil for prevention of condensation during the assay. The assay was conducted at 37 °C under anaerobic conditions. The OD_{595} readings were taken every 2 h for 48 h using an automated microplate reader (Model 550, Bio-Rad Laboratories, Hercules, CA). Some natural antimicrobials used in this study are only available in the partially purified form; therefore, we decided to express the activity of all the antimicrobials against *G. vaginalis* in arbitrary units (AU), defined as the lowest dilution of the partially purified preparation causing the full inhibition of the microorganism. One AU mL^{-1} of zinc lactate, soapnut extract, poly-L-lysine, and lauric arginate corresponded to 150, 200, 125, and 1,000 μg mL^{-1} of these preparations, respectively. The subsequent interaction analysis was conducted using arbitrary units of activity.

The Checkerboard Assay

The interactions between two antimicrobials were investigated using a microplate reader-based checkerboard assay described by Asok et al. [28] and Badaoui Najjar et al. [27]. Briefly, the aqueous solutions of each pair of antimicrobials were prepared separately. The solutions were then mixed in various proportions using a 96-well plate to produce antimicrobial mixtures containing a wide range of concentrations (the highest concentration of each antimicrobial in the mixture was above the antimicrobial's MIC). In total, each well of the microplate contained 100 μL of the antimicrobial mixture (the composition of which systematically varied throughout the plate) and 100 μL of *G. vaginalis* culture. The cells used in the assay were prepared by diluting an overnight culture of *G. vaginalis* 100 times with fresh growth medium that was incubated overnight under anaerobic conditions to rid it of oxygen. The cells were then added to the wells of the microplate containing the antimicrobials (100 μL of diluted culture per well). Changes in turbidity were monitored under anaerobic conditions for 48 h using an automated microplate reader (Model 550, Bio-Rad Laboratories, Hercules, CA). The readings at OD_{595} were taken every 2 h while the plate was incubated at 37 °C. The ultimate goal of this assay is to determine the MICs of the tested antimicrobials when they are used in various combinations.

Analysis of the Checkerboard Assay Data

The nature of interactions between lactocin 160 and other natural antimicrobials was analyzed using isobolograms. This analytical method relies on the comparison of the MICs of two antimicrobials when they are used individually to their MICs when used in

combination. The isobologram method is based on a visual comparison of these values when plotted on the same coordinate axis. Initially, the individual MICs of two antimicrobials are plotted on the *x*- and *y*-axes with the coordinates (0, *x*) and (*y*, 0). These two points are then connected by a perforated interaction line. The fractional inhibitory concentrations (FICs), defined as the ratio of the compound's MIC when used in combination with the second antimicrobial to its MIC when used individually, of each compound are then plotted as the *x* and *y* coordinates of a single point.

Statistics

All experiments were prepared at least twice in duplicates.

Results

All antimicrobials used in this study had a similar dose-dependent effect on growth kinetics of *G. vaginalis* as illustrated in Fig. 1, using poly-L-lysine as an example. The checkerboard assay allowed for simultaneous evaluation of multiple concentrations of a two-component mixture [27] to identify antimicrobials that act synergistically with lactocin 160.

According to the isobologram model, the concentration combinations located below the perforated interaction line signify synergistic interactions. Conversely, the combinations located above the interaction line indicate antagonism, while the ones located along the interaction line show an additive effect between the tested antimicrobials. Finally, the lack of microbial growth inhibition by a combination of two antimicrobials at their subinhibitory concentrations indicates the lack of interaction, i.e., the antimicrobials have no effect on each other's inhibitory activity [27].

Accordingly, several points representing concentration combinations of lactocin 160 and zinc lactate appeared below the interaction line on the isobologram, indicating synergy between these two antimicrobials (Fig. 2a). Similarly, some synergistic effect was noticed between lactocin 160 and soapnut extract (Fig. 2b). However, we did not observe any interactions between lactocin 160 and LAE; various combinations of these antimicrobials at their subinhibitory concentrations did not inhibit the growth of *G. vaginalis* (Fig. 2c). Finally, when applied in combination, lactocin 160 and poly-L-lysine had an antagonistic effect (Fig. 2d).

Discussion

Originally, we anticipated to see synergy among all four antimicrobial combinations tested in this study because of the pronounced mode of action differences between the components of each combination. Although poly-L-lysine, LAE, and soapnut extract, much like lactocin 160, target bacterial cytoplasmic membranes, the damage caused by these three antimicrobials is presumably permanent, in contrast to transient pores created by bacteriocin-like substances such as lactocin 160. However, out of the four tested antimicrobials, only zinc lactate and soapnut extract synergized with lactocin 160 against *G. vaginalis*.

Lactates may act as ionophores at pH values close to and below the pK_a of lactic acid (3.86 at 25 °C). However, under the conditions of the checkerboard assay (pH close to neutral), the antimicrobial activity of these salts is mainly due to a decrease in water activity within the bacterial environment. In contrast, lactocin 160 inhibits *G. vaginalis*' growth by depleting its various transmembrane gradients [11]; therefore, synergy between these antimicrobials with very different inhibition mechanisms is not surprising. Soapnut extract

acts as a detergent, inducing immense, permanent membrane damage that is significantly different from the transient channels formed by bacteriocins.

The antimicrobial activity of LAE is also thought to be related to the surfactant properties of this compound. Much like lactocin 160, LAE makes bacterial cytoplasmic membranes permeable to potassium ions, although little is known about the nature of the perturbance caused by this antimicrobial. The lack of interactions between these two antimicrobials can possibly be explained by a similar mode of action.

The reasons for antagonism between lactocin 160 and poly-L-lysine are unclear. It is possible that the electrostatic interactions between these two peptides reduce their activity against a target cell. The second possibility is that lactocin 160 adsorbed to the cell surface may hinder interactions between poly-L-lysine and its cellular targets. Finally, it is possible that a subinhibitory concentration of the one antimicrobial triggers some adaptive response in the target cell, making it resistant to the second antimicrobial. Interestingly, the antagonism effect was only observed at subinhibitory concentrations of lactocin 160; the effect was not evident at the inhibitory concentrations. For that reason, poly-L-lysine can, theoretically, still be included in an antimicrobial formulation involving lactocin 160, as long as both these antimicrobials are used at concentrations at or above their MICs. However, due to their synergy with lactocin 160, zinc lactate and soapnut extract are the most promising candidates for a lactocin 160-based multiple-hurdle preparation for control of *G. vaginalis*. Ultimately, BV has a complex polymicrobial nature with a significant role being played by vaginal lactobacilli; therefore, in future studies, we will also elucidate the effect of the selected antimicrobial combinations on the healthy vaginal microbiota and on the BV-related pathogens other than *G. vaginalis*.

The pH of the system used in this study was around neutral, resembling the elevated vaginal pH characteristic of BV. This system is reflective for the use of lactocin 160-based formulations for treatment of BV. However, these antimicrobials can also be potentially used to prevent recurrence of BV by suppressing the growth of *G. vaginalis* in successfully treated patients. The healthy vaginal environment has an acidic pH (<4.5). Therefore, if the formulations were used for prophylaxis of recurrent BV, the interactions between antimicrobials would take place in acidic conditions. We were unable to grow *G. vaginalis* in vitro under acidic conditions; thus, we can only speculate about the antimicrobial interactions in these conditions. Theoretically, the acidic environment should enhance the bactericidal properties of the antimicrobials, because this environment provides an additional stress for the microorganism. This is especially true for antimicrobials such as lactocin 160 and zinc lactate, which act as ionophores, making the bacterial cytoplasmic membranes permeable to traffic of hydrogen ions. However, it is also possible that the acid tolerance response (ATR) of *G. vaginalis* would induce resistance to other antimicrobials. For instance, induction of ATR in *Listeria monocytogenes* through exposure to lactic acid increased this bacterium's tolerance to the bacteriocin nisin [29, 30]. Therefore, the experimental approach is ultimately unavoidable. Accordingly, future studies can evaluate the effectiveness of the selected antimicrobial combinations against biofilms of *G. vaginalis*, which are known to have an inherent tolerance of lactic acid [31]. The biofilms can be grown to maturity at neutral pH and then can be resuspended in acidic media to test the antimicrobial interactions under acidic conditions resembling a healthy vaginal environment.

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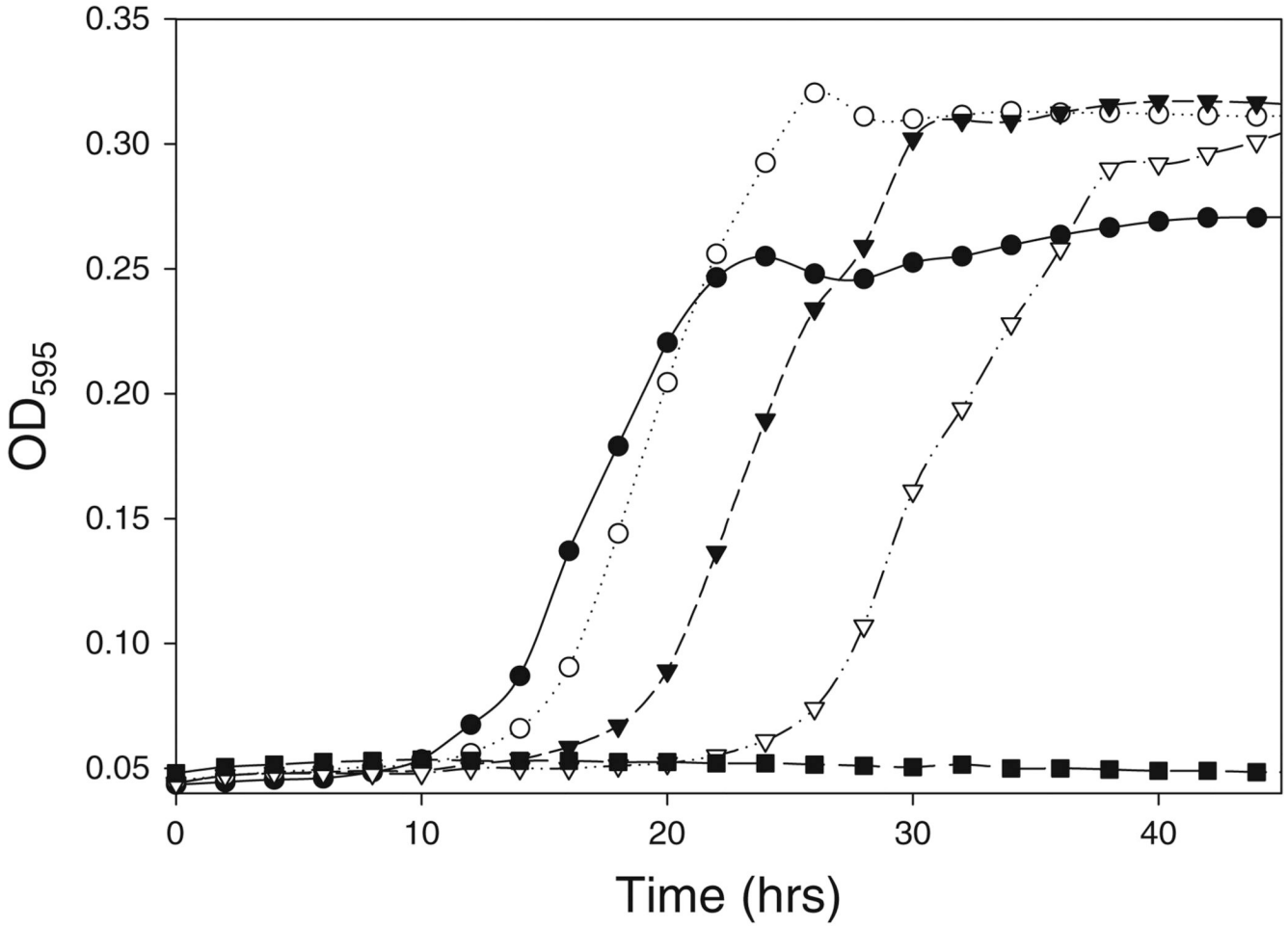

Fig. 1.

Growth kinetics of *G. vaginalis* in the presence of poly-L-lysine. The figure illustrates a typical effect of antimicrobial agents at their subinhibitory concentrations on growth kinetics of a microorganism. *G. vaginalis* grown at 0.25 AU mL^{-1} (*open circles*), 0.5 AU ml^{-1} (*closed reverse triangles*), and 0.75 AU ml^{-1} (*open reverse triangles*) of poly-L-lysine have a prolonged lag phase compared with the cells grown without the antimicrobial (*closed circles*). These effects are usually due to cells being stressed and/or due to some portion of the bacterial population being killed by the antimicrobial. By definition, 1 AU ml^{-1} (*closed squares*) is fully inhibitory to the bacterium. All the concentrations were tested in duplicates; however, the replicates of the OD values were averaged to simplify the figure

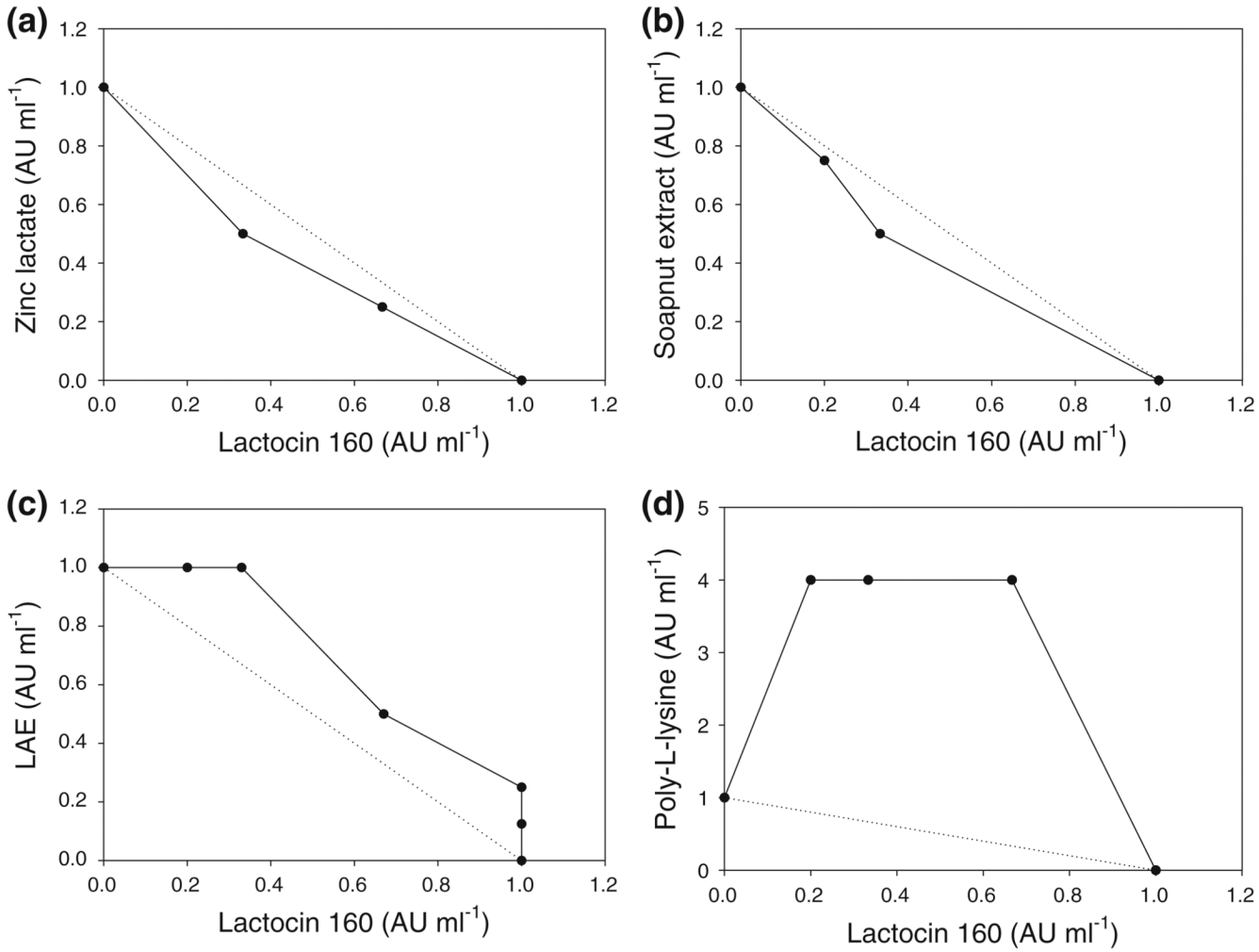

Fig. 2.

Isobolograms of interactions between lactocin 160 and four natural antimicrobials against the vaginal pathogen *G. vaginalis*. Lactocin 160 synergizes with zinc lactate (**a**) and soapnut extract (**b**). For the most part, there are no interactions between lactocin 160 and the LAE preparation (**c**). Finally, there is a marked antagonism between lactocin 160 and poly-L-lysine (**d**)